Diversity Is in My Veins
Role of Bone Morphogenetic Protein Signaling During Venous Morphogenesis in Zebrafish Illustrates the Heterogeneity Within Endothelial Cells

Jun-Dae Kim,* Heon-Woo Lee,* Suk-Won Jin

Abstract—Endothelial cells are a highly diverse group of cells which display distinct cellular responses to exogenous stimuli. Although the aptly named vascular endothelial growth factor-A signaling pathway is hailed as the most important signaling input for endothelial cells, additional factors also participate in regulating diverse aspects of endothelial behaviors and functions. Given this heterogeneity, these additional factors seem to play a critical role in creating a custom-tailored environment to regulate behaviors and functions of distinct subgroups of endothelial cells. For instance, molecular cues that modulate morphogenesis of arterial vascular beds can be distinct from those that govern morphogenesis of venous vascular beds. Recently, we have found that bone morphogenetic protein signaling selectively promotes angiogenesis from venous vascular beds without eliciting similar responses from arterial vascular beds in zebrafish, indicating that bone morphogenetic protein signaling functions as a context-dependent regulator during vascular morphogenesis. In this review, we will provide an overview of the molecular mechanisms that underlie proangiogenic effects of bone morphogenetic protein signaling on venous vascular beds in the context of endothelial heterogeneity and suggest a more comprehensive picture of the molecular mechanisms of vascular morphogenesis during development. (Arterioscler Thromb Vasc Biol. 2014;34:1838-1845.)

Key Words: bone morphogenetic protein receptors ▪ vascular endothelial cells ▪ veins ▪ zebrafish

Heterogeneity of Endothelial Cells
Endothelial cells provide the innermost lining of blood and lymphatic vessels. Although all endothelial cells share similarities, subtypes of endothelial cells can have distinct differences in morphology, gene expression, function, and developmental origin.1 For example, endothelial cells are predominantly derived from the lateral plate mesoderm, but observations from lineage tracing experiments suggest that endothelial cells can also emerge from outside of the lateral plate mesoderm.2–8 In fact, it seems that the majority of mesoderm possesses the ability to generate endothelial cells.9,10 In addition, subtypes of endothelial cells exhibit distinct molecular and cellular characteristics. Not only well characterized differences among arterial, venous, and lymphatic endothelial cells exist, even endothelial cells within the same vascular bed display distinct gene expression patterns (Figure 1). For instance, the tip and stalk cells within a single angiogenic sprout display distinct molecular profiles11; although the expression of vascular endothelial growth factor receptor 3, DLL4 (delta-like 4), APJ, and UNC5B is elevated in tip cells and attenuated in stalk cells, the expression of Notch and Robo4 displays the reverse trend.12–18 Another intriguing example of the heterogeneity within a single vessel is the recently identified hemogenic endothelium. Although it is not clear how hemogenic endothelium is initially defined, it is apparent that a small group of cells within the dorsal aorta initiate the expression of Runx1 and other key transcription factors for hematopoiesis and adopt their fate as hemogenic endothelium.19–24

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Despite their apparent heterogeneity, all endothelial cells require the aptly named vascular endothelial growth factor-A (VEGF-A) for survival.25 In conjunction with VEGF-A, additional factors including fibroblast growth factor, Notch, Wnt, and several G protein–coupled receptors seem to regulate functions and behaviors of endothelial cells. Recently, bone morphogenetic proteins (BMPs), members of the transforming growth factor-β family, have been recognized as potent modulators of angiogenesis.26 However, because of the large

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number of ligands, receptors, and interacting proteins, it has been difficult to analyze precisely the function of BMP signaling in endothelial cells. Moreover, the high level of heterogeneity within endothelial cells and apparent context dependency of BMP signaling outcomes further complicates the interpretation of the function of BMP signaling in endothelial cells. In this review, we aim to provide a comprehensive overview on the effects of BMP signaling in diverse settings to highlight the heterogeneity of endothelial cells.

**Nuts and Bolts of BMP Signaling**

BMPs represent the largest subgroup of the transforming growth factor-β superfAMILY proteins, which are essential for diverse biological processes including axis formation, osteogenesis, stem cell maintenance, and iron metabolism. Although BMPs are expressed as large precursor proteins of 400 to 500 amino acids which contain an N-terminal signal peptide domain, a prodomain for proper folding, and a C-terminal mature growth factor domain. Unlike structurally related transforming growth factor-β superfamily proteins which are secreted as latent complexes, most BMPs, with the exceptions of BMP7 and BMP9, are secreted in a mature ligand form dissociated from the prodomain by endoproteolytic cleavage. To date, >20 BMP ligands, 4 type I BMP receptors (BMPRIs), 3 type II BMP receptors, and several coreceptors have been identified. The type II receptor kinase is constitutively active in the absence of a ligand. Although it has been suggested that type I and type II receptors can exist as a preformed complex, the presence of a ligand facilitates the formation of the receptor complex, and BMPs elicit diverse cellular responses. During development, the gradient of BMP ligands is modulated by a diverse group of secreted BMP antagonists, which form disulfide bonds with BMP ligands to interfere with the ligand–receptor interaction.

On the surface of the signal receiving cells, dimeric BMP ligands induce formation of a stable tetraheteromeric receptor complex consisting of 2 BMPRIs and 2 type II BMP receptors. To date, >20 type I receptors, activin type I receptor like 1/ALK1, activin type I receptor/ALK2, BMPRIA/ALK3, and BMPRIB/ALK6, and 3 type II receptors, type II BMP receptor, ACTRIIA (activin receptor type IIA), and ACTRIIB (activin receptor type IIB), with similar structural motifs have been identified. The type II receptor kinase is constitutively active in the absence of a ligand.

Arguably, the most well-characterized downstream effectors of BMP signaling are receptor-regulated SMADs (R-SMADs) (SMAD1, 5, and 8). These receptor-activated R-SMADs are released from the receptor complex to form a heterotrimeric complex consisting of 2 R-SMADs and a co-SMAD (SMAD4) which modulate signaling outcomes. On binding to their cognate receptor complex, BMPs elicit diverse cellular responses by regulating downstream effectors.

For instance, SMADs are known to induce the expression of genes, cyclooxygenase 2 (Cox2), and Myo10 (an atypical myosin critical for filopodial formation). BMP signaling seems to activate cell type–specific targets. For example, expression of apelin and its cognate receptor APJ in pulmonary arterial endothelial cells and expression of VEGF-A in osteoblasts are regulated by BMP signaling.
shown to be induced by BMP signaling. BMP ligands are also known to elicit signaling responses by activating other downstream effectors such as Erk1/2 (extracellular signal-regulated kinases), P38, JNK (c-Jun N-terminal kinases), LIMK (LIM domain kinase 1), cofilin, AKT (protein kinase B), PKC (protein kinase C), TcTex, and a variety of Rho-GTPases signaling pathways. However, because of a large number of possible combinations of ligands, receptors, and antagonists, the detailed roles and the mechanism of activation in SMAD-independent responses in endothelial cells remain largely elusive.

Jack of All Trades? Diverse Functions of BMP Signaling in Endothelial Cells

As its name indicates, BMP was first identified as the key regulator that promotes the formation of bone. Over the past 3 decades, BMPs are not only limited to the regulation of bone formation but are indispensable for diverse biological processes including patterning and differentiation of various embryonic structures and maintenance of adult tissue homeostasis. Therefore, it may be more appropriate to consider BMPs as body morphogenesis proteins instead of BMPs.

Consistent with this idea, BMPs have been shown to play essential roles in regulating differentiation and morphogenesis of endothelial cells. During early developmental stages, BMPs are indispensable for induction of the mesoderm where endothelial cells will originate. BMPs also provide key inputs during the patterning of the lateral plate mesoderm and subsequent differentiation of endothelial cells from the lateral plate mesoderm. Perturbations of BMP activity alter the stoichiometry among lateral plate mesoderm–derived cell types, including endothelial cells. For example, ectopic expression of dominant negative Bmpr1a/Alk3 (DN-Bmpr1a) causes increased hematopoietic precursors, suggesting that BMP activity negatively regulates the emergence of endothelial cells in zebrafish, illustrating the importance of BMP signaling in differentiation of endothelial cells. During subsequent morphogenesis, intricate interactions between BMP ligands and their endogenous inhibitors dictate the patterning of developing vessels. For instance, inhibition of BMP signaling in the midline by notochord-derived Noggin creates an avascular region adjacent to the notochord. In addition, another BMP antagonist, has been shown to mediate the fusion of dorsal aortae in avian models.

In mouse, conditional inactivation of BMPRIs or type II BMP receptor within endothelial cells in mice leads to various defects during development. For instance, in mice, deletion of Alk2 or Alk3 in endothelial cells disrupts the formation of cardiac valves, deletion of BMPER, a context-dependent modulator of BMP signaling, causes failure of coronary vessel remodeling, and a lack of BMPR2 in endothelial cells predisposes pulmonary hypertension. Similarly, in zebrafish, a lack of alk3a or alk3b causes severe disruption in cardiovascular development, and mutations in alk1 causes failure in cranial vessel remodeling. More recently, it has been shown that BMP signaling also regulates the emergence of lymphatic endothelial cells during development. During development, BMP2 seems to function as an antilymphangiogenic agent by inducing expression of miRNAs which in turn negatively regulate
the stability of Prox1 mRNA. In contrast, BMP9 seems to provide prolymphangiogenic cues during development.

As shown in other vertebrates, mutations of various BMP signaling components have been linked to various pathological conditions that cause vascular dysfunction and diseases such as hereditary hemorrhagic telangiectasia (HHT) and pulmonary arterial hypertension (PAH) in humans. HHT, also known as Osler–Weber–Rendu disease, is a genetic disorder that leads to abnormal blood vessel formation in the skin, mucous membranes, and often in organs such as the lungs, liver, and brain. HHT type 1 results from pathogenic mutations in ENG (endoglin) that causes haplosufficiency of endoglin, whereas HHT type 2 is caused by a loss of function or dominant negative mutations in ALK1. Similarly, genetic manipulation of endoglin and ALK1 in mice mirrors many of the characteristics of HHT. PAH is an increase in blood pressure in the pulmonary artery leading to shortness of breath, dizziness, fainting, leg swelling, and can even lead to heart failure. The principal gene associated with PAH is BMPR2. Loss of function in BMPR2 is found in >70% of patients with hereditary PAH and 20% of patients with idiopathic PAH. Mutations in various regions of BMPR2 including the ligand-binding domain, the kinase domain, and the long cytoplasmic tail may be associated with PAH. Among them, the long cytoplasmic tail region might play an important role in PAH because mice expressing a smooth muscle–specific BMPR2 tail deletion mutation show pulmonary vascular pruning leading to elevated right ventricular systolic pressures, which is associated with PAH. The tail region is known to regulate p38 and p42/44 MAPK (mitogen-activated protein kinases) signaling, but until recently, little was known about how these signals are developed. In addition, many other BMP components are also known to be related to vascular diseases such as HHT and PAH. It is apparent that BMP signaling provides an essential function in modulating development and maintenance of endothelial cells.

**BMP Signaling in Venous Vascular Beds**

During development, venous fate is often considered the default status. Although arterial cells must undergo differentiation processes orchestrated by multiple signaling cascades, including Hedgehog, VEGF, Notch, and EphrinB2 signaling, and transcription factors, such as Forkhead box C1 and C2 (Foxc1/2), it is unclear how venous fate is determined. To date, COUP-TFII COUP transcription factor 2, an orphan nuclear receptor, is the only factor that is implicated as essential for the specification of venous endothelial cells. Moreover, the majority of widely used venous markers such as EphB4, Dab2, and NRP2 (neuropilin-2) are gradually restricted to venous endothelial cells during development and therefore are not bona fide markers at early stages, which further complicates the analyses of the specification of venous endothelial cells in early development.

However, this concept was challenged by a series of reports which collectively present a scenario, whereby venous endothelial cells are derived from a unique subset of endothelial progenitors during development. First, venous endothelial cells seem to undergo distinct morphogenetic processes to form the axial vein in zebrafish. More recently, a similar observation was reported in mouse embryos. More definite evidence indicating that venous endothelial cells may not be the default fate of endothelial cells came from lineage tracing experiments in zebrafish. This new evidence indicates that progenitors for venous endothelial cells are localized to a distinct area in developing embryos. Compared with the arterial endothelial progenitors which are preferentially located more medially within the lateral plate mesoderm, venous endothelial progenitors seem to be located laterally even in early developmental stages in zebrafish. Moreover, only arterial endothelial progenitors, not venous endothelial progenitors, migrate medially in response to VEGF and Hedgehog signaling activities forming the dorsal aorta, indicating that the signaling that governs the behaviors of venous endothelial progenitors may also be distinct.

We have previously shown that BMP2 signaling provides a venous specific cue for sprouting angiogenesis. Manipulation of BMP2 signaling during early vascular development causes severe disruption in the formation of the cardinal vein plexus, the vascular beds generated by sprouting angiogenesis of the cardinal vein. In addition, an excessive level of BMP2 signaling in zebrafish embryos leads to the formation of exuberant angiogenic sprouts from venous endothelial cells in the cardinal vein, whereas arterial...
endothelial cells in the adjacent dorsal aorta do not respond to BMP2 signaling. Interestingly, VEGF-A signaling does not seem to be essential for the morphogenesis of venous vascular beds in zebrafish. Although attenuation of VEGF-A signaling increases apoptosis in venous endothelial cells, it does not cause discernible changes in venous morphogenesis. Moreover, only arterial endothelial cells, but not venous endothelial cells, undergo sprouting angiogenesis in response to ectopic expression of VEGF-A signaling (Figure 3).117 Taken together, it seems that BMP2 signaling, not VEGF-A signaling, provides the major morphogenetic cue within venous vascular beds in zebrafish.

How does BMP signaling preferentially activate venous endothelial cells without affecting arterial endothelial cells in zebrafish? Previous reports suggest that venous endothelial cells in zebrafish may be innately sensitized to BMP signaling. First of all, both orthologs of BMP2 in zebrafish, bmpr2a and bmpr2b, are enriched in venous endothelial cells during development, possibly increasing venous endothelial cells sensitivity to BMP signaling.117,118 Moreover, a cargo-cellular function in venous endothelial cells. VEGFR indicates vascular endothelial growth factor receptor.

Beyond Venous Vascular Beds

Beyond developmental stages, BMP signaling also provides critical functions for vascular homeostasis in postnatal stages. During wound healing, it has been shown that BMP signaling is highly upregulated in keratinocytes, fibroblasts, and macrophages within the vicinity of wounds.120 In addition, BMP signaling has been implicated in angiogenesis associated with pathological conditions. For instance, expression of BMP ligands is often elevated with a concomitant decrease of BMP antagonists in renal cancer.121,122 Considering that treatments with VEGF inhibitors, such as Sorafenib (tyrosine kinase inhibitor) and Bevacizumab (monoclonal antibody for VEGF), do not provide significant long-term improvements for patients with tumor, other proangiogenic pathways such as BMP may compensate for the loss of VEGF signaling.123 Interestingly, the outcomes of BMP signaling are highly context dependent and can reflect the innate differences among endothelial cells (ie, tumor associated versus normal endothelial cells).124 Further analyses on the functions of BMP signaling within endothelial cells will enable us to develop therapeutic applications to target BMP signaling specifically in tumor-associated endothelial cells. Targeting VEGF-A has been shown to be problematic because it is essential for the survival of all endothelial cells. Therefore, targeting BMP signaling, which exerts context-dependent effects in endothelial cells, may dampen the side effects of antiangiogenic therapy while increasing the efficacy of treatment in patients with tumor.

Because of a large number of ligands and receptors, and their promiscuous interactions, the effects of BMP signaling in endothelial cells are described as highly context dependent. Inconsistent and often contradictory results among published reports further complicate the analyses on the effects of BMP signaling. Because we have just begun to interrogate diverse functions of BMP signaling in endothelial cells, detailed and comprehensive analyses on the function of BMP signaling may help us to clear up confusion and misinterpretations and suggest a more refined model of how BMP signaling works in endothelial cells which accurately describe context dependency of the signaling outcomes. For instance, it is currently unknown how pro- and antiangiogenic BMP signaling, both of which rely on SMADs activity, can be distinguished.
in signaling receiving endothelial cells. Moreover, with a notable exception with Notch and BMP signaling interaction, it is not clear how BMP signaling intersects with other signaling pathways to regulate vascular development and maintenance. With further investigation on the roles of BMP signaling in endothelial cells in health and diseases, we will be able to harness fully the potential of BMP signaling as a therapeutic target.

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