BMP Signaling Functions as a Distinct Pro-Angiogenic Cue

David Michael Wiley

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Approved by:

Dr. Victoria Bautch

Dr. Suk-Won Jin

Dr. Frank Conlon

Dr. Mark Peifer

Dr. Ellie Tzima

ABSTRACT

DAVID MICHAEL WILEY: BMP Signaling Functions as a Distinct Pro-Angiogenic Cue (Under the direction of Dr. Victoria L Bautch and Dr. Suk-Won Jin)

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is critical for the growth and survival of tissues in both normal and pathological scenarios. Vascular networks observed in vertebrates display a vast array of morphological and functional diversity. Large blood vessels rapidly transport blood to more highly branched capillaries where circulation is slowed and oxygen and nutrients are able to more effectively diffuse into the surrounding tissue. Unfortunately, our understanding of how this morphological diversity is generated is largely unknown. During zebrafish development, we found that angiogenic sprouts from the dorsal aorta are dependent on vascular endothelial growth factor A (VEGFA) signaling, and do not respond to bone morphogenetic protein (BMP) signals. In contrast, the highly branched angiogenic sprouts from the axial vein are regulated by BMP signaling independently of VEGFA signals, indicating that BMP is a vein-specific angiogenic cue during early vascular development. Responding to distinct angiogenic cues, the neighboring dorsal aorta and the axial vein are able to regulate distinct programs of sprouting angiogenesis. When we analyzed the effects of BMP in mammalian systems we found that BMP signaling is a potent and selective regulator of branching morphogenesis. These effects are distinct from VEGFA which has pleiotropic effects on vessel morphogenesis. Upon closer analysis, we find that BMP signaling regulates tip cell morphology during angiogenesis. Interestingly, inhibiting pathways that are known to establish tip cell identity prevents BMP-induced branching in zebrafish. Taken together, our

results support a paradigm whereby BMP regulates branching morphogenesis and network diversity via its effects on tip cell responses.

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Bone Morphogenetic Protein Functions as a Context-Dependent Angiogenic Cuein Vertebrates

This chapter is adapted from a solicited review published in Seminars in Cell and Developmental Biology in 2011. I wrote the first draft of the manuscript and made fig. 1.1 and 1.4. Dr. Suk-Won revised and added to my original draft and made fig. 1.2 and 1.3.

ABSTRACT

Bone Morphogenetic Protein (BMP) signaling has been implicated in diverse biological processes. Although how BMP signaling regulates behaviors of endothelial cells during angiogenesis are not fully understood, increasing evidence indicate functions of BMP signaling components are essential in developmental and pathological angiogenesis. Here we review recent advances in delineating the functions of BMP signaling during angiogenesis. In addition, we discuss downstream pathways that transduce BMP signaling in endothelial cells, and factors that modulate BMP signaling response in endothelial cells. Finally, we provide recent insight on how BMP signaling functions as a context dependent angiogenic cue.

INTRODUCTION

1. General Background

Bone Morphogenetic Protein (BMP) signaling is involved in diverse morphogenetic processes during development including bone and cartilage formation, early embryonic patterning along the dorsal-ventral axis, specification of endodermal organs [1], as well as pathological situations. However, its function during angiogenesis, the process by which new blood vessels form from pre-existing vessels, remains largely unknown. Although the Vascular Endothelial Growth Factor (VEGF) signaling pathway is well established as a major regulator of angiogenesis [2-3], complex vascular networks require input from multiple signaling pathways to pattern properly. Therefore, understanding the role of additional regulators of angiogenesis, such as the Bone Morphogenetic Protein (BMP) pathway, will help elucidate the complex mechanisms involved during angiogenesis.

2. Overview of BMP signaling cascade

BMP growth factors are members of the TGF-β super-family [4]. BMP ligands dimerize and bind to a tetraheteromeric receptor complex composed of two type I and two type II BMP receptors. Additionally, Type III receptors, such as Endoglin, can interact and modulate ligand affinity for type I and type II receptors. Once the signaling complex forms, the kinase domain of the type I BMP receptor phosphorylates and activates SMAD1, SMAD5, and SMAD8 (R-SMADs). Activated R-SMADs bind SMAD4 (co-SMAD) and translocate to the nucleus to initiate transcription of downstream target genes (Fig. 1.1). In addition to activating the SMAD signaling cascade, BMP signaling can also act through SMAD-independent mechanisms. For instance, BMP signaling can activate MAPK such as Erk and p38 [5].

3. Human pathological conditions caused by BMP signaling dysregulation:

In humans, mutations of various BMP signaling components have been linked to various pathological conditions affecting the vascular system. Mutations in the *ENG* gene and *ACVRL1, (*ENG encodes for the type III receptor, Endoglin; and *ACVRL1 encodes for* a type I receptor, ALK1) cause Hemorrhagic Hereditary Telangiectasia (HHT) 1 and HHT2 respectively [6-7]. HHT is an autosomal dominant vascular dysplasia characterized by recurrent nose bleeds, mucocutaneous telangiectases (small dilated blood vessels), and arteriovenous malformations (AVMs) [8]. Similarly, genetic manipulation of Endoglin and ALK1 in mice replicates many of the characteristics of HHT [9-13].

In addition, several components of BMP signaling pathway have been linked to pulmonary arterial hypertension (PAH) in humans. PAH is a progressive disorder thought to arise from abnormal endothelial cell growth and maintenance. PAH causes an increase in arterial pressure, occlusions in pulmonary arteries, and can even lead to heart failure. The primary gene associated with PAH is *BMPR2*. However, mutations in *ALK1*,*Endoglin,* or *SMAD8* have also been implicated in PAH [14-16]. Genetic manipulations in murine models also recapitulate the pathological symptoms found in humans. Global deletion of one copy of the BMPRII gene exhibited increased pulmonary vascular resistance and thickened arteries in mice [17]. Interestingly, global reduction of *BMPR2* by shRNA transgene caused a mucosal hemorrhages and incomplete mural cell coverage, phenotypes which are the common characteristics of HHT [18]. This suggests that BMP signaling is critical in the pathophysiology of PAH.

4. Ligand-receptor complexes

The BMP signaling pathway contains multiple BMP ligands which are subdivided in to groups based on sequence and function [19]. BMP2 and BMP4 form the BMP2/4 subgroup; BMP5, BMP6, BMP7, and BMP8 form the BMP7 subgroup; Growth Differentiation

Factor (GDF) 5, GDF6, and GDF7 form the GDF5 subgroup, and BMP9 and BMP10 form a fourth subgroup.

BMP ligands, once secreted, readily form a homodimer via a disulfide bond and are stabilized. Homodimers of various BMP ligands are capable of signaling. However, recent studies suggested that heterodimeric BMP ligands can induce more robust downstream activation than homodimeric BMP ligands. For instance, during zebrafish development, Bmp2b/7 ligand is a more potent regulator for dorsoventral patterning than Bmp2b or Bmp7 homodimer [20].

There are at least four type I receptors and three type II receptors that BMP ligands can interact with; Alk1, Alk2, Alk3, and Alk6 are the type I receptors, and BMP receptor type II (BMPRII), Activin Receptor type IIA and B (ACTRIIA, and ACTRIIB) are the type II receptors. Additionally, Type III receptors, such as Endoglin, can interact and modulate ligand affinity for its type I and type II receptors.

The sequence in which BMP ligands bind type I and type II receptors depends on the relative binding affinity. BMP2 and BMP4 have a high affinity for Alk3 and Alk6 type I receptors which recruit BMPRII/ActRII and ActRIIB type II receptors [21-22]. BMP6 and BMP7 interact with ActRII/ActRIIB and recruit Alk2, Alk3 and Alk6 [22-24]. GDF5 and GDF6 interact with Act RII, Act RIIB and BMP RII and Alk6 [25-26]. BMP9 and BMP10 interact with ALK1 and BMPRII/ActRII [27-28]. The composition of the Bmp signaling complexes likely underlies the diverse effects observed through BMP signaling (Fig. 1.1).

5. Ligands

In the mammalian system, there are at least 20 BMP ligands present. During development, diverse BMP ligands are widely expressed and many of them show overlapping yet distinct pro- and/or anti-angiogenic properties.

5.1. BMP2

Multiple *in vivo* studies have analyzed the affects of BMP2 and BMP4 on angiogenesis. BMP-2 induces blood vessel formation in tumors formed from A549 cells and enhanced angiogenesis in Matrigel plugs containing these cells [29]. Other study independently showed that BMP2 overexpression in MCF-7 breast cells also induced vessel formation in tumors, demonstrated that BMP2-induced vessel formation in the mouse sponge assay [30].

In vitro, BMP2 stimulates proliferation of human aortic endothelial cells (HAEC) [29] and pulmonary aortic endothelial cells (PAEC) [31]. BMP2 increases the migration of microvascular endothelial cells (HMEC) [32], and increases tube formation in human aortic endothelial cells (HAEC), human umbilical vein endothelial cells (HUVEC) [29] and HMECs [32].

5.2. BMP4

Over-expression of BMP4 in the paraxial or lateral plate mesoderm [33] and grafts of BMP-4 beads in the paraxial mesoderm of quail embryos induced ectopic vessel formation [34]. In addition, BMP4 stimulus induced angiogenesis in the chicken chorioallantoic membrane (CAM) assay [35].

BMP4 induced the proliferation of mouse embryonic stem cell-derived endothelial cells (MESEC) and HMECs [36]. BMP4 increased cell migration in quail embryonic endothelial cells (QEECs), HUVECs, [33], HMECs [32], and MESECs [36]. Bmp4 increased tube formation of HMEC [36] and mouse aortic endothelial cells (MAEC) [35].

5.3. BMP6, 7

BMP6 induced microvessel outgrowth in aortic rings [37], proliferation in mouse embryonic endothelial cells (MECs) [37], migration in bovine aortic endothelial cells (BAEC)

[38] and MECs [37], and tube formation in BAECs [38]. Similarly, BMP7 induced angiogenesis in the CAM assay [39].

5.4. GDF5

GDF5 increased vessel formation in the CAM assay and in the rabbit cornea assay and increased migration of BAECs [40].

5.5. BMP9,10

BMP9 inhibited vessel growth in the mouse sponge assay [41] and blocked new vessel growth in a metatarsal angiogenesis assay [42]. Bmp9 inhibited proliferation and migration of BAECs [42]. BMP9 and the closely related BMP10 inhibit the proliferation and migration of HMECs [28].

6. Receptors:

Similar to BMP ligands, both BMP Type I and Type II receptors are widely expressed in vertebrate embryos during development, and many of them modulate angiogenesis.

6.1. Type I receptors:

The majority of type I receptors of BMP signaling appear to have pro-angiogenic functions. Constitutively active forms of ALK2, ALK3 and ALK6 promoted endothelial cell migration and tube formation in BAECs [38].

Interestingly, the function of ALK1, another type I receptor, appears to be more complicated. Lesions in the zebrafish *alk1* gene caused an increase in endothelial cell number and dilated cranial vessels, indicating that ALK1 in this setting may inhibit certain aspects of angiogenesis [43]. Similarly, expression of constitutively active ALK1 (ALK1CA) inhibited endothelial sprouting from embryoid bodies [44], and inhibited proliferation and

migration of HMECs and HUVECs [45]. However, ALK1CA over-expression increased the cell migration of MEECs [46]. Given that ALK1 can associate with multiple TGF-β/BMP ligands and receptors, it is likely that different associations may account for complex ALK1 deficient phenotypes and inconsistencies between these studies.

6.2. Type II receptors:

In humans and mice, lack of BMPR2 caused aberrant growth of endothelial cells in pulmonary vessels and increased arterial pressure, symptoms frequently found in PAH. In zebrafish, two BMPR2 orthologs, Bmpr2a and Bmpr2b, have been identified. The morpholino-mediated knockdown of either of these genes inhibited angiogenesis [47].

6.3. Additional receptor regulation:

BMP receptor complexes can exist as preformed complexes (PFC), which become activated upon ligand binding. Alternatively, BMP receptor complexes can be induced by ligand binding to a single receptor, which recruits other subunits of BMP receptor complexes, forming a BMP induced signaling complex (BISC). While PFC is internalized by Clathrin mediated endocytosis and preferentially activate SMAD pathway, BISC is internalized by caveosome and activates the Mitogen Activated Protein Kinase (MAPK) pathway [48-50].

Bmp receptors also interact with a number of co-receptors ranging from GPI-anchored proteins to receptor tyrosine kinase (RTK) [51-55]. Co-receptors such as Dragon (a member of repulsive guidance molecules) and c-Kit (a well characterized RTK) can enhance Bmp signaling by physically binding to Bmp ligands and receptors simultaneously. Others receptors that signal through Bmp receptors include the RTKs TrkC and Ror2, and decoy receptor Bambi. Bambi can attenuate Bmp signaling by competing with Bmp type I receptor [56].

Unlike other TGF-β receptors, BMPRII has a long carboxy terminal tail, which is regulated independent of type I receptors. The BMPRII cytoplasmic tail has been implicated in regulating many processes by directly interacting with many factors including LIMK1, (a kinase which regulates of actin dynamics) [57], Tctex-1 (a light chain of dynein) [58], c-Src (a tyrosine kinase) [59], and Jiraiya (a membrane protein) [60] . Therefore, the carboy terminal of BMPRII may play a critical role in regulating angiogenesis independent of the heteromeric receptor complex.

7. Antagonists:

Several secreted proteins that function as antagonists of BMP signaling have been identified. Like BMP ligands, these proteins contain multiple Cysteine residues used to form disulfide bonds with BMP ligands, which in turn interfere with the ligand-receptor recognition [61]. Based on the number of the Cysteine residues and the size of the resulting Cystine-knot structure, BMP antagonists can be divided into three main subgroups. Members of differential screening-selected gene aberrative in neuroblastoma (DAN) family of BMP antagonists include Cerberus [62-63], Gremlin [64], and Sclerostin [65], and a have eight-membered ring Cystine-knot. Proteins related to Twisted gastrulation [66] have a nine-membered ring Cystine-knot, and Chordin [67]and Noggin [68] have a ten-membered ring Cystine-knot [61]. Members within each subgroup appear to be more phylogenetically related, suggesting that each subgroup of BMP antagonists is evolutionarily distinct [69]. While most of BMP antagonists function as homodimers rings [61], recently identified BMP antagonists, Sclerostin and related Uterine Sensitization Associated Gene-1 (USAG-1), function as monomers [70-71].

Bmp antagonists appear to play a pivotal function during angiogenesis. Recent studies have demonstrated that the area near the midline of embryos remains avascular since the nearby notochord inhibits the migration of endothelial cells by secreting two main

BMP antagonists, Noggin and Chordin [72-73]. Furthermore, Noggin and Chordin also appear to be critical for regulating the fusion of the developing dorsal aorta in avians [74].

One BMP antagonist, Crossveinless-2 (Cvl2), also known as Bmper [75-76], is selectively express in an endothelial specific manner during development [75]. Interestingly, unlike the majority of BMP antagonists, Cvl2 can function as a BMP agonist and/or antagonist in a context dependent manner [77-79]. A lower concentration of Cvl2 can facilitate phosphorylation of SMAD-1/5/8, while a higher concentration attenuates the efficacy of BMP signaling[80]. Accordingly, in developing vessels, lower concentration of Cvl2 activates sprouting and angiogenesis, while higher concentration inhibits this process [78]. By investigating the effects of Cvl2 on BMP4 signaling, Kelley and colleagues have shown that Clv2 can directly bind to BMP4 and can interfere with its interaction with BMP Type II receptor. This leads to decreased Clathrin-mediated internalization of BMP ligand-receptor complexes [78]

8. Downstream signaling cascade and cross-talk with other pathways:

Binding to BMP ligand to heteromeric BMP receptor complexes trigger activation of diverse downstream signaling cascades, resulting in transcriptional activation of target genes. For instance, BMP6 transcriptionally activated ID1 through the SMAD signaling cascade. Over-expression of ID1 induced EC migration and tube formation in BAECs, mimicking the affects of BMP6 [38]. Microarray analysis found that treating MECs with BMP6 caused a transcriptional increase in Cox2 (a gene that catalyzes the conversion of Arachidonic acid to Prostaglandins) and MyoX (an atypical myosin critical for filopodial formation). Cox2 mediated BMP6-induced proliferation, migration, and network assembly of MECs as well as microvessel outgrowth in aortic rings [37]. In addition, MyoX induction through BMP6 is necessary for filopodial formation, cell alignment, directed migration, and tube formation in MECs [81].

BMP signaling also stimulates MAPK pathways such as, ERK, JNK, p38 [82]. BMP4-induced HUVEC sprouting is dependent on ERK [83]. In addition, small molecule inhibitors demonstrated that Bmp2-mediated angiogenesis in zebrafish requires both Smad and Erk activation (Fig. 1.2)

Furthermore, the Wnt pathway appears to interact with BMP signaling. Active Wnt signaling inhibits GSK3 which allows the accumulation of β-catenin and downstream gene activation. MAPK and GSK3 phosphorylate the SMAD1 linker region leading to the polyubiquitinylation and degradation of SMAD1 [84]. BMP2 signaling through BMPRII inhibited GSK3-β, which lead to increased PAEC survival and proliferation. In addition, BMP2 signaling recruited Disheveled (a noncononical Wnt receptor) which promoted RhoA–Rac1 signaling and PAEC motility [31].

BMP signaling also cooperates with VEGF-A signaling. The over-expression of BMP2, BMP4, BMP6, and BMP7 increased VEGF-A transcription in various cell types [85-87]. VEGF-A neutralizing antibodies inhibited the BMP mediated angiogenic responses in preosteoblast-like cells and in fetal metatarsal assay [85]. However, *bmp2b* over-expression in zebrafish induced a robust angiogenic response that is not affected by morpholino inhibition of Vegf-A signaling [Aramaki, #57]. Furthermore, the interactions between BMP and VEGF signaling appear to go beyond transcriptional regulation. Dorsomorphin is a small molecule inhibitor that was first reported to be a selective inhibitor of BMP signaling by inhibiting BMP type I receptors and was later shown to also be a potent inhibitor of VEGF signaling by inhibiting the VEGFR2 function [88] . The ability of dorsomorphin to interact and inhibit both type I BMP receptors and VEGF receptors suggests that they might share structural similarities.

9. Arterial and venous differences in BMP signaling:

Both human vascular diseases associated with BMP signaling, HHT and PAH, affect a distinct subset of endothelial cells. PAH selectively affects the arteries connecting the lungs to the heart. The HHT pathology causes aberrant vascular growths that fail to form proper arterial and venous connections (AVMs) in the skin, digestive tract, lungs, liver and brain. ALK1 linked to both of these vascular disorders [6, 14]. Interestingly, ALK1 is selectively expressed in arterial endothelial cells during murine development and is highly expressed in the lung endothelium of adults [89]

During mammalian eye development the the pupillary membrane regresses and capillaries in this tissue undergo apoptosis (programmed capillary regression) [90]. BMP4 induces endothelial cell apoptosis during programmed capillary regression in rats [91]. While capillary and venous endothelial cells were responsive to BMP4-induced apoptosis, arterial endothelial cells were resistant to BMP4-induced apoptosis. This differential responsiveness to BMP4-mediated apoptosis was shown to be caused by the increased arterial expression of inhibitory SMADs (SMAD6 and SMAD7) [91]. Arterial endothelial cells experience higher levels of shear stress than venous endothelial cells. Fluid mechanical stimulation of cultured endothelial cells induces I-SMAD expression [92], suggesting that shear stress may account some BMP-responsive differences between arterial and venous endothelial cells.

BMP signaling also differentially regulated arterial and venous angiogenesis during zebrafish development. The vascular network of early zebrafish embryos contains a dorsal aorta and an axial vein which extend angiogenic sprouts [93-94]. This simple vascular network is useful system for studying the arterial and venous differences during vertebrate angiogenesis. Conditional over-expression of *noggin3* and morpholino inhibition of *bmpr2a* or *bmpr2b* selectively inhibited venous angiogenesis, while the overexpression of *bmp2b* induced ectopic sprouts from the axial vein but not the axial artery (Fig. 1.3) [47]. Analogs of dorsomorphin, DMH1 and DMH4, were created which selectively inhibited ALK2, ALK3 or

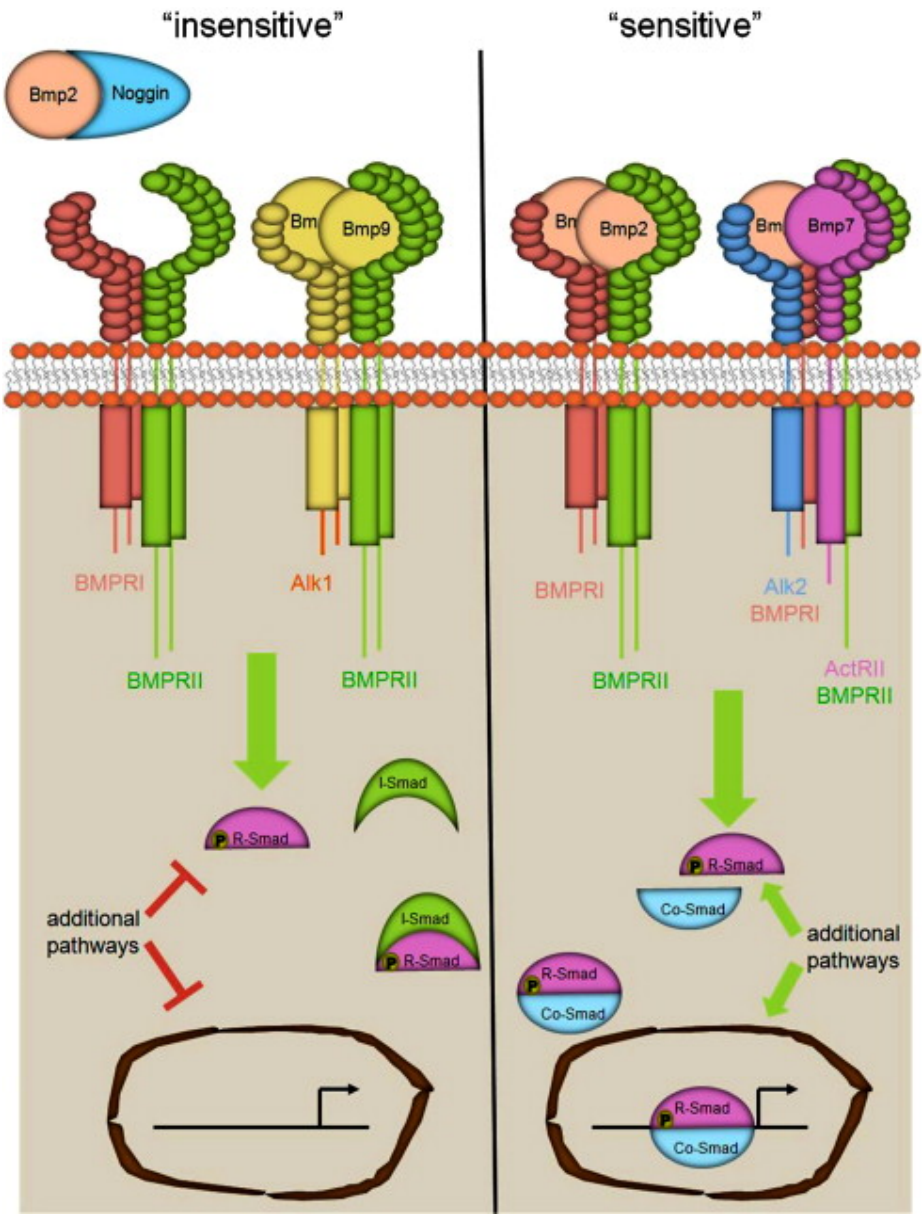
VEGFR2 function, respectively [88]. In accordance, DMH1 (a BMP inhibitor) selectively inhibited venous angiogenesis while DMH4 (a VEGF inhibitor) selectively inhibited arterial angiogenesis in zebrafish [47] . Most interestingly, BMP signaling can promote angiogenesis independent of VEGF-A signaling in zebrafish, and elicit distinct angiogenic responses. Collectively, these findings support a model in which VEGF-A and BMP signaling promotes angiogenesis from venous and arterial endothelial cells respectively (Fig. 1.4) [47]. The factor(s) which make(s) the axial vein responsive and the dorsal aorta refractory to BMP stimulus during early zebrafish development remain to be identified. Discovering these factors will help elucidate the function and relevance of BMP signaling during mammalian angiogenesis.

10. Concluding Remarks:

As discussed above, both pro-angiogenic and anti-angiogenic functions of BMP signaling have been reported. Considering numerous pathway components, dynamic expression pattern, as well as potential redundancies of BMP signaling, it is not surprising that BMP signaling can elicit diverse responses from endothelial cells. Therefore, it is essential to identify factors confers the context dependent pro- and/or anti-angiogneic effects of BMP signaling and delineate cellular and molecular mechanisms that mediate BMP signaling within endothelial cells to develop a consensus model for the role of BMP signaling in endothelial cells.

FIGURES

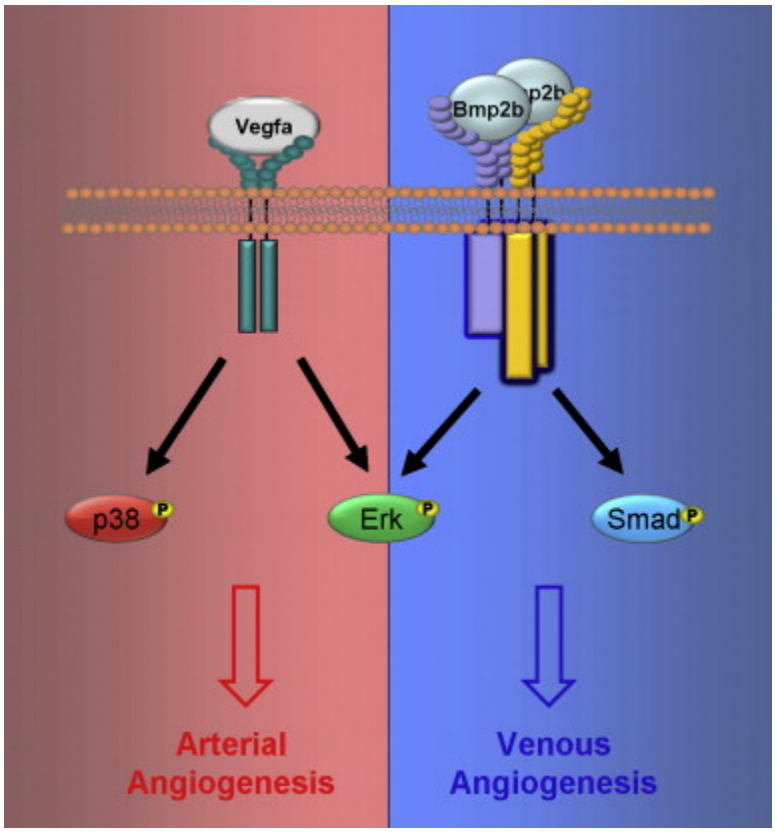
Figure. 1.1. Methods of regulating BMP responsiveness.



Extracellular antagonists bind to BMP ligands and prevent the ligands from interacting with receptors. BMP9 BMP10 bind to non-angiogenic heteromeric receptor complexes consisting of BMPRII and ALK1, which may limit the availability of angiogenic ligand-receptor complexes. In contrast, when angiogenic ligand-receptor complexes are formed, and inhibitory BMP ligands are absent, co-SMAD is translocated into the nucleus and

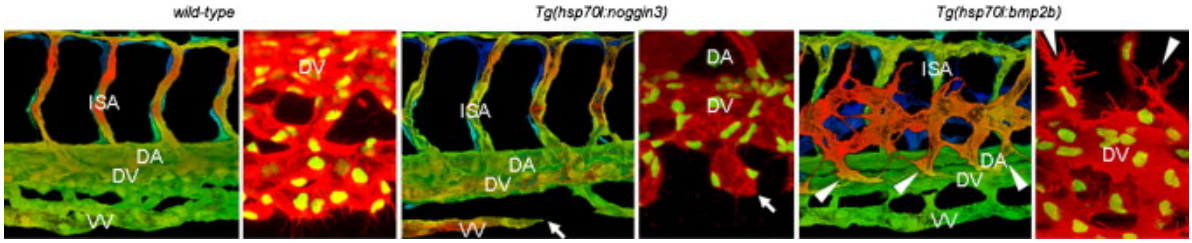
promotes the transcription of BMP target genes within endothelial cells. Alternative signaling pathways may also have important roles in regulating the intracelluar responses to BMP stimulus.

Figure 1.2. VEGFA and BMP pathways have distinct as well as overlapping intracellular targets.



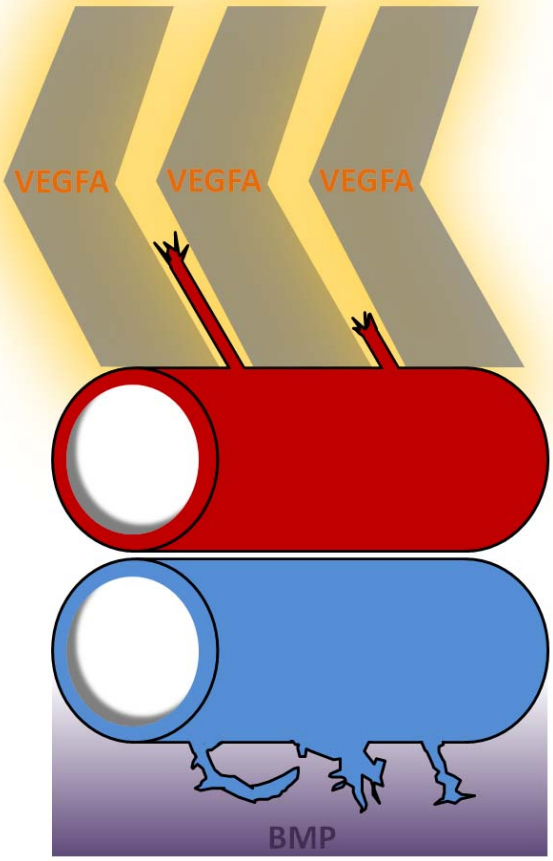
The VEGFA pathway is known to phosphorylate and activate, among other factors, MAPK signaling cascades, which includes both p38 and ERK. Canonical BMP signaling activates Smads however BMP stimulation has been shown to effectively activate MAPK signaling cascades as well. During angiogenesis, BMP requires activation of ERK and not p38 [60].

Figure 1.3. Bmp signaling is necessary and sufficient for sprouting from the axial vein.



Blood vessels in wild-type (A and B), *Tg(hsp70:noggin3)* (C and D)and *Tg(hsp70:bmp2b)* (E and F) embryos in the *Tg(kdrl:GFP)* transgenic background (A, C and E). 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 (A, C, and E), Filopodia formation of *Tg(fli1:nGFP);Tg(kdrl:ras-mCherry)* embryos starting at 32hpf (B, D, and F). Arrows in panels c and d show sprouts from the axial vein that fail to make connections in *Tg(hsp70:noggin3)* embryos. Arrowheads in panel e and f point to ectopic sprouts that branch from the axial vein in *Tg(hsp70:bmp2b)* embryos. Abbreviations: DA, dorsal aorta; VV, ventral vein; DV, dorsal vein; NC, notocord; NT, neural tube; ISA, intersegmental artery.

Figure 1.4. Distinct angiogenic cues regulate arterial and venous angiogenesis during early zebrafish development.



VEGFA regulates sprouting from the Dorsal Aorta, while BMP signaling is the predominate angiogenic cue during venous angiogenesis off the Axial Vein. VEGFA expressed dorsally within somites attracts sprouts from the Dorsal Aorta. Meanwhile, ventral expression of BMP regulates sprouting from the axial vein.

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CHAPTER 2

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

This chapter is adapted from a manuscript published into Nature Cell Biology in 2011. I designed the experiments with the intellectual contribution of Dr. Suk-Won Jin and Dr. Victoria Bautch. I carried out the experiments. Jun-Dae Kim did the in situ hybridization for Fig. 2.9b. Jijun Hao and Dr. Charles C. Hong provided key reagents for Fig. 2.13. I created the figures. I wrote the original manuscript which was revised multiple times by Dr. Suk-Won Jin and Dr. Victoria Bautch.

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.

RESULTS AND DISCUSSION

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 (ISAs) [1] (arrows, Fig. 2.6a), while those from the posterior AV extend ventrally (arrowheads, Fig. 2.6a) to form a honeycomb-like network termed the caudal vein plexus (CVP), which is composed of a dorsal and ventral vein with interconnecting vessels (Fig. 2.1a). 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 failed to segregate [4] (Fig 2.1a), endothelial cell apoptosis was significantly increased [5] (Fig. 2.6b), and ISA sprouts were blocked [5] (Fig. 2.1a). 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. 2.1b) (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. 2.1 and Fig. 2.6c). 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. 2.1c and Fig. 2.9). 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. 2.1c and Fig. 2.6d).

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 (Fig. 2.7a). We next analyzed the effects of decreased Bmp activity on sprouting of 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. 2.2a). 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. 2.2a, Fig. 2.8a). 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 (Fig. 2.7b). Since CVP patterning was perturbed while ISA patterning was largely unaffected, these results suggest 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. 2.2a, Fig. 2.8b). 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, Fig. 2.9a). The Bmp-induced plexus highly expressed a venous marker, *dab2*, indicating that their venous identity (Fig. 2.9b). Although Bmp over-expression induced robust sprouting from the AV, ectopic sprouts were never observed from the DA (Fig. 2.2a). 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, Fig. 2.9c). The SIVP in *bmp2b* over-expressing embryos was shifted dorsally (arrows, Fig. 2.9c) and contained ectopic vessels (arrowhead, Fig. 2.9c), 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. 2.2b). However, *Tg(hsp70l:noggin3)* embryos contained atypical angiogenic sprouts that failed to make connections and never formed a proper plexus (Fig. 2.2b). 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. 2.2b).

To better characterize the *noggin3* and *bmp2b* over-expression phenotypes, we counted venous endothelial nuclei and performed venous 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 *bmp2b* over-expressing embryos, we

observed a slight but significant increase in endothelial cell numbers (Fig. 2.8c). Venous branch points, however, were significantly altered in *noggin3* or *bmp2b* over-expressing embryos (Fig. 2.8d). We found that the number of branch points was decreased more than 7 fold by *noggin3* over-expression, while increased approximately 2.5 fold by *bmp2b* over-expression (Fig. 2.8d). In addition, *bmp2b* over-expression caused a significant increase in the number of filopodia (Fig. 2.9d-e), and randomized their direction (Fig. 2.9f). 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. 2.3a-d and Fig. 2.10). 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. 2.3a-b and Fig. 2.10c). Moreover, knock-down of *bmpr2a* or *bmpr2b* in *bmp2b* over-expressing embryos inhibited formation of ectopic sprouts (Fig. 2.3c-d and Fig. 2.10d). 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. 2.3e). In contrast, the *DNBmprI-GFP*-expressing cells were unable to extend sprouts from the CV, and they failed to connect with neighboring endothelial cells to form a honeycomb-like plexus (Fig. 2.3f).

The segments that contained *DNBmprI-GFP*-expressing cells had fewer branch points than *GFP* expressing control cells (Fig. 2.3g), indicating that Bmp signaling within

endothelial cells is important during branching morphogenesis. In addition, the frequency in which the branches connect to form a plexus was significantly reduced in *DNBmprI-GFP*-expressing cells, suggesting that Bmp signaling within the endothelial cells is critical for the formation of endothelial networks (Fig. 2.3h). 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]. 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 (Fig. 2.11a-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 [12]. 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 the formation of the CVP without affecting ISAs and inhibition of Erk activity blocked the formation of both the CVP and ISAs (Fig. 2.4a-b). Moreover, inhibiting R-Smad or Erk activation in Bmp over-expressing embryos efficiently inhibited the percentage of segments with ectopic vessels, while inhibiting p38 had no effect on the percent of segments with ectopic vessels (Fig. 2.4c-d). Interestingly, the Erk inhibitor also drastically attenuated the length and progression of the ectopic sprouts (Fig. 2.4e). 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)* were compared to WT at 2 and 5 hours post heat-shock induction of *bmp2b*. *id2a*, a downstream transcription target of Bmp signaling, was used as a positive control. *vegfa*, *vegfc* (also a major stimulus of lymphangiogenesis[13]), *vegfr3*/*flt4* (a venous marker and receptor for Vegf-C), and *dll4* (an arterial marker and tip cell marker[13-14]) were also tested. Bmp over-expression upregulated *id2a* by over three fold at 2 hours post heat-shock while *vegfa*, *vegfc*, *flt4*, and *dll4* were either marginally affected or not affected at all (Fig. 2.12). In addition *vegfa*, *vegfc*, *flt4*, and *dll4* transcript levels were unaffected 5 hours post heat-shock induction of *bmp2b* (Fig. 2.12).

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 axial vessel at 2dpf (Fig. 2.5a). 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. 2.5a-b). We next analyzed the effects of Bmp and Vegf-A small molecule inhibitors during sprouting angiogenesis of the axial vessels12. 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 (Fig. 2.13). 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 (Fig. 2.13). 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, it suggests 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 *vegfa*$_{121}$ by heat-shock treatment of *Tg(hsp70l:bmp2b)* or *Tg(hsp70l:vegfa*$_{121}$*)* transgenic lines, respectively. As expected, *bmp2b* over-expression induced robust ectopic sprouts along the AV, but not from the DA (Fig. 2.5c). In contrast, *vegfa*$_{121}$ over-expression did not induce ectopic sprouts from the AV, but increased sprouting along the DA was observed (Fig. 2.5c). The distinct angiogenic responses between *bmp2b* over-expressing embryos and *vegfa*$_{121}$ 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. 2.5d). 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 [15-16]. Collectively our results suggest a model of Bmp mediated angiogenesis in which Bmp2b binds Bmpr2a/b and Alk2/Alk3 hetero-tetrameric receptor complex in venous endothelial cells and activates R-Smad and Erk, which elicits various angiogenic responses, including sprout migration and fusion (Fig. 2.5e).

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[17-20]. In addition, several types of carcinomas express high levels of BMP growth factors [21], and anti-angiogenesis cancer drugs that singularly antagonize VEGF-A activity are only partially effective [22]. Therefore, future studies that target both Bmp and Vegf-A signaling may be more successful at manipulating blood vessel growth

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METHODS

Zebrafish husbandry

Zebrafish (*Danio rerio*) embryos were raised as previously described[27]. The following transgenic lines were used: *Tg(fli1:nEGFP)^{y7}* [23], *Tg(kdrl:GFP)^{s843}* [24], *Tg(kdrl:ras-mCherry)^{s896}* [25], *Tg(hsp70l:bmp2b)^{fr13}* [26], *Tg(hsp70l:noggin)^{fr13}* [26], *Tg(hsp70l:dnbmprI-GFP)^{w30}* [27], 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 [24]. 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 CV (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 [28]. 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

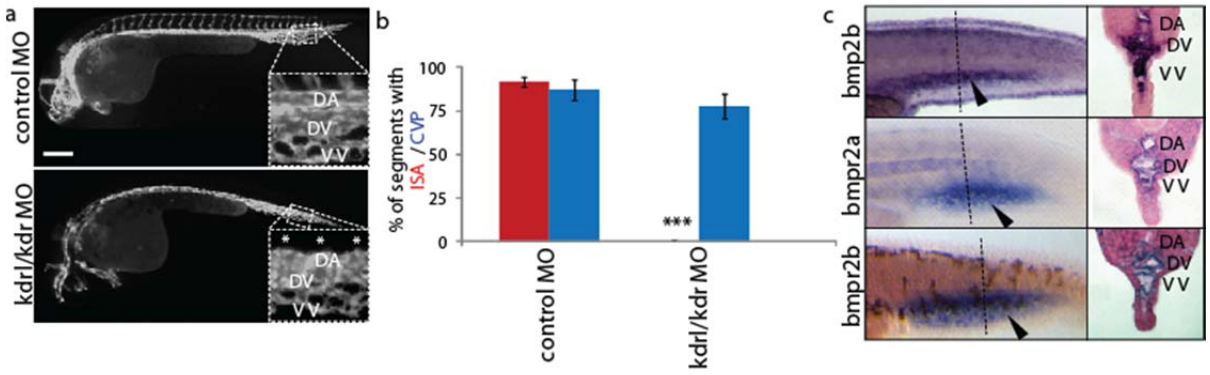
The *vegfa* gene was amplified from cDNA of 32hpf embryos. The PCR product was ligated into the pCR8 vector (Invitrogen). The *vegfa* gene was sequenced and found to be the *vegfaa*$_{121}$ splicing isoform. The gateway tol2 kit [29] was used to create the *hsp70l:vegfaa*$_{121}$ construct, which was 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.

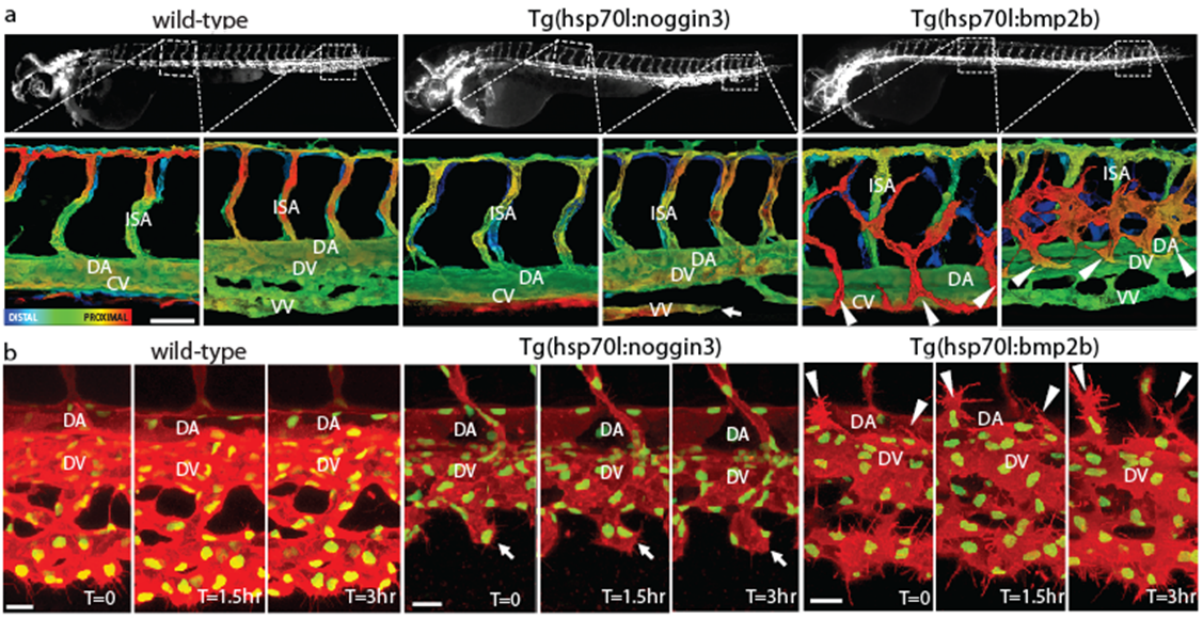
FIGURES

Figure 2.1. The CV forms angiogenic sprouts despite loss of Vegf receptor activity, and expresses Bmp pathway components.



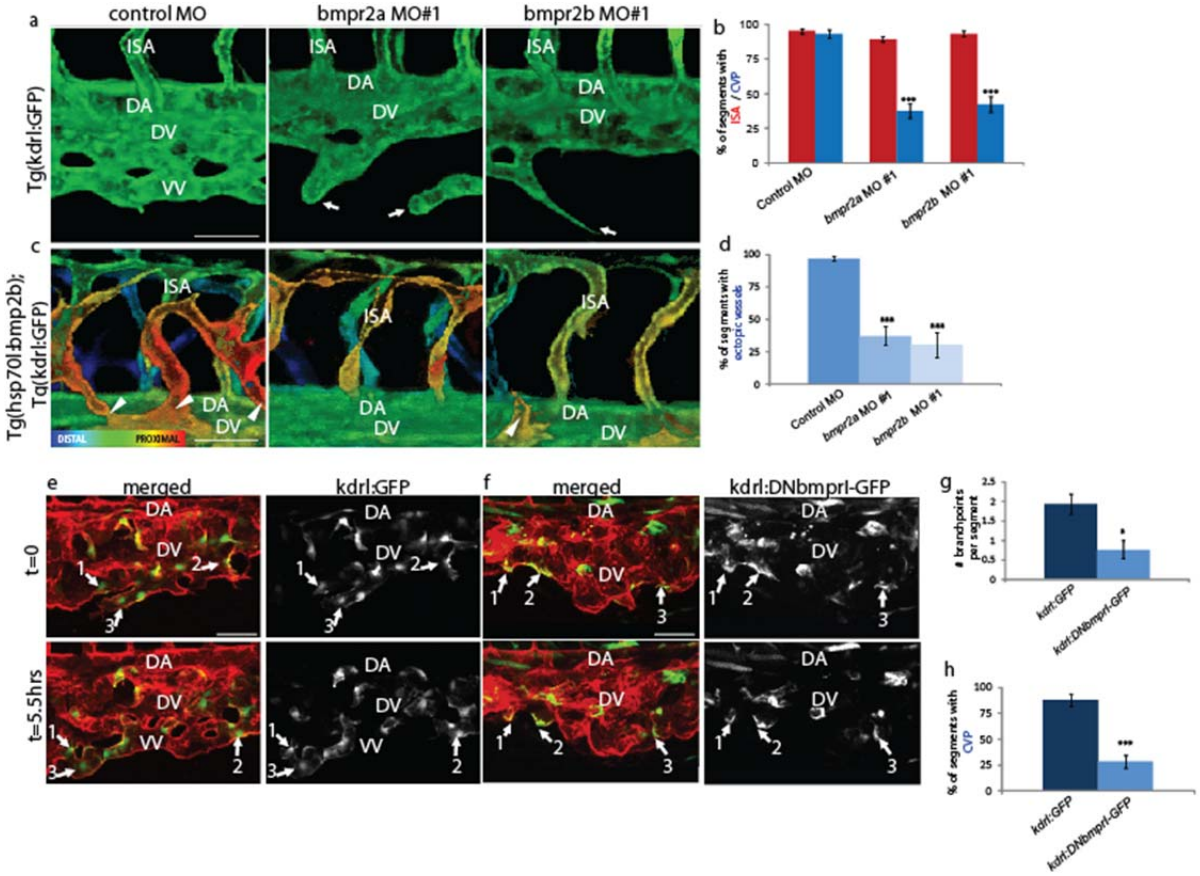
(a) Epiflourescent images of 34hpf *Tg(kdrl:GFP)* control and *kdrl/kdr* MO injected embryos; insets show higher magnification of the CVP region. Asterisks denote the lack of intersegmental arteries in *kdrl/kdr* MO injected embryos. Scale bar, 250μm. (b) The percentage of segments that contain an ISA (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 2.2. Bmp signaling is necessary and sufficient for sprouting from the CV.



(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 CV that fail to make connections in *Tg(hsp70:noggin3)* embryos. Arrowheads in panel a and b point to ectopic sprouts that branch from the CV 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.

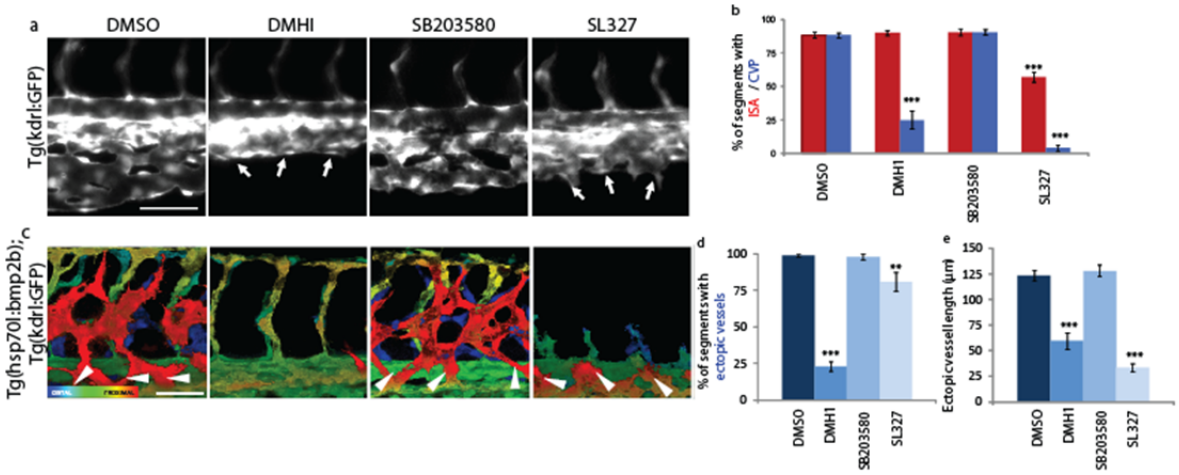
Figure 2.3. Angiogenesis from the CV requires *bmpr2a* and *bmpr2b* and involves endothelial cell autonomous activation of Bmp signaling.



(a) Confocal monochrome projections of *Tg(kdrl:GFP)* embryos injected with a standard control, *bmpr2a*, or *bmpr2b* MO. The sprouts from the CV 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

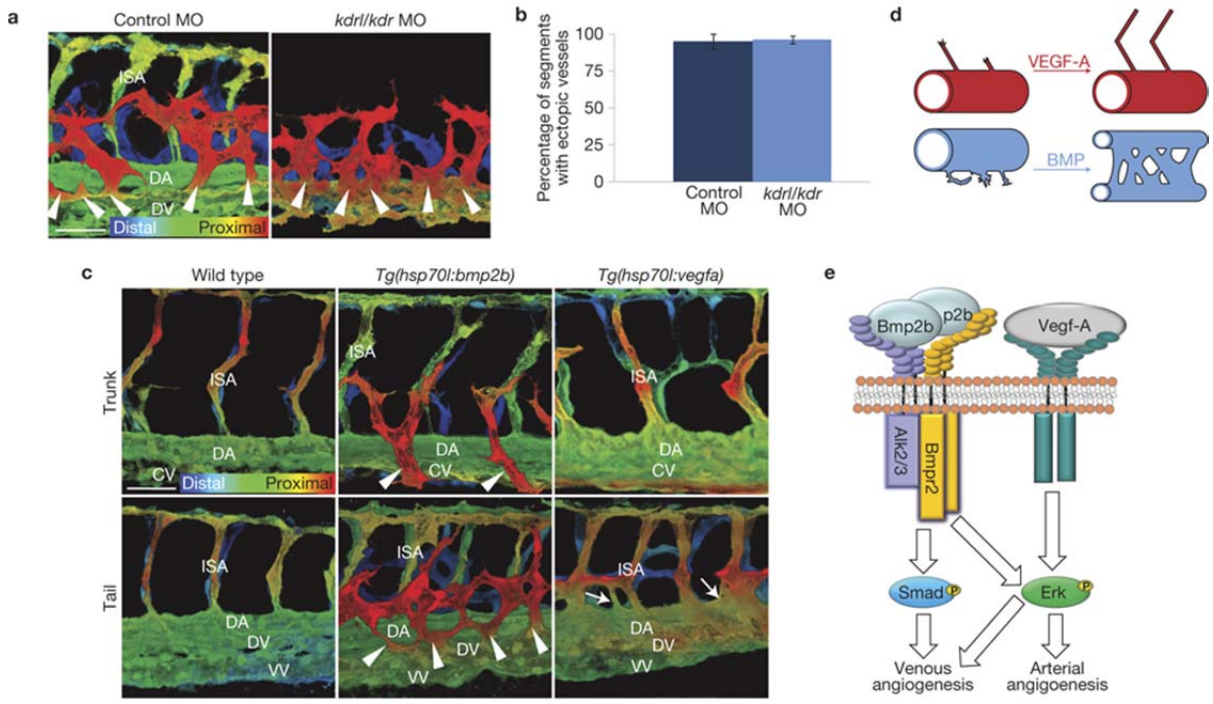
images of *Tg(kdrl:GFP)* (e) and *Tg(kdrl:DNBmpr1-GFP)* (f) mosaic embryos in a *Tg(kdrl:ras-mCherry)* background. Numbered arrows indicate mosaic endothelial cells. (g) The number of branch points and (h) the percent of segments containing a CVP were quantified in mosaic segment containing *GFP* or *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 2.4. 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 CV. (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.

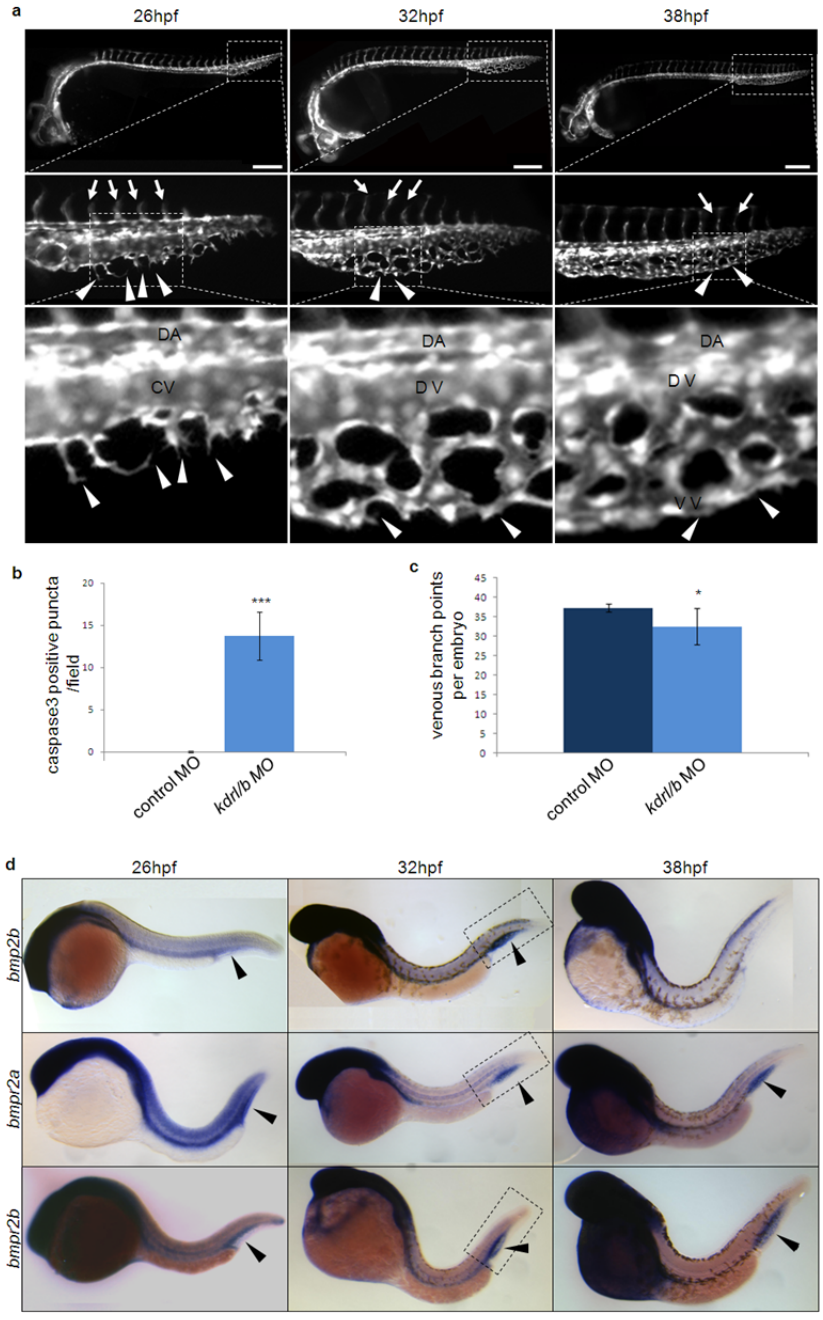
Figure 2.5. Bmp signaling regulates CV angiogenesis independent of Vegf receptor activity.



(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 CV 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.

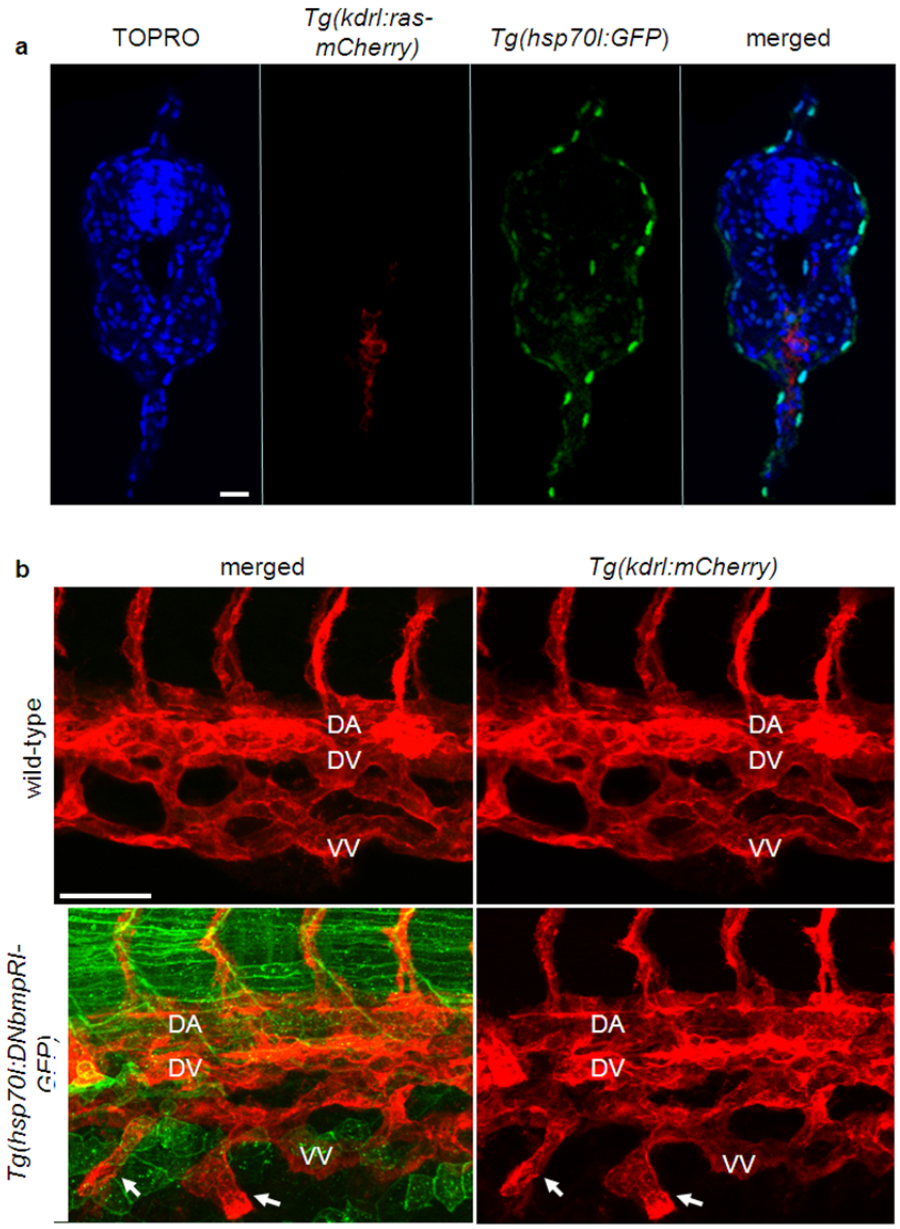
Figure 2.6. Vegf-A signal is dispensable for the formation of Caudal Vein Plexus (CVP), and endothelial cells within the angiogenic region of the axial vein highly express Bmp pathway components.



(a) Epiflourescent micrographs of *Tg(kdrl:GFP)* embryos at 26hpf, 32hpf, and 38hpf. Areas within dashed rectangles are shown with higher resolution in the panel below. Endothelial cell sprouts from the dorsal aorta form the ISAs (arrows). Angiogenic extensions sprout from

the caudal vein (CV) at 26hpf and establish connections with neighboring sprouts by 32hpf (arrowheads). These endothelial cell connections are stabilized leading to the formation of a mature CV plexus by 38hpf (arrowheads). The CV plexus is a fenestrated network composed of a dorsal (DV) and ventral vein (VV) with interconnecting vessels. Scale bar, 250μm. Abbreviations: DA, dorsal aorta; VV, ventral vein; DV, dorsal vein. (**b**) *Tg(kdrl:GFP)* embryos were injected with either control or *kdrl/b* MOs and stained for cleaved-Caspase3 at 34hpf. A 40x objective was to analyze endothelial cells in the tail. The number of cleaved-caspase3 positive endothelial cell puncta per field of view was counted and quantified in control (n=5) and *kdrl/b* (n=4) MO injected embryos. No cleaved-Caspase3 positive puncta in endothelial cells were observed in the control injected embryos, while *kdrl/b* morphant embryos contained on average 13.75 cleaved-Caspase3 positive endothelial cells per field. Error bars represent mean ± SEM. ***P<0.001 versus control, Student's *t* test. (**c**) *Tg(kdrl:GFP)* embryos were injected with either control or *kdrl/b* MOs and the number of venous branch points were counted in control (n=5) and *kdrl/b* (n=7) MO injected embryos. The number of venous branch points in *kdrl/b* MO injected embryos was marginally decreased compared to control MO injected embryos (37.2 in control vs 32.4 in *kdrl/kdrb* MO injected embryos). Error bars represent mean ± SEM. *P<0.05 versus control, Student's *t* test. (**d**) Micrographs are of embryos at 26hpf, 32hpf, and 38hpf after *in situ* hybridization with *bmp2b*, *bmpr2a*, or *bmpr2b*. Bmp signaling components show high reactivity within the developing CV plexus (black arrow heads). Dashed boxes indicate the areas magnified in Fig. 1.

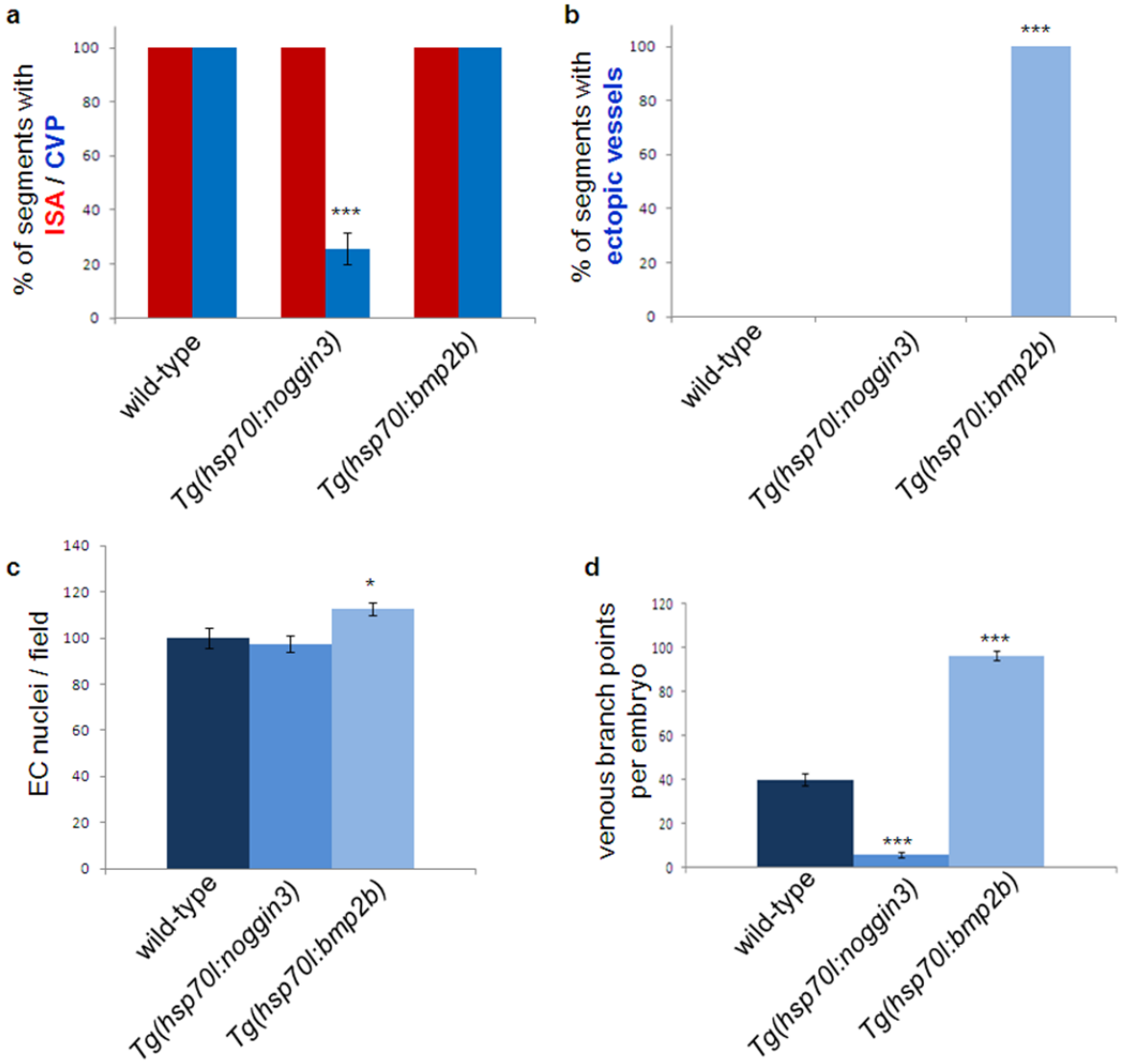
Figure 2.7. Activation of the *hsp70l* promoter drives quasi-ubiquitous expression of GFP in *Tg(hsp70l:GFP)* embryos, and effectively blocks CVP formation in *Tg(hsp70l:DNbmprI-GFP)* embryos.



(a) *Tg(hsp70l:GFP)* embryos were heat-shocked and sectioned at 42hpf. Merge image indicates that GFP is expressed in the majority of non-neural cells. (b) Wild-type and *Tg(hsp70l:DNbmprI-GFP)* embryos were heat-shocked at 23hpf for 30minutes at 42°C. Wild-type embryos do not express DNbmprI-GFP and contain a properly formed CV plexus.

Tg(hsp70l:DNbmprI-GFP) embryos express DNbmprI-GFP following heat-shock and contain CV plexus defects (arrows). Scale bar, 50μm. Abbreviations: DA, dorsal aorta; VV, ventral vein; DV, dorsal vein.

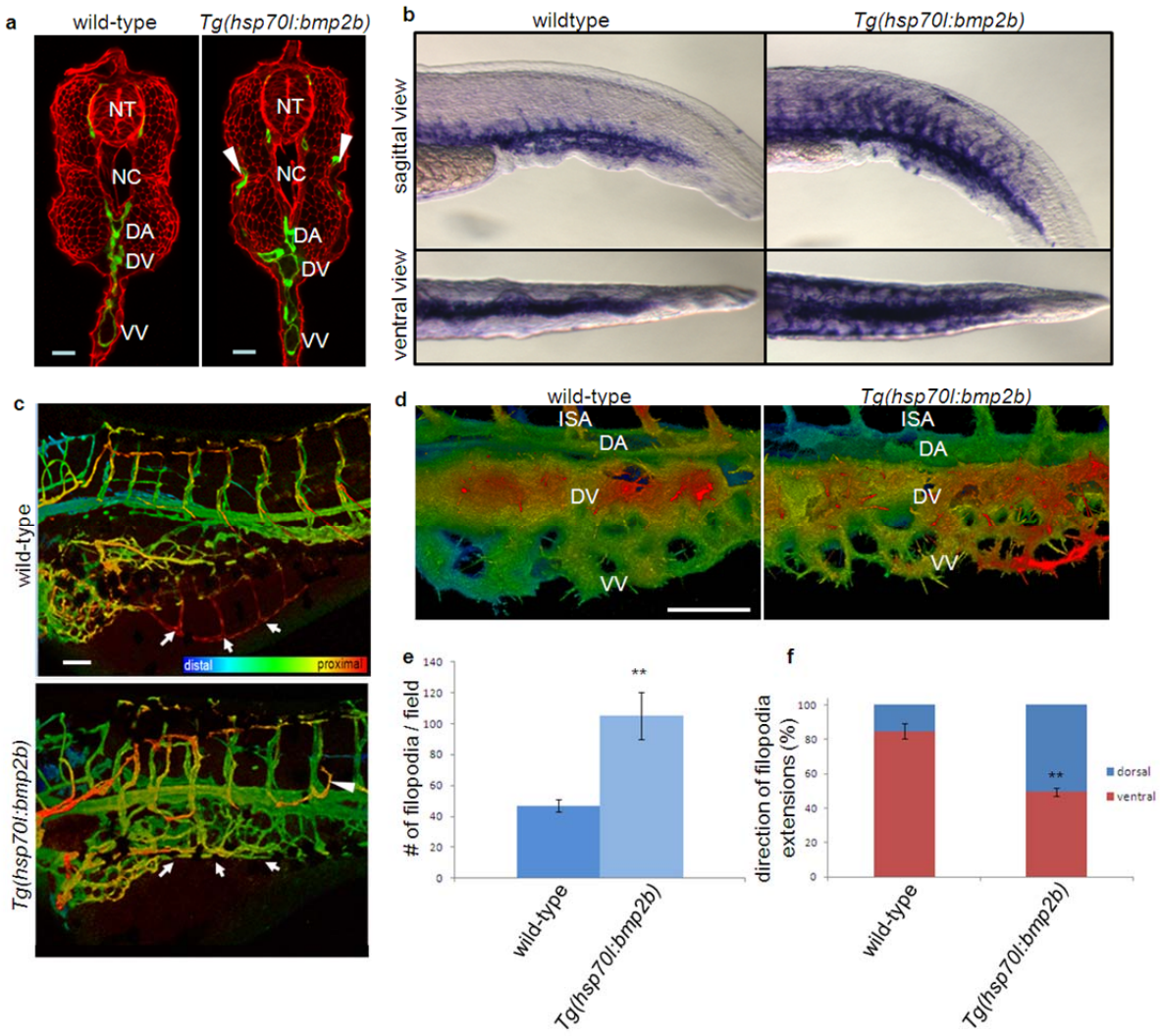
Figure 2.8. Bmp signaling regulates venous plexus formation by affecting endothelial cell number and venous branching.



(a) The percentage of segments that contain an ISA (red bars) or a CV plexus (blue bars) was quantified in wild-type (n=6), *Tg(hsp70l:noggin3)* (n=6), and *Tg(hsp70l:bmp2b)* (n=6) embryos. Over-expression of *noggin3* blocked the formation of veins but not the arteries. (**b**) The percentage of segments containing ectopic vessels was quantified in wild-type (n=6), *Tg(hsp70l:noggin3)* (n=6), and *Tg(hsp70l:bmp2b)* (n=6). *bmp2b* over-expression causes robust ectopic vessel formation. Error bars represent mean ± SEM. ***P<0.001 versus control, Student's *t* test. (**c**) The number of endothelial cells in the CV plexus region of

Tg(fli1:nEGFP) embryos with wild-type (n=6), *Tg(hsp70l:noggin3)* (n=6), *Tg(hsp70l:bmp2b)* (n=6) background was quantified. The number of endothelial cell nuclei per field of view is displayed. The average number of endothelial cells in *noggin3* over-expressing embryos was not significantly decreased, but the average number of endothelial cells in *bmp2b* over-expressing embryos was increased by 12.5%. (**d**) Branch point analysis of venous networks demonstrated that *noggin3* over-expressing embryos exhibited decreased branching, while *bmp2b* over-expressing embryos exhibited increased venous branching (n=3 for wild-type, n=4 for *Tg(hsp70l:noggin3)*, and n=4 for *Tg(hsp70l:bmp2b)*). Error bars represent mean ± SEM. *P<0.05 and ***P<0.001 versus control, Student's *t* test.

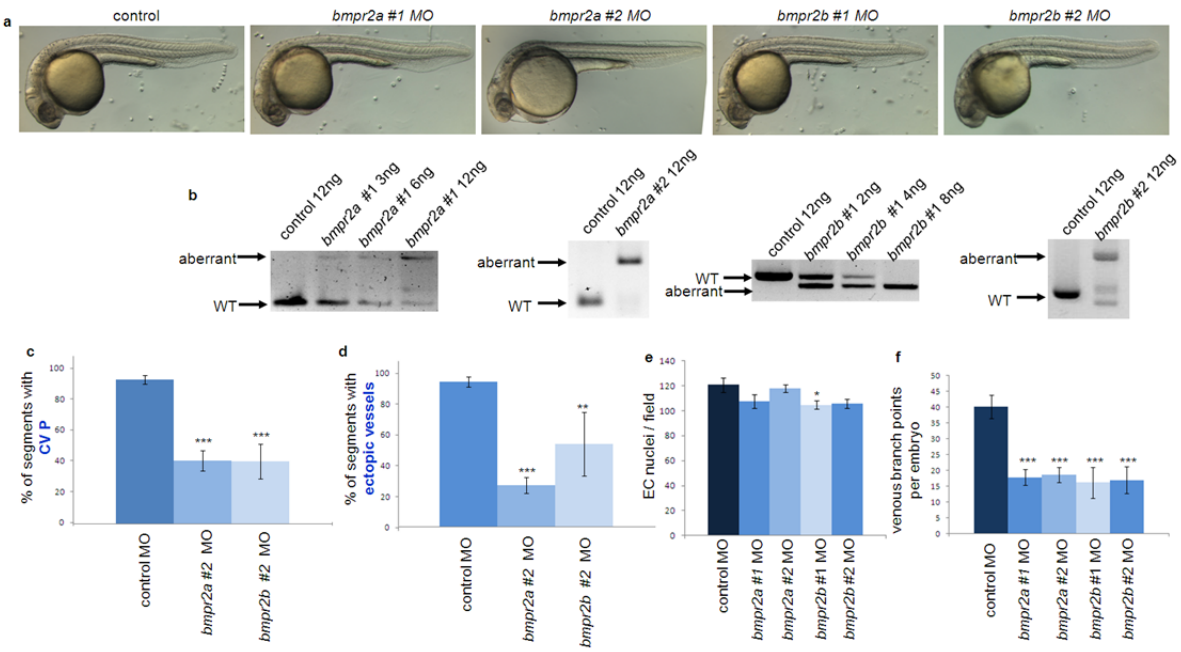
Figure 2.9. Bmp signaling promotes angiogenesis from venous endothelial cells.



(**a**) Wild-type and *Tg(hsp70:bmp2b)* embryos were cross sectioned at 48hpf and stained with β-tubulin (red) to outline cells. Ectopic vessels in *Tg(hsp70:bmp2b)* embryos formed between the epithelial surface and the somite boundary (arrows). Scale bars, 20μm. Abbreviations: DA, dorsal aorta;VV, ventral vein; DV, dorsal vein; NC, notocord; NT, neural tube. (**b**) Wild-type and *Tg(hsp70:bmp2b)* embryos were heat-shocked and subsequently fixed at 30 hpf. A marker of venous endothelium, *dab2*, was strongly expressed in the ectopic vessels that emanated from the axial vein in *Tg(hsp70:bmp2b)* embryos. (**c**) Representative images of the subintestinal vein (SIV) plexuses of 84hpf *Tg(kdrl:GFP)* and *Tg(hsp70l:bmp2b); Tg(kdrl:GFP)* embryos that were heat-shocked at 60hpf. Confocal Z-

stacks were converted into heat-map projections and scale bars represent proximity of vessels. The SIV plexus in *Tg(kdrl:GFP)* embryos contains stereotypical ventral projections (arrows)*; bmp2* over-expression shifted SIV vessels dorsally (arrows) and induced ectopic sprouts (arrowheads). Scale bar, 50μm. (**d**) Wild-type and *Tg(hsp70l:bmp2b)* embryos were analyzed 4 hours post heat-shock. Filopodia were imaged in the *Tg(kdrl:ras-mCherry)* transgenic background, and Z-stacks were assembled in a heat map as descrive in previous legends. (**e**) The number of filopodia was quantified. *bmp2b* over-expressing embryos contained more filopodia per field compared to control. (**f**) The angle of the filopodia projections relative to the dorsal aorta was analyzed. While the majority of wild-type filopodia extended in the ventral direction (84.8 percent), filopodia in *bmp2b* over-expressing embryos was randomized. wild-type, n=4; *Tg(hsp70l:bmp2b)* n=4 embryos. Error bars represent mean ± SEM. **P<0.01 versus control, Student's *t* test. Abbreviations: DA, dorsal aorta; ISA, intersegmental artery; VV, ventral vein; DV, dorsal vein.

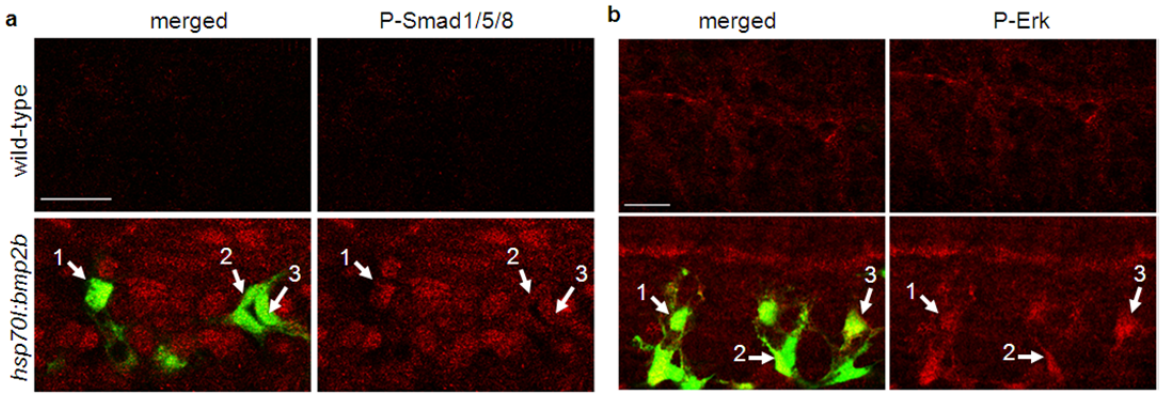
Figure 2.10. Both *bmpr2a* and *bmpr2b* are necessary for venous angiogenesis.



(**a**) Brightfield images of 32hpf embryos injected with control, *bmpr2a #1*, *bmpr2a #2, bmpr2b* #1, or *bmpr2b* #2 MO. (**b**) PCR analyses from morphant cDNA demonstrate the efficiency of each splicing MO. (**c**) WT embryos or (**d**) *Tg(hsp70l:bmp2b)* embryos were injected with *bmpr2a* #2 and *bmpr2b* #2 splicing MOs. (**c**) The percentage of segments that contain a CV plexus was quantified in control (n=48), *bmpr2a* #2 (n=40), and *bmpr2b* #2 (n=20) MO injected embryos. (**d**) The percentage of segments that contain an ectopic vessel was quantified in control (n=32), *bmpr2a* #2 (n=38), and *bmpr2b* #2 MO (n=15) injected embryos. *bmpr2a* #2 and *bmpr2b* #2 splicing MOs inhibited the formation of the CV plexus as well as the ectopic vessels. Error bars represent mean ± SEM. **P<0.01 and ***P<0.001 versus control, Student's *t* test. (**e**) The number of endothelial cells in the CV plexus region of *Tg(fli1:nEGFP)* embryos was quantified by counting the number of endothelial cell nuclei per field of view in control (n=7), *bmpr2a* #1(n=7), *bmpr2a* #2 (n=7); *bmpr2b* #1 (n=7), and *bmpr2b* #2 (n=7) MO injected embryos. (**f**) Branch point analysis of venous networks was performed in control (n=11), *bmpr2a* #1 (n=13), *bmpr2a* #2 (n=24), *bmpr2b* #1 (n=10), and

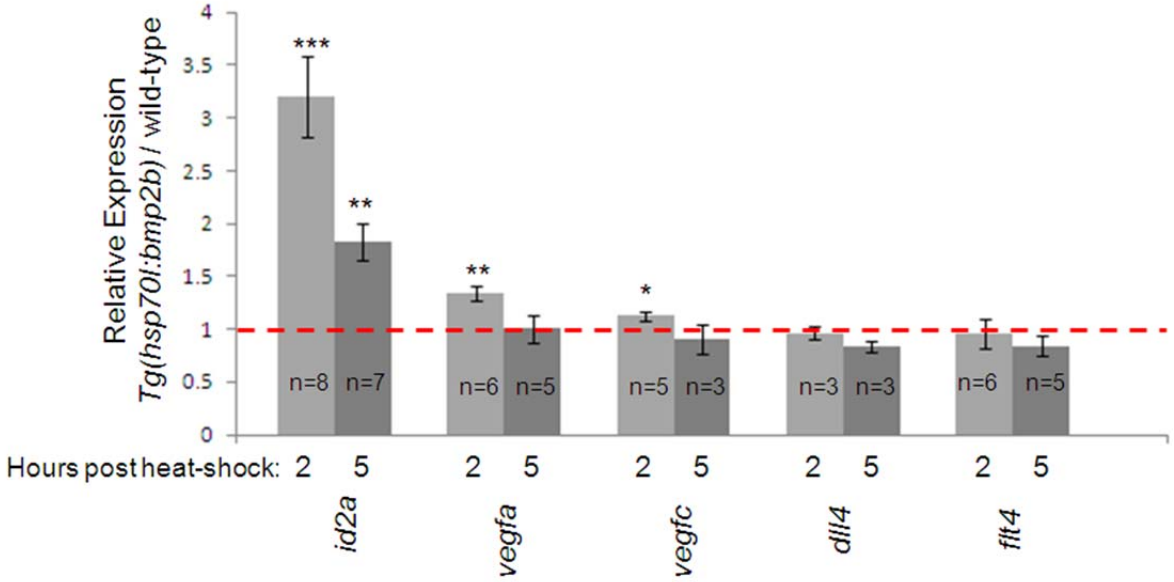
bmpr2b #2 (n=12) MO injected embryos. *bmpr2a* and *bmpr2b* morphants exhibited significantly decreased branching. Error bars represent mean ± SEM. *P<0.05 and ***P<0.001 versus control, Student's *t* test.

Figure 2.11. P-Smad and P-Erk are expressed in Bmp-induced sprouts.



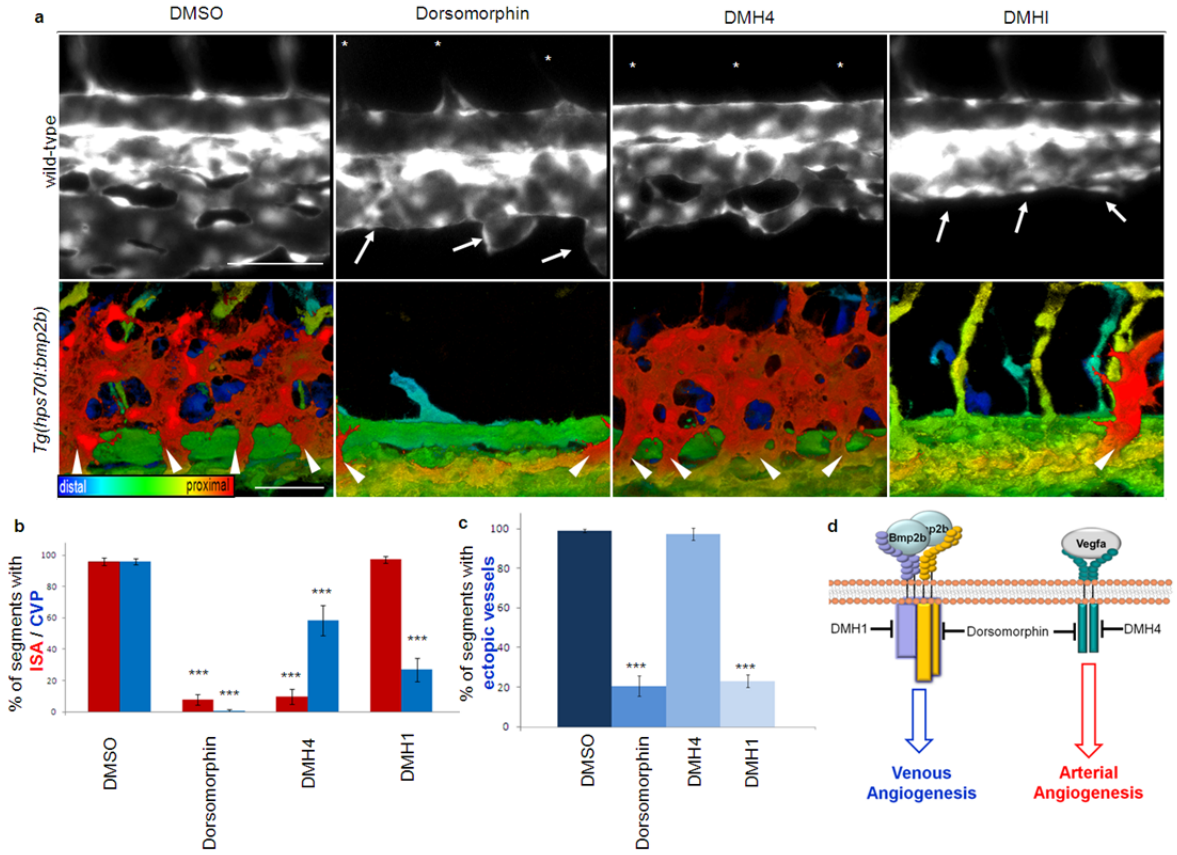
Tg(kdrl:GFP) and *Tg(hsp70l:bmp2b);Tg(kdrl:GFP*) heat-shocked embryos were stained with (a) phospho-Smad1/5/8 or (b) phospho-Erk. Confocal images were taken between the epithelial surface and somite boundary, where Bmp-induced ectopic sprouts form. Numbered arrows indicate Bmp-induced ectopic endothelial cells which express either phospho-Smad1/5/8 or phospho-Erk

Figure 2.12. The effects of *bmp2b* over-expression on transcription levels of selected genes.



Gene expression level of *bmp2b* over-expressing embryos was compared to wild-type embryos using quantitative RT-PCR. At 2 hr post heat-shock (light gray bars), transcription of a known Bmp target gene, *id2a*, was increased by 3.2 fold (P<0.001), while those of *vegfa* and *vegfc* were moderately increased (P=0.0042 and P=0.483 respectively), and transcription of *dll4* and *flt4* was unaffected. At 5 hr post heat-shock (dark gray bars) *id2a* was the only transcript increased (P=0.0029). n = the number of independent RNA samples/experiments (Error bars represent mean ± SEM. *P<0.05, **P<0.01, **P<0.001, one sample *t* test.)

Figure 2.13. Distinct functions of Bmp and Vegf-A signaling during angiogenesis.



(a) Epiflourescent micrographs of 38hpf *Tg(kdrl:GFP)* embryos (top panel) and *Tg(hsp70l:bmp2b); Tg(kdrl:GFP)* embryos (bottom panel) treated with DMSO, dorsomorphin (blocking both Vegf-A and Bmp signaling), DMH4 (blocking Vegf-A signaling), and DMH1 (blocking Bmp signaling). Arrows in the top panel point defective formation of venous sprouts ventrally, asterisks point defective formation of arterial sprouts dorsally, and arrowheads in the bottom panel point ectopic venous sprouts. (b) The percentage of segments that contain an ISA (red bars) or a CV plexus (blue bars) was quantified in DMSO (n=14), dorsomorphin (n=13), DMH4 (n=6), and DMH1 (n=13) treated embryos. In dorsomorphin-treated embryos, formation of both arteries and veins was significantly reduced. Treatment with DMH4, a specific inhibitor of Vegf-A signaling preferentially blocks the formation of arterial sprouts/vessels. Addition of DMH1, a specific inhibitor of Bmp

signaling, selectively blocks the formation of venous sprouts. (c) The percentage of segments that contain an ectopic vessels (green bars) was quantified in DMSO (n=11), dorsomorphin (n=6), DMH4 (n=5), DMH1 (n=13) treated embryos. The formation of Bmp-induced venous sprouts is inhibited by dorsomorphin or DMH1 treatment, but not by DMH4 treatment. (d) Schematic diagram showing the specific targets of each small chemical inhibitor used in this study. Error bars represent mean ± SEM. ***P<0.001 versus control, Student's *t* test.

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CHAPTER 3

BMP Signaling Selectively Affects Branching Morphogenesis during Angiogenesis via Tip Cell Responses

This chapter is adapted from a manuscript currently in preparation. I designed the experiments with the intellectual contribution of Dr. Suk-Won Jin and Dr. Victoria Bautch. I carried out the experiments, except where stated otherwise. Stryder Meadows and Dr. Ondine Cleaver preformed the experiment for Fig. 3.2.a-b. Diana Chong and Andrew T Barber helped in the experiments for Fig. 3.1.b. Andrew Barber helped with the quantification in Fig. 3.1d-f, and Fig. 3.4.b, c, and e. I created all of the figures. I wrote the original version of the draft and Dr. Victoria Bautch edited and contributed to the text.

ABSTRACT

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is critical for the growth and survival of tissues in both normal and pathological scenarios. Vascular networks observed in vertebrates display a vast array of morphological and functional diversity. Large blood vessels rapidly transport blood to more highly branched capillaries where circulation is slowed and oxygen and nutrients are able to diffuse into the surrounding tissue. Unfortunately, our understanding of how this morphological diversity is generated is largely unknown. We find here that BMP signaling is a potent and selective regulator of branching morphogenesis in multiple angiogenic systems. These effects are distinct from VEGFA which has pleiotropic effects on vessel morphogenesis. Upon closer analysis, we find that BMP signaling regulates tip cell morphology during angiogenesis.

Interestingly, inhibiting pathways that are known to establish the tip cell identity prevents BMP-induced branching in zebrafish. Taken together, our results support a paradigm whereby BMP regulates branching morphogenesis and network diversity via its effects on tip cell responses.

INTRODUCTION

During both developmental and pathological angiogenesis, new blood vessels form from preexisting vessels via proliferation, sprouting, and anastamosis [1]. This process provides new conduits for delivering oxygen and nutrients to developing tissues via blood circulation. In pathological scenarios such as cancer, the tumor is hypoxic and requires new blood vessels to survive and expand [2-4]. Thus being able to understand and manipulate the pathways involved in angiogenesis has important therapeutic applications.

The VEGFA signaling pathway is a prominent angiogenic pathway [5, 6]. VEGFA signaling is activated when the VEGFA ligand binds VEGFR2, a tyrosine kinase receptor that is selectively expressed in endothelial cells. This interaction leads to the phosphorylation and activation of multiple downstream effector pathways that ultimately result in the cellular responses that lead to angiogenesis. Specifically, the VEGFA signaling pathway regulates endothelial cell migration, proliferation, and sprouting to form new blood vessels.

Angiogenic sprouts are led by a tip cell, which extends multiple filopodia to sense its environment and guide the nascent sprout [7-9]. The tip cell expresses elevated levels of the Notch ligand Dll4 compared to neighboring stalk cells. The heightened expression of Dll4 in the tip cell activates Notch receptors in neighboring cells, which induces the stalk cell phenotype. The prevailing model is that Notch activation maintains the stalk cell phenotype by repressing the expression of VEGFR2, FLT4,and other pro-angiogenic factors, and induces the expression of anti-angiogenic factors, such as VEGFR1 [10].

While the pro-angiogenic effects of the VEGFA signaling pathway are well established, the mechanisms by which “non-canonical” angiogenic factors, such as the Bone Morphogenetic Proteins (BMPs), operate are much less understood [11]. BMPs are part of the highly conserved TGF-β super-family. BMP ligand dimers bind to two type I and two type II receptors to form a complex that induces the phosphorylation and activation of R-

Smads (Smad 1,5, and 8). R-Smads bind to the co-Smad (Smad4) and translocate to the nucleus to activate transcriptional targets. This signaling is inhibited by extracellular antagonists, such as Chordin and Noggin, which bind BMP ligands and prevent them from interacting with the receptor [12]. The Smad signaling cascade activates the transcription factor Id1, which induces endothelial cell migration and angiogenic responses *in vitro* [13]. BMP signaling also activates MEK/ERK signaling, which is a critical mitogenic and morphogenic pathway [14].

BMPs have diverse functions during development in vertebrates. For example, BMPs are important in the development of ventral structures and tissues such as kidney and blood, and in the initial stages of endothelial cell specification from stem cells [15]. BMPs are implicated in the patterning of early blood vessels, and endogenous BMP antagonists such as Chordin and Noggin are associated with the initial separation of the paired dorsal aorta, and their down-regulation is associated with the fusion process [16-18]. BMPs also affect the formation of filopodia in endothelial cells, and the unconventional Myosin X has been implicated as a downstream effector of BMP-mediated filopodia formation [19]. In human disease, pulmonary arterial hypertension (PAH) is associated with mutations in BMPR2 [20, 21]. In addition, mutations in ALK1, a BMP type I receptor, or Endoglin, a BMP co-receptor, cause an autosomal dominant vascular dysplasia called hereditary hemorrhagic telangiectasia (HHT) that is characterized by arteriovenous malformations [22-24]. These findings indicate that BMPs are critical to both vascular development and diseases involving the vasculature. However, a unifying cellular model describing how BMP regulates angiogenesis remains to be elucidated.

Using the zebrafish model system, we recently demonstrated that Bmp signaling selectively regulates sprouting angiogenesis from the axial vein, while the classical VEGFA signaling pathway regulates intersomitic vessel sprouting from the dorsal aorta during early development [25]. Although BMP-responsive vessels do not appear restricted to the venous

compartment in mammals, we hypothesized that mechanisms that regulate BMP responsiveness in zebrafish would be relevant in understanding how BMP affects mammalian vessels. Here we show that BMP affects branching morphogenesis in distinct ways from VEGFA in mammalian vessels. BMP both increases and lateralizes branching, and it predominantly affects tip cell responses. Notch signaling is antagonistic to BMP signaling in both zebrafish and mammalian vessels, suggesting a mechanism to regulate BMP responsiveness and integrate BMP and VEGFA signaling during mammalian angiogenesis.

RESULTS

Given our findings in zebrafish, we wanted to determine if and how BMP signaling regulates mammalian angiogenesis. To determine the effects of BMP signaling on developing mammalian blood vessels, we used mouse ES cells which undergo a programmed differentiation to produce multiple tissues, including lumenized vessels [26, 27]. On days 6-8 of differentiation the developing blood vessels undergo angiogenic expansion. Therefore we incubated ES cell cultures on day 6 and 7 with either Noggin or BMP. Addition of Noggin caused a decrease in blood vessel branching frequency relative to the control cultures, while BMP incubation led to a significant increase in branching frequency (Fig. 3.1a-b). These results show that BMP affects branching morphogenesis of developing blood vessels.

To begin to address whether the effects of BMP on vessel branching were endothelial cell specific, BMP signaling was manipulated in a fibrin bead assay, in which human umbilical vein endothelial cells (HUVECs) were attached to microcarrier beads and embedded in a fibrin matrix [28]. As vascular sprouts emerged at 2 days post-embedding cultures were treated with Noggin or BMP. In this assay endothelial cells sprout in an extracellular matrix without the direct contact of other cell types, so any morphological effects of BMP and Noggin are likely caused directly on the endothelial cells. While Noggin treated vascular sprouts had long relatively unbranched sprouts that appeared “bamboo-like”, the BMP-treated sprouts appeared more branched and had a “bush-like” appearance (Fig. 3.1c).

To quantify these phenotypic differences, we quantified multiple parameters. We quantified the branching frequency and found that the number of branches per vessel length was significantly decreased with Noggin and significantly increased with BMP (Fig. 3.1d). In addition to regulating branching frequency, BMP manipulation appeared to affect the angle at which branches extended from their parent vessel. To quantify this observation, we

measured the maximum branch angle at junctions. Treating sprouts with Noggin significantly decreased this angle making the sprouts extend more distally while treatment with BMP significantly increased this angle causing vessels to extend more laterally (Fig. 3.1e). The sprout length, the distance from the base of the bead to the most distal end of a sprout, was measured. Noggin treatment significantly increased this distance while BMP treatment significantly decreased this distance (Fig. 3.1f). Taken together, these results show that increasing BMP signaling affects multiple branching parameters which lead to a more highly branched lateral growth of vessels at the expense of distal growth.

To determine the effects of BMP signaling *in vivo* we used the mouse retina as a model system. We analyzed the developing vasculature of the postnatal retina, which expands via sprouting angiogenesis from the optic nerve to the periphery of the retina during the first post-natal week. This angiogenic sprouting occurs along a vascular front in which arterial and venous identity are still largely indistinct. Behind the vascular front, distinct arteries and veins become morphologically and molecularly distinguishable.

To determine when and where BMP signaling impacts angiogenesis the mouse retina, we used BRE:eGFP transgenic mice that express eGFP in response to activation of a BMP Responsive Element (BRE). BRE is strongly expressed in both arteries and veins (Fig. 3.2a), and in both tip and stalk cells at the vascular front (Fig. 3.2b). Although detection of extremely dynamic changes in expression levels within a tissue is technically limiting due to perdurance of eGFP protein, the strong and selective expression of the BRE:eGFP reporter in endothelial cells of developing retinal vessels indicates that BMP signaling is active during angiogenesis.

Given that mammalian retinal vessels experience BMP signaling, we hypothesized that manipulations of BMP signaling would perturb developing vascular networks. To inhibit BMP signaling in mice we used the selective small molecule inhibitor of BMP signaling, DMH1 [29] and analyzed the effects in the postnatal retina. In control embryos the periphery

of the retinal vasculature contains angiogenic sprouts that extend outward from the base to give the vascular front a “spiky” appearance. Injection of DMH1 resulted in fewer sprouts at the vascular front, and these sprouts did not extend as far as controls (Fig. 3.2c). Because treatment with DMH1 may have indirect effects on angiogenesis, we next used a genetic approach to manipulate BMP signaling in vivo. The *Bmpr2* gene was conditionally deleted using a *Tie2-Cre* driver, which is expressed in endothelial cells and some hematopoietic cells. The vascular front of *Bmpr2*$^{f/f}$; *Tie2-Cre* retinas resembled that of the DMH1-treated embryos and were significantly less branched than controls. In addition, the *Bmpr2*$^{f/+}$*; Tie2-Cre*, heterozygous retinas, displayed an intermediate phenotype (Fig. 3.2d-e). Thus BMP signaling blockade via DMH1 exposure and the vascular-selective genetic deletion of *Bmpr2* inhibited branching of developing retinal vessel networks in the mouse.

Since manipulating BMP signaling has these strong effects on branching morphogenesis, we wanted to directly compare it to VEGFA. Sprouts off fibrin beads were stimulated with Noggin, BMP, or VEGFA. While the Noggin treatment induced longer and less branched vessels, BMP treated vessels were more branched. In contrast, VEGFA stimulation induced large vascular sheets (Fig. 3.3a). The *Tg(hsp70l:bmp2b), Tg(hsp70l:noggin),* and *Tg(hsp70l:vegfaa*$_{121}$*)* zebrafish transgenic lines were used to manipulate BMP and VEGFA signaling. Conditional over-expression of *noggin3* induced long unbranched vessels in the zebrafish posterior cardinal vein (PCV) plexus, while *bmp2b* over-expression induced highly branched ectopic networks from the PCV plexus (Fig. 3.3b). Thus the effects of BMP signaling on vessel branching in the fibrin bead assay are consistent with those seen in the zebrafish vein. Conditional overexpression of *vegfaa* in zebrafish resulted in a single large dilated vein in the PCV plexus region and induced large dysmorphic sprouts from the dorsal aorta. Thus, the effects of *vegfaa* overexpression in zebrafish phenocopy the effects of VEGFA stimulation in the fibrin bead assay. Taken together, these results are consistent with the idea that BMP potently and selectively affects

branching morphogenesis, while VEGFA signaling has pleiotropic effects on vessel morphogenesis which causes unregulated protrusive growth when misregulated (Fig. 3.3c).

The hyper-branching vessel phenotype observed with BMP mimics manipulations that lead to changes in tip cell numbers and responses [30]. Therefore, we hypothesized that BMP signaling affects tip cell properties. High resolution analysis of BMP- and Noggin-treated sprouts generated in the fibrin bead assay revealed that tip cells that experienced more or less BMP signaling were morphologically different from controls. The Noggin-treated tip cells were thinner and more “pointed”, while the BMP-treated tip cells were more “splayed” (Fig. 3.4a). To quantify these morphological distinctions, the distance between the nucleus and the distal end of the tip cell was measured. Noggin treatment significantly lengthened this parameter of tip cells, while BMP stimulation significantly shortened this distance (Fig. 3.4b). In addition, the width at the distal end of the tip cell was significantly wider in the BMP-stimulated sprouts (Fig. 3.4c). The net effect of these differences is that lower levels of BMP signaling increase the apical extension of tip cells while high levels of BMP increase the lateral extensions of tip cells (Fig. 3.4f). Interestingly, the effects of BMP signaling on tip cell morphology are paralleled at the network level, where Noggin sprouts are longer and less branched and BMP sprouts are shorter and more highly branched (Fig. 3.1c-f). In addition to affecting tip cell morphology, we calculated the percent of cells that have the tip cell morphology. Cells were considered to have the tip cell morphology if they were at the tip cell position or if they were stalk cells that contained extracellular extensions. BMP blockade via Noggin decreased the percent of cells with tip cell morphology in sprout while BMP stimulus increased this percentage (Fig. 3.4d-e). Thus BMP signaling affects both the number of tip cells and their morphology, suggesting that BMP may affect branching morphogenesis via modulation of tip cell numbers and dynamics.

BMP signaling has a dramatic effect on tip cell responses in mammalian vessels. In addition, these effects appear to underline the increased branching and lateral growth

caused by BMP stimulus. Thus we reasoned the effects of BMP signaling on tip cell responses in mammalian vessels might help to elucidate the pro-angiogenic effects of BMP signaling in zebrafish. Therefore, we analyzed factors and pathways that regulate tip/stalk cell dynamics using the zebrafish model.

Notch signaling co-ordinates tip/stalk cell phenotype. Notch activation induces the stalk cell phenotype. To determine where Notch is active we used the *Tg(Tp1bglob:eGFP)* transgenic zebrafish line that uses a Notch-responsive element to drive eGFP expression [31]. We find that the Notch reporter is selectively expressed in zebrafish arterial endothelial cells (Fig. 3.5a). To test the hypothesis that Notch activity inhibits BMP-mediated angiogenesis, we treated zebrafish embryos with DAPT, a small molecule that prevents the cleavage of Notch to produce NICD and thus inhibits Notch activity. DAPT treatment had no observable effect on venous angiogenesis (data not shown), which is predicted given the absence of Notch activity in the vein. However, Notch inhibition increased ectopic branching in the intersegmental vessels that sprout from the artery, as was previously shown [32] (Fig. 3.5b). Over-expression of BMP induced robust ectopic branching from the vein, while intersegmental vessels were largely unaffected, as we previously reported [25]. However, simultaneous over-expression of BMP and inhibition of Notch signaling caused significantly more ectopic intersegmental vessels than did inhibition of Notch signaling alone, and the ectopic vessels were more numerous in DAPT-treated embryos that also over-expressed BMP compared to embryos treated with only DAPT (Fig. 3.5c-d). This finding suggests that one of the consequences of Notch activation is to inhibit BMP responsiveness in zebrafish arteries during development.

To further test this hypothesis, we asked whether Notch activation inhibits BMP-mediated angiogenesis. The *Tg(UAS:NICD); Tg(hsp70l:gal4)* transgenic line was used to conditionally over-express NICD and activate Notch signaling. Heat shock treatment of *Tg(hsp70l:bmp2b)* transgenic embryos induced BMP activation [25], while heat shock

treatment of *Tg(hsp70l:bmp2b);Tg(UAS:NICD); Tg(hsp70l:gal4)* embryos simultaneously activated BMP and Notch signaling. This simultaneous activation of BMP and Notch disrupted intersegmental vessel formation and also significantly reduced the number of BMP-induced ectopic venous sprouts relative to controls that only over-expressed BMP (Fig. 3.5e-f). Taken together, these results demonstrate that Notch signaling inhibits Bmp-mediated angiogenesis.

Notch signaling also co-ordinates tip/stalk cell decisions, at least in part by repressing pro-angiogenic factors and inducing the expression of anti-angiogenic factors, thus making cells less responsive to angiogenic cues [33]. One of the pro-angiogenic factors inhibited by Notch activity is the Flt4 (fms-related tyrosine kinase 4, also called VEGFR-3) receptor [34-36]. In the developing retinal vessel network FLT4 expression is elevated at the vascular front and is important in angiogenic sprouting [36]. In zebrafish, Flt4 is selectively expressed in veins with the exception of the tip cells of intersegmental vessels [34]. Inhibiting Notch activity leads to ectopic Flt4 expression in arterial endothelial cells in zebrafish, indicating that Notch activity in arteries appears to inhibit Flt4 expression and limit its expression to veins in zebrafish [34, 37]. Flt4 is also uniquely required for venous angiogenesis in zebrafish [34, 37]. In short, Notch activity inhibits Flt4 which is a critical regulator of venous angiogenesis in zebrafish.

Given the selective expression and function of Flt4, we wondered whether Flt4 regulates BMP-mediated angiogenesis. Control or antisense morpholinos (MO) to Flt4 were injected into *Tg(hsp70l:bmp2b)* embryos, and Bmp2b was conditionally over-expressed. Knockdown of Flt4 dramatically reduced the number of BMP-induced ectopic sprouts, while the intersegmental vessels were largely unaffected (Fig. 3.6a-b). We previously showed that knockdown of the zebrafish Vegfr2 orthologs, Kdr and Kdrl, did not inhibit BMP-induced ectopic sprouts [25]. These findings highlight that Flt4 but not Kdr and Kdrl is required for

Bmp-mediated angiogenesis. Taken together, these results demonstrate Notch and FLT4 play important roles in regulating the specificity of BMP responses in zebrafish.

Zebrafish arterial and venous endothelial cells have contrasting levels of Notch activity and Flt4 expression during early development. The vein has low Notch activity and high Flt4expression, while the arteries have high Notch activity and low Flt4 expression (Fig. 3.6c). Our findings demonstrate that low Notch activity and high Flt4 expression permit BMP-mediated branching morphogenesis, while high Notch activity and low *flt4* expression restricts BMP-mediated branching morphogenesis. This paradigm is likely relevant in mammalian vessels, where tip cells have low Notch activity and elevated FLT4and are responsive to BMP, while stalk cells have elevated Notch activity and reduced FLT4and are refractory to BMP (Fig. 3.6d).

DISCUSSION

Our findings demonstrate that BMP signaling is required for proper branching morphogenesis in mammalian vessels. The effects of BMP on vessel branching are distinct from those of VEGFA in several ways. First, the major effect of BMP signaling is on sprouting morphogenesis, while VEGFA is both a morphogen and a mitogen. The effects of elevated BMP signaling on sprouting vessels lead to more productive sprouting and branch formation, whereas the effects of elevated VEGF signaling lead to unregulated protrusive activity that does not lead to productive branching. Finally, loss or blockade of BMP changes only the morphogenetic patterning of mammalian vessel networks, while the requirement of VEGFA for endothelial cell survival leads to a complete loss of developing vessel networks upon loss of VEGFA [38]. Taken together, these findings suggest that during the development of mammalian vessel beds, VEGFA and BMP integrate signaling to regulate the extent of vessel arborization and thus generate the diverse morphology seen in mammalian vessels. These findings also suggest that targeting the BMP pathway therapeutically could allow for more “fine-tuning” of vascular responses.

How is BMP responsiveness regulated in mammalian vessels? The presence of BMP receptor complexes and ligand availability will be clearly involved in responses to BMP. However, endothelial BMP responsiveness is also likely regulated by non-BMP mechanisms. Here we show that Notch and FLT4 regulate the ability of sprouting vessels to respond to BMP. Loss of Notch activity rendered arterial sprouting sensitive to BMP over-expression, while excess Notch activity and inhibition of Flt4 led to suppression of ectopic BMP-induced sprouting from the vein.

Our findings lead to a model of BMP-induced vessel sprouting that highlights the responsiveness of tip cells to BMP signaling and suggests how BMP regulates the patterning of vessel networks (Fig. 3.7). Our results predict that tip cells will be responsive to a BMP stimulus, since they have reduced Notch activity and elevated Flt4 expression

relative to neighbors (blue cell, Fig. 3.7a). Interestingly, our results demonstrate that BMP signaling regulates tip cell morphology by increasing the lateral extensions of tip cells (blue cells, Fig. 3.7b). In addition to tip cells, we hypothesize that other cells in extending sprouts are potentially responsive to BMP via reduced Notch activity and elevated Flt4 expression (green cells, Fig. 3.7b). In the absence of BMP or in situations where endogenous BMP antagonists prevent BMP signaling, these cells remain quiescent and act as stalk cells. However, in situations where BMP is available, these cells respond to BMP signals with filopodia extensions and adopt a tip cell phenotype (green cells, Fig. 3.7b). These new tip cells migrate out at angles approximating 90 degrees from the parent spout, and this process is predicted to increase both the lateral spread of the vessel plexus and the overall degree of branching. We thus propose that the input provided by BMP refines and fine-tunes the basic branching pattern to provide the numerous types of blood vessel networks found in mammals (Fig. 3.7c).

MATERIALS AND METHODS

Cell culture maintenance and treatment

The maintenance and differentiation of ES cells was performed as previously described [27]. To manipulate BMP signaling levels human BMP-2 (RnD; 355-BEC), BMP-2/BMP-7 (R and D; 3229-BM), or human Noggin (RnD; 6057-NG) was added at day 6 and day 7 of ES cell differentiation at a concentration of 200ng/mL.

The fibrin bead assay was performed as previously described [39]. Recombinant BMP, Noggin, and VEGF-A (Peprotech) were added to fibrin bead assay on 2, 4, and 6 days after embedding in beads fibrin at a concentration of 200ng/mL.

Sample processing and staining

ES cells were fixed and processed on day 8 as previously described [40]. ES cell cultures were stained with rat anti-mouse PECAM-1 (BD Biosciences) at 1:1000 and goat anti-rat IgG Alexa Fluor 488 (IgG; H+L) at 1:200 (Invitrogen).

Fibrin bead cultures were fixed and processed on day 8. Fibrin beads were fixed in 2% PBS for 30 minutes and stained with Alexa Fluor 488 phalloidin (Molecular Probes) at 1:200 and DRAQ7 (Biostatus) at 1:1000 in PBS with 1% Triton and 4% BSA.

Retinas were fixed and processed for staining with isolectin GS-IB4 conjugated to Alexa Fluor 488 (Molecular Probes) at 1:100 as described [30, 41].

Mouse experiments

The following transgenic lines were used: *BRE:eGFP* [42], *Tie2-Cre* [43], and *Bmpr2*$^{f/f}$ [44]. To generate conditional knockouts, *Bmpr2*$^{f/+}$*; Tie2-Cre* mice were generated and crossed to *Bmpr2*$^{f/f}$ mice. The resulting *Bmpr2*$^{f/f}$*, Bmpr2*$^{f/+}$, *Tie2-Cre; Bmpr2*$^{f/+}$*,* and *Tie2-Cre; Bmpr2*$^{f/+}$ mice were sacrificed at P4-5, and retinas were processed, imaged and quantified.

DMH1 (Sigma, D8946) was resuspended in 20% ethanol and 80% sunflower seed oil (Sigma). DMH1 was injected intraperitoneally into P2 mice at a final concentration of 0.173 mg per kg of mouse weight. Retinas were harvested and processed at P4.

Zebrafish experiments

Zebrafish (*Danio rerio*) embryos were raised as previously described. The following transgenic lines were used: *Tg(kdrl:GFP)*^{s843} [45], *Tg(hsp70l:bmp2b)*^{fr13} [46], *Tg(hsp70l:noggin)*^{fr13} [46], *Tg(hsp70l:vegfaa121;cmlc2:EGFP)*^{nc2} [25], *Tg(Tp1bglob:eGFP)* ^{um14} [31], *Tg(UAS:myc-Notch1a-intra)*^{kca3} [47], and *Tg(hsp70l:Gal4)1.5*^{kca4} [47].

Zebrafish embryos were treated with 100uM of DAPT in 2% DMSO from 10hpf until they were processed and imaged. Embryos were heat-shocked at 25–26 hpf for 30 min at 40 °C.

Micro-injections of morpholino oligonucleotides were carried out as previously described [48]. Briefly, embryos were injected at the single-cell stage with 8 ng of control morpholino (Gene Tools) and 8 ng of flt4 MO: 5' - TTAGGAAAATGCGTTCTCACCTGAG - 3' [49].

Quantification

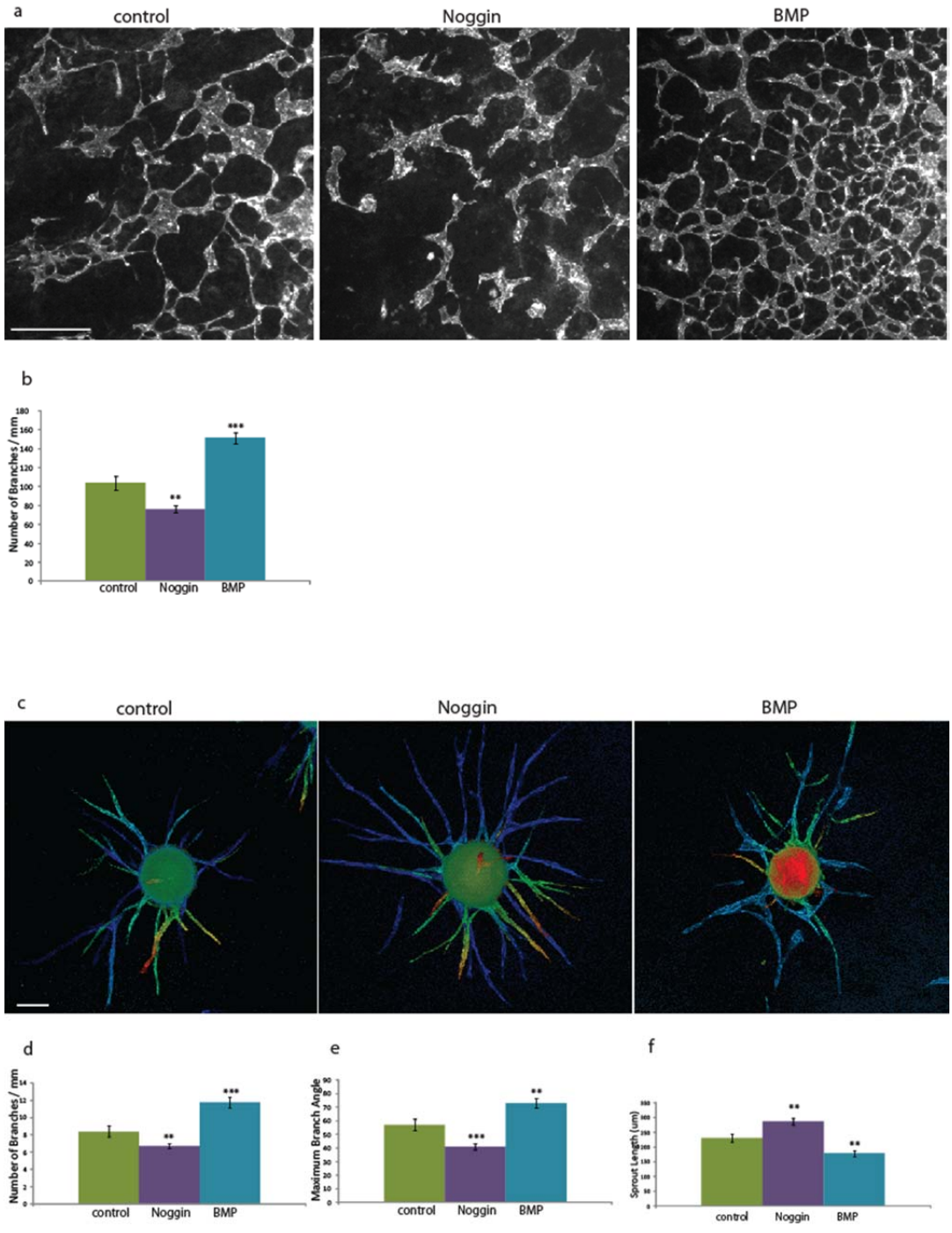
The branching frequency was measured by skeletonizing the ES cell derived vessel networks using the Image J software (NIH). The number of branches per mm of vessel length was subsequently calculated. In the mouse retina, the branching frequency was calculated by skeletonizing the branches at the vascular front. Specifically, the most distal vessel along the angiogenic front (red line, Fig. 3.2c-d) and the immediate and distal sprouts from that vessel were analyzed. In the fibrin bead assay individual sprouts from the fibrin bead were skeletonized and measured. Nascent sprouts < 50 microns were excluded from analysis.

More detailed analysis of sprouting from the fibrin bead assay was as follows. The maximum branch angle was generated by calculating the branch angle at sprout junctions. For example, if an angiogenic sprout contained a branchpoint with more than one branch, the maximum angle between the branches was calculated. For this measurement, branches that fused with other sprouts were excluded, as these interactions may alter the branch angle. The sprout length was measured by tracing the distance from the base of the sprout to the most distal end of the sprout. The nucleus to tip measurements were taken from the middle of the cell nucleus to the distal end of the tip cell and the tip cell width was calculated by measuring the greatest width within the first 10um from the distal end of the tip cell (see schematic, Figure 3.4b-c).The percent of cells with extracellular projections was calculated by counting the number of tip cells and stalk cells which have extracellular projections and dividing it by the total number of cells in the sprout (see schematic, Fig. 3.4e). Sprouts that were analyzed had at least 7 nuclei.

To quantify the frequency of sprouting in zebrafish, the percentage of segments that contained angiogenic sprouts were calculated. Each segment is defined as the area on the anterior–posterior 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 calculate the percent of segments with ectopic arteries each segment that contained an ectopic arterial vessel was given a value of 1, and each segment that lacked an ectopic arterial sprout was given a value of 0. The percentage of segments with ectopic arteries was subsequently calculated (Fig. 3.5d). Similarly, to calculate the percent of segments with ectopic veins in *bmp2b*-overexpressing embryos, segments that contained an ectopic vein were given a value of 1, and each segment that lacked an ectopic vein was given a value of 0. The percentage of segments with ectopic veins was subsequently calculated (Fig. 3.5f and Fig. 3.6b).

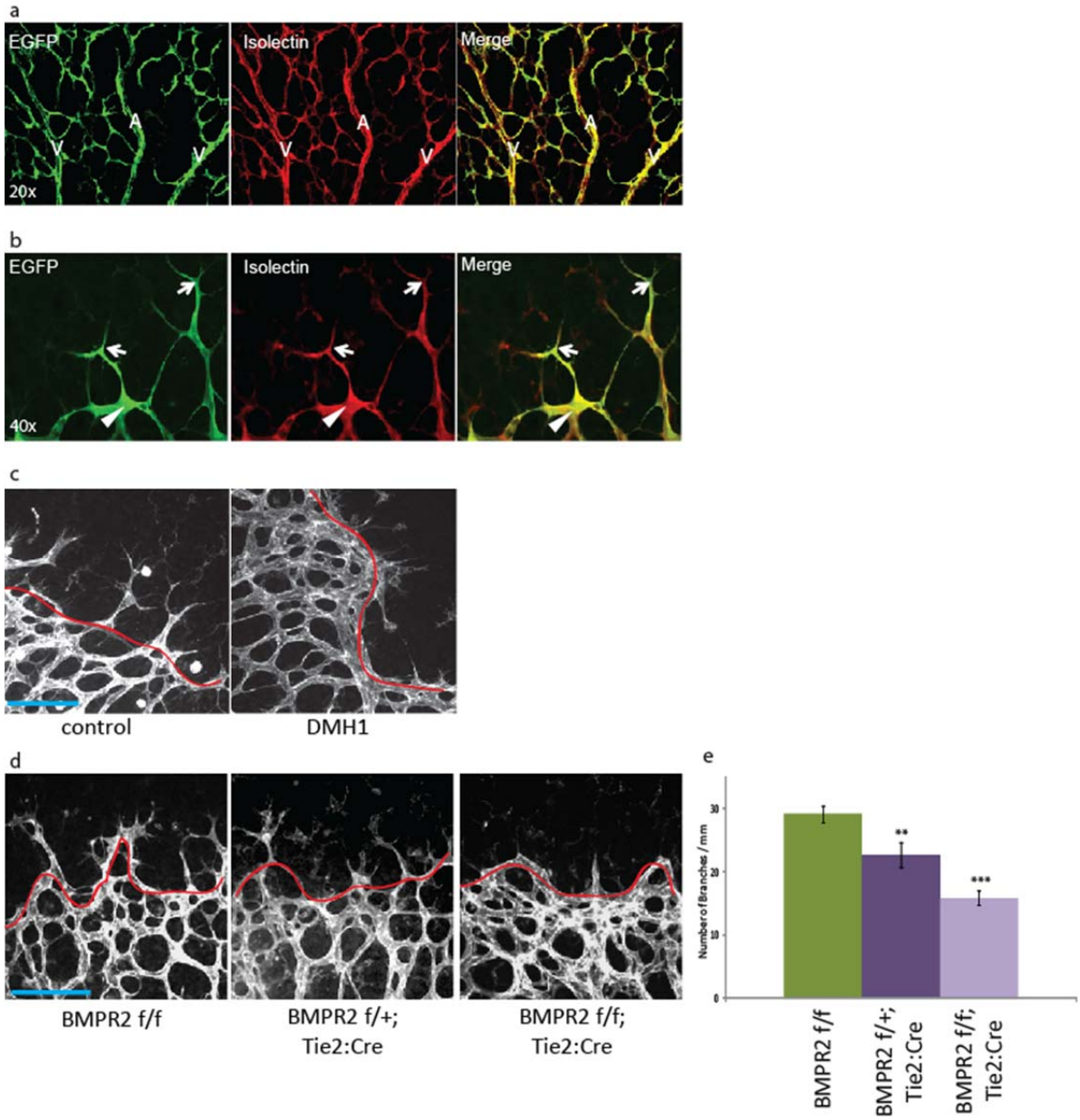
FIGURES

Figures 3.1. BMP signaling increases branching morphogenesis and lateral growth.



ES cell cultures were stimulated with either BMP or Noggin on days 6 and 7 of differentiation. Noggin treated blood vessels appeared larger and less branched, while BMP treated vessels formed highly branched networks (a). Scale bar is 250um. Addition of Noggin caused a decrease in number of branches per mm, while BMP significantly increased the branching frequency relative to the control (b). BMP and Noggin were added to the fibrin bead assay. Sprouts treated with Noggin formed longer less branched sprouts than control, while BMP stimulation lead to shorter more highly branched networks (c). Scale bar is 100um. Noggin stimulation significantly decreased the number of branches per mm while addition of BMP significantly increased the frequently of branching relative to control sprouts (d). The maximum branch angle was measured at branch junctions. Noggin significantly reduced this angle while BMP significantly increased this angle (e). The sprout length was significantly increased with Noggin and significantly decreased with BMP (f). Error bars represent mean ± SEM. **P<0.01 and ***P<0.001 versus control, Student's *t* test.

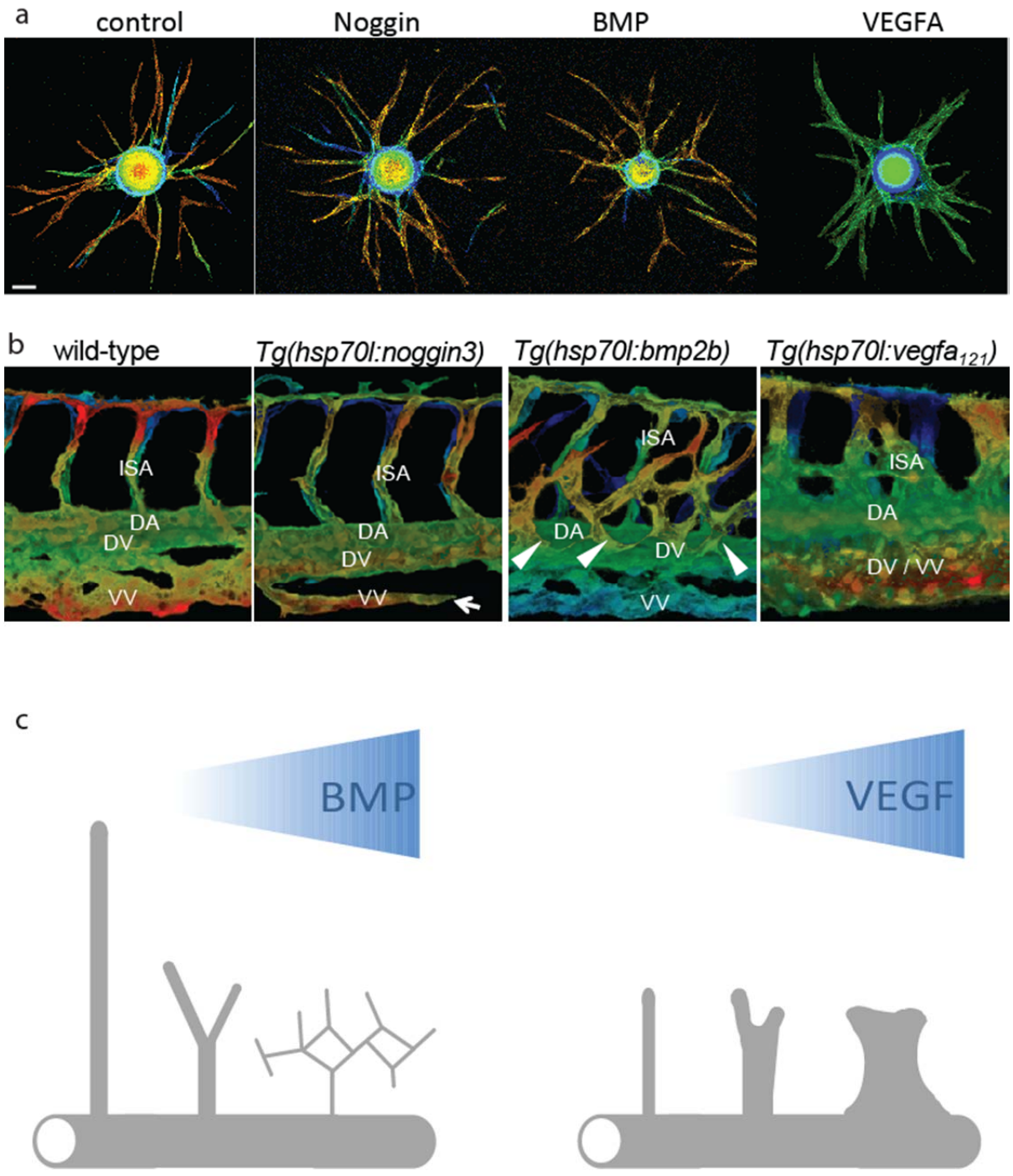
Figure 3.2. BMP regulates branching morphogenesis in mice.



Retinas from BRE:eGFP transgenic mice (eGFP=green) were stained with isolectin B4 (red) to visualize the vessels. In the P4-5 postnatal retina eGFP highly colocalizes with isolectin. BRE is strongly expressed in both arteries and veins (a), and is expressed in both tip (arrows) and stalk cells (arrowheads) at the vascular front (b). Vehicle and DMH1 were administered to mice at P2 and retinas were harvested at P4. DMH1 decreased branching

at the angiogenic front (red line) and resulted in a “smooth” appearance (c). *Bmpr2* was conditionally deleted using the endothelial *Tie2-Cre* driver. The vascular front of *Bmpr2*$^{f/f}$*; Tie2-Cre* retinas was significantly less branched than controls. In addition heterozygous mice had an intermediate phenotype (d-e). Scale bar are 100um. Error bars represent mean ± SEM. **P<0.01 and ***P<0.001 versus control, Student’s *t* test.

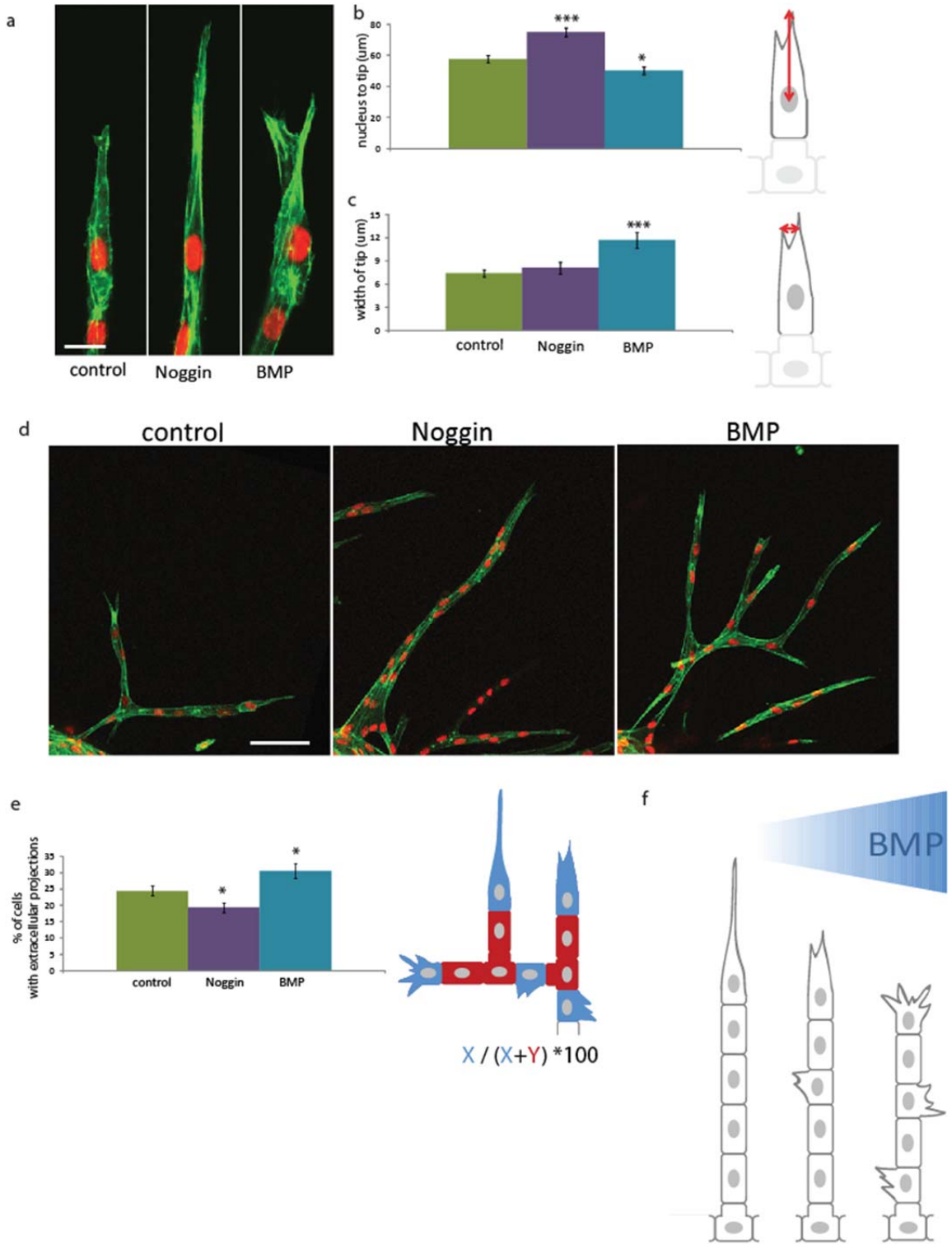
Figure 3.3. BMP and VEGF-A have distinct morphological effects on angiogenesis.



3D color projections of fibrin beads stimulated with Noggin, BMP2, and VEGF-A (a) and zebrafish in which either *noggin3, bmp2b*, or *vegfa* are overexpressed (b). Over-expression of *noggin3* induced long unbranched vessels (arrows), *bmp2b* oxer-expression induced the

formation of highly branched ectopic networks (arrowheads), and *vegfa* over-expression induced dysmorphic vascular sheets (c). DA, dorsal aorta; VV, ventral vein; DV, dorsal vein; ISA, intersegmental artery. Scale bar is 100um.

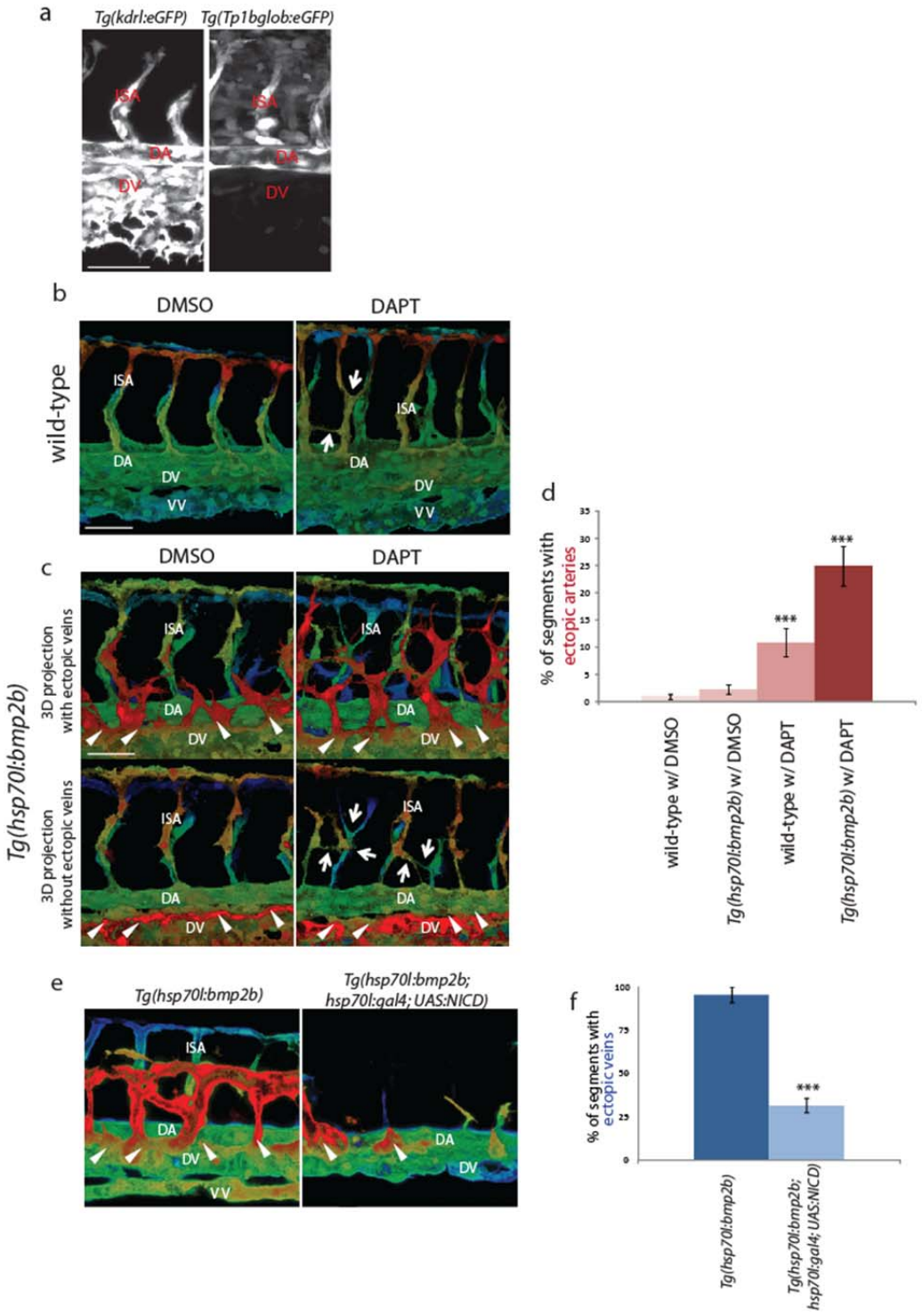
Figure 3.4. BMP signaling regulates tip cell morphology.



The fibrin bead assay was used to analyze the effects BMP or Noggin on tip cell morphology. BMP signaling was manipulated by addition of recombinant BMP or Noggin.

Noggin treatment caused tip cells to have a more pointed morphology while BMP treatment caused tip cells to have a more splayed out morphology (a). Scale bar is 20um. The distance between the center of the nucleus and the distal end of the tip cell was measured. This distance was significantly longer with addition of Noggin and significantly shorter in BMP stimulated tip cells (b). The width at the distal end of the tip cell was also quantified. BMP-stimulated tip cells were significantly wider than control (c). The percent of cells with extracellular projections was quantified for each condition. BMP treatment significantly increased this percentage while Noggin treatment significantly decreased this percentage (d-e). Scale bar is 100um. Bmp signaling increases the number of cells with extracellular extensions and causes tip cells to have more lateral extensions (f). Error bars represent mean ± SEM. **P<0.05 and ***P<0.001 versus control, Student's *t* test.

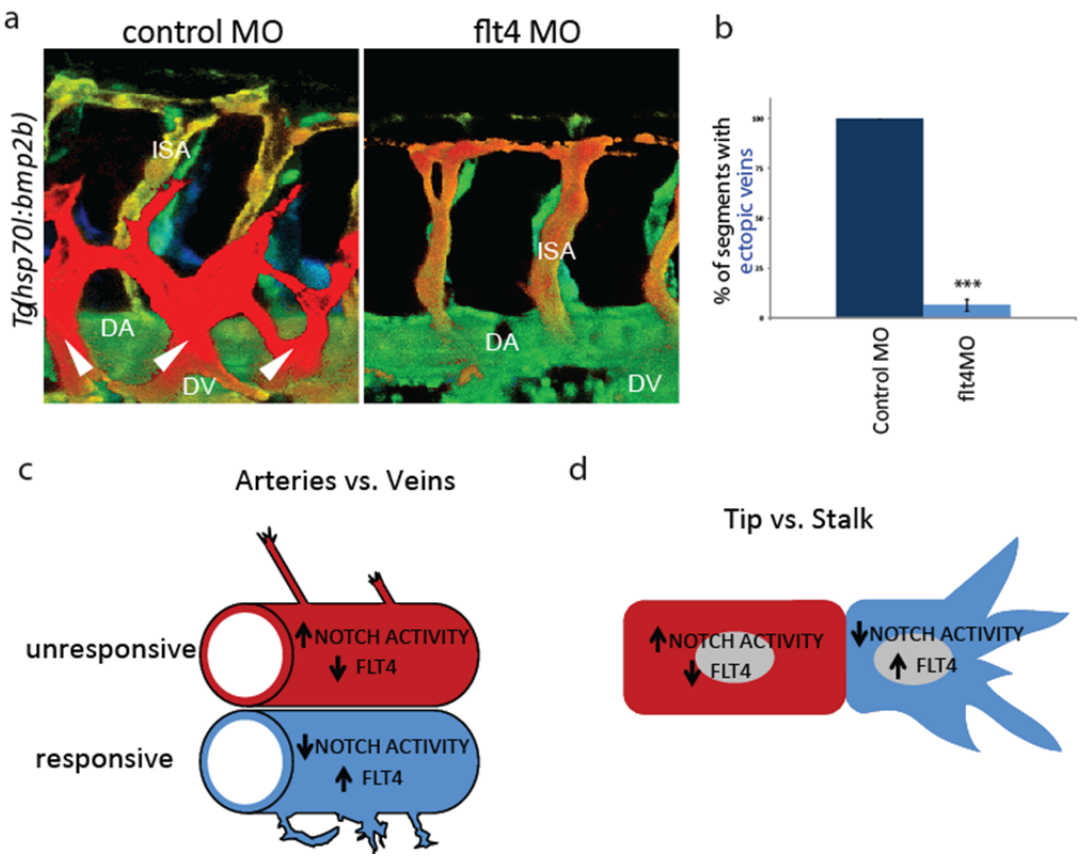
Figure 3.5. Notch activity inhibits Bmp-induced angiogenesis in zebrafish.



The *Tp1bglob:eGFP* transgenic zebrafish line was used to determine where Notch is active. The expression was compared to the pan-endothelial reporter line *kdrl:GFP*. The Notch

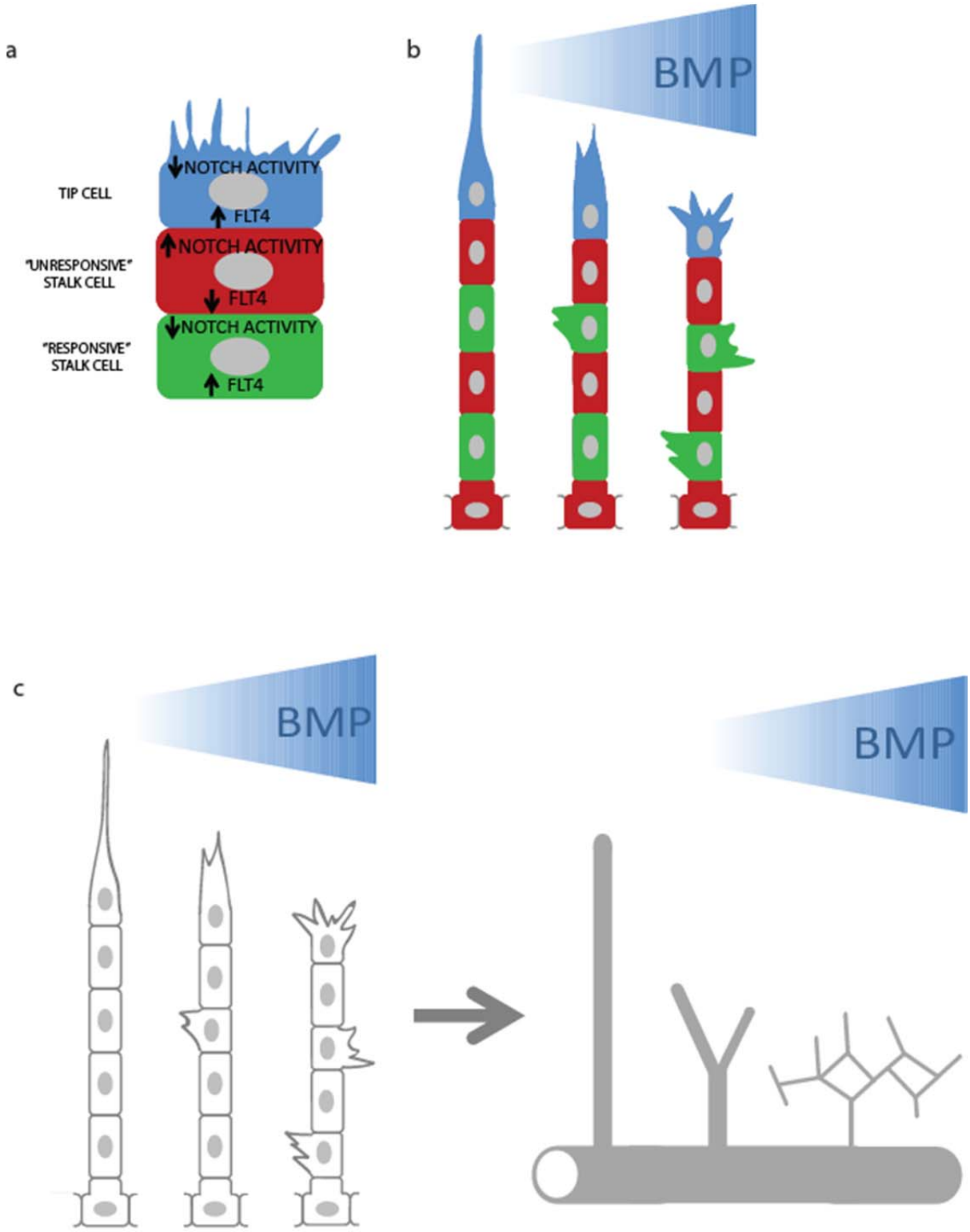
reporter is highly expressed in arterial cells and not expressed in venous endothelial cells at ~28hpf (a). Wild-type embryos were treated with DMSO or DAPT. DAPT treatment caused a significant increase in the number of ectopic ISAs relative to the DMSO control (b and d). *bmp2b* over-expressing embryos were also treated with DMSO and DAPT. *bmp2b* over-expression in DMSO treated embryos induces robust ectopic sprouts form the axial vein in both DMSO and DAPT treated embryos (arrowheads). To visualize the ISAs in Bmp-overexpressing embryos the z stacks containing the ectopic sprouts (arrowheads) were removed from the 3D projection. The ISAs are largely unaffected in *bmp2b* over-expressing embryos treated with DMSO. However, the combination of DAPT and *bmp2b* over-expression significantly increased the number of ectopic ISAs (arrows) relative to DAPT treatment alone (c-d).To test the effects of Notch gain-of-function on Bmp-induced ectopic sprouts, we compared the ectopic sprouts in *Hsp70l:bmp2b*; *UAS:NICD; Hsp70l:gal4* transgenic embryos to *Hsp70l:bmp2b* embryos. Heat-shock induction of either *bmp2b* over-expression alone induced ectopic venous sprouts, while over-expression of or *bmp2b* and *NICD* together significantly inhibited BMP induced ectopic sprouts (e-f). DA, dorsal aorta; VV, ventral vein; DV, dorsal vein; ISA, intersegmental artery. Scale bar is 50um.

Figure 3.6. Flt4 is required for BMP-mediated angiogenesis.



flt4 was knocked down using antisense MOs. Embryos injected with *flt4* MO had significantly fewer Bmp-induced ectopic sprouts (arrowheads) than controls (a-b). DA, dorsal aorta; VV, ventral vein; DV, dorsal vein; ISA, intersegmental artery. Our result support a model in the low Notch activity and high *flt4* expression in veins makes the responsive to Bmp stimulus, while high Notch activity and low *flt4* expression in the arteries makes them unresponsive (c). In mammalian vessels, tip cells have low Notch activity and elevated Flt4 and stalk cells have elevated Notch and low Flt4, which suggests that tip cells will be selectively responsive to BMP stimulus (d). Error bars represent mean ± SEM. ***P<0.001 versus control, Student's *t* test.

Figure 3.7. A working model for how BMP signaling affects angiogenesis.



Our results predict that tip cells will be responsive to BMP stimulus, since they have reduced Notch activity and elevated Flt4 expression relative to neighbors (blue cells, a). Our results demonstrate that low BMP signaling causes tip cells to have a long apical extension, while BMP stimulation increases the lateral extensions of these tip cells (blue cells, b). We hypothesize that, in addition to tip cells, other cells in extending sprouts are potentially responsive to BMP via reduced Notch activity and elevated Flt4 expression relative to neighbors (green cells, a). Without BMP stimulus these cells remain quiescent and act as stalk cells. However, in situations where BMP is available, these cells respond to BMP signals with extracellular extensions and adopt a tip cell phenotype. Thus, we propose that the combinatorial effects of BMP signaling on tip cell morphology and tip cell induction increases the branching and lateral spread of the vessels (c).

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CHAPTER 4

Conclusions and Perspectives

As the field has progressed, our understanding of the mechanisms involved in regulating angiogenesis has become more intricate and complex. In a simplified angiogenic model, the VEGFA signaling pathway is the predominant chemoattractant for endothelial cells. Hypoxic tissues upregulate HIF, which transcriptionally increases VEGFA expression and leads to the formation of new vessels [1]. This pattern provides oxygen to the hypoxic tissue, which ends the HIF-induced angiogenic process. However, this simplified model does not explain how the movement or attraction of endothelial cells is co-ordinated. In other words, if all endothelial cells in a tissue responded identically to a stimulus, we would expect to see a uniform ball or sheet of cells that surrounded the stimulus. However, the vascular networks that form *in vivo* are highly intricate and organized, and thus are predicted to require complex regulatory mechanisms. In fact, recent evidence demonstrates that Notch signaling plays an important role in refining or “fine-tuning” angiogenesis: Lateral inhibition through Notch signaling coordinates which cells respond to the angiogenic stimulus (i.e. which cells are tip cells and which cells are stalk cells) [2].

Alternative angiogenic pathways have also been found, and my research helped to elucidate the role of BMP signaling during angiogenesis. My findings demonstrate that the BMP is a critical angiogenic factor for vascular development in both zebrafish and mammals [3]. Importantly, BMP signaling adds more layers of complexity to regulation of angiogenesis that may help to elucidate this intricate morphological process. The experiment that initiated this entire project involved manipulating the BMP signaling pathway during zebrafish

angiogenesis. Surprisingly, this experiment showed that BMP over-expression robustly induces ectopic angiogenesis. The dramatic BMP-induced angiogenic response was independent of the VEGFA signaling pathway in zebrafish, and restricted to sprouting from the axial vein and other venous beds.

Additional angiogenic stimuli may permit the vascular system to make more sophisticated networks. In a scenario in which two adjacent vessels need to form distinct networks, being responsive to different stimuli may be important. For example, the dorsal aorta and axial vein are spatially juxtaposed during early zebrafish development. My findings demonstrate that VEGFA signaling regulates sprouting from the dorsal aorta while BMP signaling regulates sprouting from the axial vein. In this context, blood vessels are able to make distinct and more complex networks by selectively responding to BMP signaling in the vein during early zebrafish development.

Angiogenesis involves many complex cellular processes. Endothelial cells in an angiogenic vessel must sense the local environment and migrate toward the chemoattractive cues. Many of these cells extend cellular processes and initiate branching, and some of these cells also proliferate. It seems unlikely that one pathway or stimulus is responsible for coordinating these diverse cellular responses. Thus, having growth stimuli that affect different aspects of morphogenesis can increase the flexibility and complexity of responses during tissue morphogenesis.

BMP and VEGFA affect endothelial cell morphogenesis in different ways during angiogenesis. To compare the angiogenic effects of BMP and VEGFA, we manipulated BMP and VEGFA in multiple systems that assay aspects of angiogenesis. Increasing BMP signaling in both zebrafish and in vitro assays of mammalian sprouting lead to the formation of highly branched vascular networks, while blockade of BMP signaling via addition of the BMP antagonist, Noggin, lead to longer vessels that branched less frequently. These findings demonstrate that BMP signaling functions as an important branching

morphogenetic cue. On the other hand, VEGFA over-expression provoked a very different angiogenic response. In general, VEGFA over-expression induced the formation of large dysmorphorphic vascular sheets in both zebrafish and in mammalian assays in vitro. The VEGFA signaling pathway regulates multiple aspects of angiogenesis, such as proliferation, migration, survival and permeability; in contrast our results suggest that BMP signaling has very selective effects on branching morphogenesis.

When we look at tissues *in vivo*, we can make predictions about what the morphology of vessels will be based on the amount of BMP agonist or antagonist present. In the zebrafish, BMP antagonists are strongly expressed in the somites and in the notochord [4-6]. Intersegmental vessels branch from the dorsal aorta and migrate dorsally between these somites. Consistent with predictions, these vessels have a long unbranched morphology. In contrast, sprouts from ventral vein experience low levels of Bmp antagonists and high levels of Bmp2b ligand. Consistent with our model, these vessels form a highly branched vascular plexus (Fig. 4.1).

Another pathway that has been shown to selectively affect branching morphogenesis at the network level is Notch signaling, which exerts these effects through its effects on the tip/stalk cell morphology [7]. Inhibiting Notch increases expression of pro-angiogenic factors such as FLT4, VEGFR2, and Neuropilin while activation of Notch increases expression of anti-angiogenic factors such as Flt1, thereby affecting the cell's relative "sensitivity" to VEGF inputs [8-10]. Interestingly, during early zebrafish development Notch is selectively active in arteries, which are refractory to BMP stimulation. Inhibiting Notch signaling increases the BMP mediated responses in arteries, and Notch activation inhibits BMP-induced angiogenesis in the zebrafish vein. Taken together, these results show that Notch activation restricts the angiogenic effects of BMP. If these findings are extended to the cellular level, it suggests that tip cells should be more responsive to BMP than stalk cells, because tip cells have decreased levels of Notch activity relative to stalk cells [11, 12] .

Notch activity induces the stalk morphology in part by inhibiting FLT4, which we find to be critical during BMP-induced angiogenesis [9, 13, 14]. FLT4, also known as VEGFR3, is highly related to KDR / VEGFR2 and is most well-known for binding to VEGFC to regulate lymphangiogenesis [15]. How does FLT4 regulate BMP-mediated angiogenesis? The easiest explanation is that BMP signaling transcriptionally regulates FLT4 or its ligands. Our morpholino analysis in zebrafish suggests that FLT4 is required for BMP mediated angiogenesis in a VEGFC- and VEGFD-independent manner (data not shown). BMP and FLT4 signaling components may also directly or indirectly interact via post-translational modifications. FLT4 can heterodimerizes with KDR and at the cell membrane in certain contexts [16]. While the presence of these heterodimers is intriguing yet the biological significance is largely unknown. These interactions with KDR have caused me to speculate that FLT4 may also form receptor complexes with BMP receptors. If FLT4 signaling is post translational regulated by BMP signaling, it could serve as a way for endothelial cells to respond to tip/stalk cell decisions faster than through transcriptional regulation alone. In this hypothetical context, Notch would transcriptionally regulates FLT4 expression and BMP signaling could post transcriptionally stimulate FLT4 activity. Future experiments aimed at determining the post translational effects of BMP on FLT4 could provide helpful insight into their interactions.

Notch signaling may intersect directly with the BMP signaling pathway by transcriptionally inhibiting BMP signaling components. Id1 is an important transcription target downstream of BMP signaling. In addition, Id1 itself has been shown to be sufficient at inducing angiogenic responses [17]. Recent studies have shown that Hey1, a downstream transcriptional target of Notch, is regulated by BMP stimulation and inhibits Id1 expression and migration *in vitro* [18]. However, if Id1 is a biologically relevant link between BMP and Notch signaling, remains to be fully elucidated.

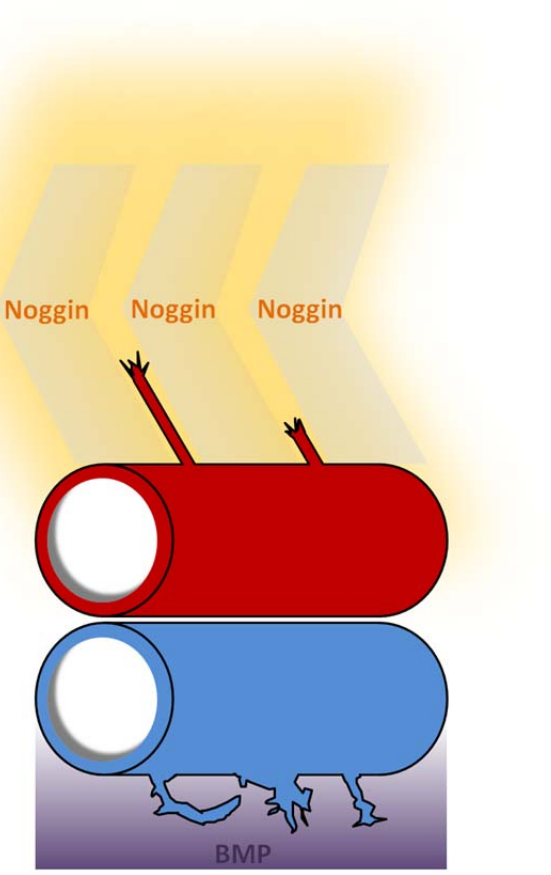
Currently, there are no known interactions between the BMP receptors and the VEGF receptors. However, the small molecule inhibitor, dorsomorphin, has been shown to effectively inhibit ALK2, 3 and VEGFR2 in luciferase and kinase assays [19, 20]. It is formally unknown how this small molecule inhibitor is thought to act, but it is thought to bind in the ATP pocket of receptors. The selective sensitivity of BMP and VEGF signaling to dorsomorphin suggests that they may share intracellular components. Thus the BMP and VEGF signaling pathways function as distinct angiogenic cues that activate similar downstream effectors. Determining which downstream factors are shared between BMP and VEGF signaling pathways may have important therapeutic applications.

BMP stimulus induces robust filopodia and extracellular extensions that likely underlie the morphological effects at the network level. These finding imply that BMP is directly or indirectly regulating cytoskeleton dynamics. BMP has been shown to induce filopodia by transcriptionally increasing Myosin X [21]. In addition, the cytoplasmic tail of BMPR2 directly interacts with many factors including LIMK1, (a kinase which regulates of actin dynamics) [22], Tctex-1 (a light chain of dynein) [23], c-Src (a tyrosine kinase) [24], and Jiraiya (a membrane protein) [25]. Determining what cells and which factors regulate extracellular extensions during sprouting angiogenesis will likely provide interesting insight into the tip/stalk cell paradigm.

In conclusion, my thesis work has helped elucidate important angiogenic roles for BMP in shaping the complex vascular networks observed in vertebrates. These findings helped to elucidate the basic role or BMP signaling during blood vessel morphogenesis, and suggest that applied approaches aimed at manipulating vessel morphology should consider the role BMP signaling during angiogenesis.

FIGURES

Figure 4.1. *In vivo* expression of BMP and BMP antagonists during zebrafish development



In zebrafish embryos Noggin is expressed in somites and the notochord located above the dorsal aorta, and Bmp is expressed below the axial vein. High concentrations of BMP correlate with highly branched angiogenesis while high concentrations of Noggin correlate with long unbrached angiogenic ISA sprouts.

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