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Bone Morphogenetic Proteins in Vascular Homeostasis and Disease

Marie-José Goumans,[1] An Zwijsen,[2,3] Peter ten Dijke,[1,4] and Sabine Bailly[5,6,7]

[1]Department of Molecular Cell Biology, Leiden University Medical Center, 2300 RC Leiden, The Netherlands

[2]VIB Center for the Biology of Disease, 3000 Leuven, Belgium

[3]KU Leuven Department of Human Genetics, 3000 Leuven, Belgium

[4]Cancer Genomics Centre Netherlands, Leiden University Medical Center, 2300 RC Leiden, The Netherlands

[5]Institut National de la Santé et de la Recherche Mécale (INSERM), U1036, 38000 Grenoble, France

[6]Laboratoire Biologie du Cancer et de l'Infection, Commissariat à l'Énergie Atomique et aux Energies Alternatives, Biosciences and Biotechnology Institute of Grenoble, 38000 Grenoble, France

[7]University of Grenoble Alpes, 38000 Grenoble, France

Correspondence: m.j.t.h.goumans@lumc.nl; sabine.bailly@cea.fr

It is well established that control of vascular morphogenesis and homeostasis is regulated by vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), Delta-like 4 (Dll4), angiopoietin, and ephrin signaling. It has become clear that signaling by bone morphogenetic proteins (BMPs), which have a long history of studies in bone and early heart development, are also essential for regulating vascular function. Indeed, mutations that cause deregulated BMP signaling are linked to two human vascular diseases, hereditary hemorrhagic telangiectasia and pulmonary arterial hypertension. These observations are corroborated by data obtained with vascular cells in cell culture and in mouse models. BMPs are required for normal endothelial cell differentiation and for venous/arterial and lymphatic specification. In adult life, BMP signaling orchestrates neo-angiogenesis as well as vascular inflammation, remodeling, and calcification responses to shear and oxidative stress. This review emphasizes the pivotal role of BMPs in the vascular system, based on studies of mouse models and human vascular disorders.

Bone morphogenetic proteins (BMPs) were originally discovered based on their capacity to induce bone and cartilage formation at ectopic sites (Urist 1965). As their pleiotropic functions have become apparent, it has been proposed to call them body morphogenetic proteins (Reddi 2005). BMPs comprise a dozen of the 33 known polypeptides of the transforming growth factor-β (TGF-β) family (Bragdon et al. 2011; Katagiri and Watabe 2016). Most BMPs have been reported to elicit some effects on vascular cells. Based on their sequence similarity, receptor affinities and function, the BMP ligand polypeptides are classified into several subgroups including the BMP-2 and -4 group; BMP-5, -6, -7, -8a, -8b group; BMP-9, -10

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group; and BMP-3, -3b, -11, -12, -13, -14, -15, and -16 group (Katagiri and Watabe 2016; Morikawa et al. 2016). Among the different BMPs, BMP-2, BMP-4, BMP-6, and BMP-7 have been shown to function in vascular biology. Additionally, BMP-9 and -10 have emerged as crucial factors in endothelial function and vascular diseases (David et al. 2009).

Several reviews discuss the emerging role of BMPs in vascular remodeling, focusing on vascular biology (Garcia de Vinuesa et al. 2016; Morrell et al. 2016) and their molecular targets (Luo et al. 2015). Here, we will describe human vascular disorders and animal models that have contributed to our understanding of the role of BMP signaling in cardiovascular diseases and that may help us to find more effective treatment modalities. A review on TGF-β signaling in control of cardiovascular function has also been published (Goumans and ten Dijke 2017).

VASCULAR DEVELOPMENT IN A NUTSHELL

The vascular system circulates blood and lymph throughout the body and is critical for tissue growth, homeostasis and repair. Blood vessels supply tissues with oxygen and nutrients, whereas lymphatic vessels absorb and filter interstitial fluids from these tissues. Blood vessels comprise endothelial cells (ECs) and mural cells, such as vascular smooth muscle cells (VSMCs) and pericytes. Interactions and interplay between ECs and mural cells play pivotal roles in vascular biology (Risau 1997; Carmeliet and Jain 2011).

Vessel formation during embryonic development begins with vasculogenesis (i.e., the de novo formation of blood vessels from locally differentiating ECs). This is followed by angiogenesis, which entails remodeling of the honeycomb-like pattern of the primary plexus into a stable branched network of vessels. The vasculature further expands through sprouting angiogenesis (i.e., formation of new blood vessels from pre-existing ones) and vascular pruning (Eilken and Adams 2010; Korn and Augustin 2015). Capillary sprouting requires interactions among three different EC subtypes—tip cells, stalk cells, and quiescent phalanx cells—that each perform highly specific functions (De Smet et al. 2009). ECs can transition between subtypes, which is termed EC plasticity. During the activation phase of angiogenesis, increased vascular permeability and basement membrane degradation allows EC migration and proliferation into the extracellular space. These new sprouts are spearheaded by the tip cell and elongated by proliferation of the trailing stalk cells. In the resolution phase of angiogenesis, EC migration and proliferation are stalled, the basement membrane is reconstituted, and vessel maturation is promoted. Lumen formation will occur via fusion of neighboring sprouts (Gebala et al. 2016). Next, mesenchymal cells are recruited and subsequently differentiate into pericytes or VSMCs, which are attached to the newly formed vessel (Eilken and Adams 2010; Korn and Augustin 2015). Although blood vessels predominantly remain quiescent throughout adult life, they retain the capacity to rapidly form new vasculature in response to injury or pathological conditions. During this process, transitions between the resolution phase and the activation phase of angiogenesis are determined by an intricately regulated balance between the inducers and the inhibitors of these phases.

Angiogenesis requires the coordinated integration of multiple signaling cascades, including the vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), Notch/Delta-like protein 4 (Dll4), angiopoietin, ephrin, TGF-β, and BMP pathways. The BMP pathway plays an important role in maintaining vascular homeostasis and affects several processes, including the endothelial responses to shear stress, hypoxia, and inflammation.

BONE MORPHOGENETIC PROTEINS

BMP ligands are synthesized as dimers of large proproteins that include an amino-terminal signal peptide, a long propeptide, and a carboxy-terminal mature peptide that contains a cystine knot (Mueller and Nickel 2012). These BMP proproteins are proteolytically cleaved by proconvertases to generate the active dimeric form (Miyazono et al. 2010). Although BMP process-

ing is predominantly intracellular, it might also occur at the plasma membrane or extracellularly (Yadin et al. 2016). Dimerization to generate the active ligand requires a covalent disulphide bridge at the seventh conserved cysteine within each monomer outside of the cystine knot structure formed by the six other cysteines. Active BMPs are homodimeric but some heterodimers (i.e., BMP-2/5, BMP-2/6, and BMP-2/7) are more potent than the corresponding homodimers in certain experimental settings (Sieber et al. 2009). Mature BMP dimers remain noncovalently associated with their cleaved prodomains. The fact that prodomains confer latency to TGF-β (and some other family members) but not to the BMPs remains unresolved and is currently being investigated for certain BMPs, including BMP-10 (Sengle et al. 2011; Jiang et al. 2016b; Yadin et al. 2016). The prodomain of BMP-9 does not inhibit its biological activity and is rapidly released from the mature protein on binding to the receptors (Kienast et al. 2016). Posttranslational modifications, including N- and O-glycosylation, strongly influence BMP activity, because glycosylation is important for BMP secretion, prolongs a BMP's half-life, and modulates BMP receptor binding and function (Saremba et al. 2008).

Regulation of cell differentiation, proliferation, and apoptosis require appropriate ligand concentration and activity, as well as tight regulation of the local availability of BMPs (Umulis et al. 2009). Upon secretion, ligand binding to extracellular matrix proteins may induce a conformational change that prevents ligand access to the BMP receptors (Wohl et al. 2016). Some prodomains (e.g., of BMP-2, -4, -7, and -10, and growth and differentiation factor [GDF-]5) form stable complexes with extracellular proteins (e.g., fibrillin-1 or collagen IV), thereby restricting their diffusion in the extracellular space (Sengle et al. 2008; Wang et al. 2008; Ramirez and Rifkin 2009). BMP-4, BMP-6, BMP-9, and BMP-10 are detected in the circulation, and can therefore affect tissues and organs at a distance, as well as the endothelial lining of the vessels, through luminal activation of BMP receptors (David et al. 2008; Souza et al. 2008; Herrera and Inman 2009; Bidart et al. 2012).

A well-established mechanism for regulating the extracellular availability of BMPs involves a gradient of BMP antagonists that prevents BMP binding to receptors and BMP action (Chang 2016; Hinck et al. 2016). Multiple secreted proteins (e.g., noggin, chordin, cerberus, matrix Gla protein [MGP], and follistatin) can sequester BMP ligands and impair their interactions with receptor complexes. Other interacting proteins, such as cross-veinless 2, also known as BMP-binding EC precursor-derived regulator or BMPER, may exert more complex effects by either decreasing or increasing BMP signaling (Yao et al. 2012). BMPER was originally identified in a screen for proteins that are differentially expressed in embryonic endothelial precursor cells (Moser et al. 2003), suggesting its potential role in vascular remodeling (see below). These different antagonists and agonists bind to BMPs with different affinities, and can preferentially interfere with one family member and not others. For example, noggin binds with strong affinity to BMP-2, BMP-4, and BMP-7, with lower affinity to BMP-6, and not to BMP-9 and BMP-10 (David et al. 2008; Song et al. 2010). Also, MGP can either inhibit or enhance the BMP-2 and BMP-4 activity, depending on its concentration (Zebboudj et al. 2002; Yao et al. 2006).

BMP SIGNALING

BMPs bind as dimers at the cell surface to heterotetrameric receptor complexes that include two types of dual specificity kinase receptors (Table 1, Fig. 1): the BMP type I receptors (i.e., activin receptor-like kinase [ALK]-1, ALK-2, ALK-3, and ALK-6) and the BMP type II receptors (i.e., BMPRII, ActRIIA, and ActRIIB) (Yadin et al. 2016). These type I and type II receptors bind a variety of BMP ligands, whereas the activin type II receptors also bind activins (Upton and Morrell 2009). Regardless of the receptor combination, the type II receptor contains a constitutively active kinase domain, even in the absence of ligand. Binding of intracellular FK506-binding protein (FKBP) 12 acts as a gatekeeper mechanism, setting a threshold for type I receptor activation by the

Table 1. Bone morphogenetic protein (BMP) ligands and their receptors

BMP	Type II	Type I	R-Smad	Coreceptor
BMP-2	BMPRII	ALK-3	Smadl, 5, 8	RGM
BMP-4		ALK-6		
	ACTRIIA/B	ALK-3	Smadl, 5, 8	
		ALK-6		
BMP-6	BMPRII	ALK-3	Smadl, 5, 8	RGM
BMP-7		ALK-6		
	ACTRIIA/B	ALK-2	Smadl, 5, 8	
		ALK-3		
		ALK-6		
BMP-9	BMPRII	ALK-1	Smadl, 5, 8	Endoglin
BMP-10		ALK-2		
	ACTRIIA/B	ALK-1	Smadl, 5, 8	
			Smad2	

BMPs bind to a combination of type I and type II receptors resulting in the carboxy-terminal phosphorylation and activation of Smad proteins and Smad-mediated modulation of gene transcription. Although there is redundancy, BMPs have a preference related to which receptor complex they bind and which coreceptor may modulate the signal.

BMP, bone morphogenetic protein; ALK, activin receptor-like kinase, BMPRII, BMP type II receptor; ActRIIA/B, activin type II receptor A or B; RGM, repulsive guidance molecules.

type II receptor in the absence of ligand (Huse et al. 1999).

BMP Receptors

BMPs are classified into different subgroups based on their sequence similarity and affinities for specific receptors (Table 1) (Bragdon et al. 2011; Katagiri and Watabe 2016). BMP-2 and BMP-4 preferentially bind to ALK-3 and ALK-6 (also known as BMPRIA and BMPRIB, respectively), and BMPRII or ActRIIA/B. BMP-6 and BMP-7 bind to ActRIIA and form a complex with ALK-2, also known as ActRI. BMP-9 and BMP-10 bind with high affinity to ALK-1, also known as TSR-I or SKR3, in combination with BMPRII, ActRIIA, or ActRIIB. Moreover, BMP-9 binds ALK-2 only in the presence of a type II receptor, whereas BMP-10 cannot bind to ALK-2 (Scharpfenecker et al. 2007; Olsen et al. 2014). Interestingly, ALK-2 can form a heterodimer with other type I receptors, thereby increasing the signaling complexity (Yadin et al. 2016). Various BMPs show different affinities for their receptors. The binding affinities of BMP-9 and BMP-10 for ALK-1 are in the picomolar range, which is exceptionally high (Townson et al. 2012; Kienast et al. 2016) compared with the binding affinities of the other BMPs for their type I receptors (Mahlawat et al. 2012). On the other hand, BMP-9 shows substantial differences with BMP-10 in type II receptor binding. BMP-9 binds most strongly to ActRIIB, and shows weaker binding to BMPRII and weakest binding to ActRIIA, although these data remain to be confirmed in ECs (Townson et al. 2012; Kienast et al. 2016). In contrast, BMP-10 does not show such a clear preference for the different type II receptors (Townson et al. 2012).

BMP Coreceptors

The binding of a BMP to its receptor can be modulated by coreceptors, which lack an intrinsic signaling motif (Table 1, Fig. 1). Two transmembrane receptors, betaglycan, also called the TGF-β type III receptor or TβRIII, and endoglin play roles in the vasculature. Betaglycan increases the binding of BMP-2, -4, -7, and -14 to ALK-3 and ALK-6 (Kirkbride et al. 2008), whereas endoglin has been shown to promote or repress signaling via distinct mechanisms that depend on the levels of endoglin and the concentrations of receptors and ligands (ten Dijke et al. 2008). Endoglin interaction with ALK-1 increases the responses to BMP-9

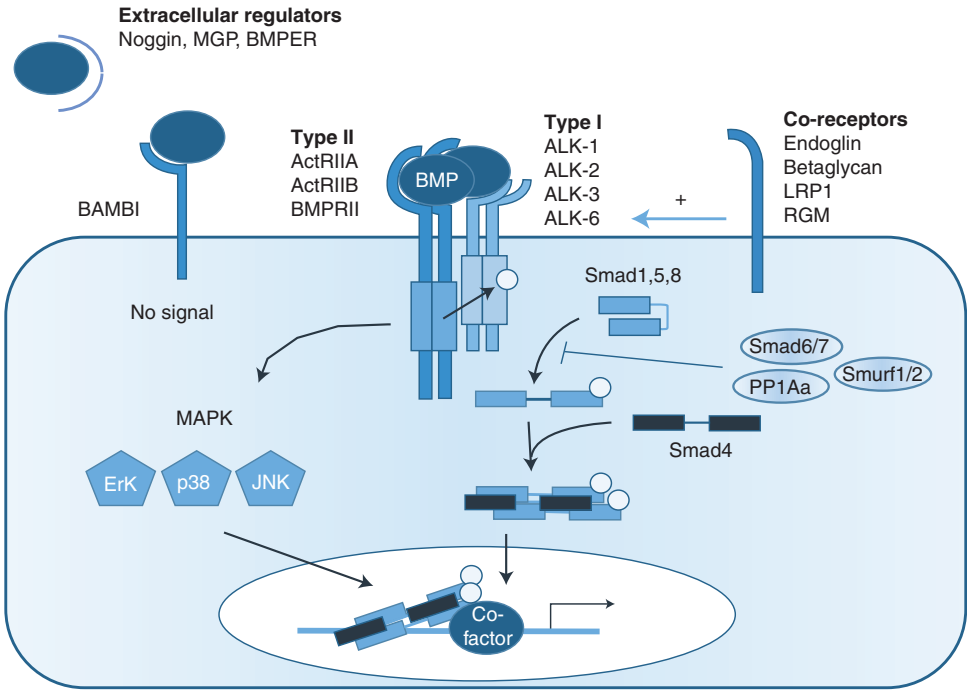

Figure 1. Bone morphogenetic protein (BMP) signaling pathway. Schematic representation of the BMP signaling pathway. BMPs interact with specific type I and type II receptors to form a heterotetrameric complex. Complex formation and ligand binding can be potentiated by a coreceptor—that is, endoglin, betaglycan, lipoprotein receptor-related protein 1 (LRP-1), or repulsive guidance molecule (RGM). After complex formation, the type II receptor phosphorylates the type I receptor, which then carboxy-terminally phosphorylates Smad1, Smad5, and Smad8 (canonical BMP signaling). Phosphorylated Smads propagate the signal via complex formation with Smad4, translocation into the nucleus, and regulation of the expression of target genes. Besides Smad-dependent signaling, BMPs can also transduce signals by mitogen-activated proteins kinases (MAPKs) (noncanonical BMP signaling). Canonical BMP signaling is intracellularly inhibited by inhibitory Smads—that is, Smad6 and/or Smad7, E3 ubiquitin ligases, such as Smurf1 or Smurf2, or phosphatases (PPA1α). BMPs are extracellularly inhibited from binding to the receptor complex by secreted inhibitors, like noggin, matrix Gla protein (MGP), and the decoy receptor BAMBI. BMP signaling can be extracellularly stimulated or inhibited by BMPER.

and BMP-10 (Blanco et al. 2005; David et al. 2007; Scharpfenecker et al. 2007; Castonguay et al. 2011; Alt et al. 2012). The present model is that endoglin and ALK-1 at the cell surface act together to bind and capture BMP-9, and that subsequent BMP-9 binding to the type II receptor displaces the bound endoglin to form a ligand-bound type I and type II receptor signaling complex. The affinity of BMP-9 for endoglin is in the nanomolar range (Castonguay et al. 2011; Kienast et al. 2016). Endoglin can also form a complex with the TGF-β type II receptor, and either promote or inhibit TGF-β-induced Smad1 and Smad5 phosphorylation by ALK-1 (Lebrin et al. 2004; Pece-Barbara et al. 2005). The interaction of endoglin with ALK-1 is also enhanced by fibronectin and integrin $\alpha_5\beta_1$ leading to increases of BMP-9- and TGF-β-induced phosphorylation of the BMP signaling effectors Smad1, Smad5, and Smad8 (Tian et al. 2012). Endoglin can be shed from the cell surface through the actions of matrix metalloproteinases, and the soluble endoglin ectodomain may thereby act as a released receptor trap for BMPs that can affect vascular remodeling (Venkatesha et al. 2006; Hawinkels et al. 2010; Rathouska et al. 2015; Gallardo-Vara et al. 2016). Soluble endoglin was also shown to scavenge

BMP-9 and prevent BMP-9-induced Smad1, Smad5 and Smad8 phosphorylation, whereas it cannot bind TGF-β (Gregory et al. 2014).

BMP signaling is potentiated by the glycosylphosphatidylinositol-anchored repulsive guidance molecule (RGM) proteins (Corradini et al. 2009) and low-density lipoprotein receptor-related protein 1 (LRP-1) (Pi et al. 2012), whereas the decoy receptor BMP and activin membrane-bound inhibitor (BAMBI) sequesters ligands from type I receptors and inhibits BMP signaling in ECs (Onichtchouk et al. 1999; Guillot et al. 2012).

Heterotetrameric BMP Receptor Complexes

Although BMP receptors are widely expressed, not all cell types express all BMP receptors, and the type I–type II receptor combinations for each BMP may therefore be cell-type specific. The available type I, type II, and type III receptors will determine which BMP will preferentially signal and what the cellular response will be. ALK-2 and ALK-3 are widely expressed, whereas ALK-1 is more selectively expressed on ECs, and ALK-6 is expressed by only a few cell types (Garcia de Vinuesa et al. 2016). BMPRII and ActRIIA are ubiquitously expressed, whereas ActRIIB expression is restricted to certain cell types, including ECs. Among these type II receptors, only BMPRII is highly expressed in ECs. The high binding affinity of BMP-9 and BMP-10 for ALK-1 and BMPRII, together with the high ALK-1 and BMPRII expression levels in ECs, may explain the major role of these two BMPs in angiogenesis. ALK-1 may also form a complex with ActRIIA or ActRIIB enabling BMP-9- or BMP-10-induced activation (David et al. 2007; Scharpfenecker et al. 2007). BMP-2 and BMP-4 bind to ALK-3 or ALK-6; however, because ALK-3 and ALK-6 are not highly expressed in ECs, BMP-2 and BMP-4 are unlikely to induce strong responses in these cells. BMP-6 and BMP-7 bind to ActRIIA and ALK-2, thus inducing activation of Smad1 and Smad5. VSMCs express BMPRII and ALK-3 at high levels, whereas ALK-1 and endoglin are not highly expressed in VSMCs under physiological conditions (Upton et al. 2008). BMPRII and ALK-3 mediate responses to BMP-2 and BMP-4 in pulmonary arterial (PA) smooth muscle cells (SMCs). ALK-1, ALK-2, BMPRII, ActRIIA, and ActRIIB are also expressed in cultured murine VSMCs, and treatment with BMP-9 induces calcification (Zhu et al. 2015).

BMP Signal Transduction

On formation of a ligand–receptor complex, type II receptors activate type I receptors through phosphorylation of serine and threonine residues in their glycine-serine-rich (GS) domain. Activated type I receptors phosphorylate and thus activate the receptor-regulated Smads (R-Smads), which then form heteromeric complexes with the common mediator Smad, Smad4 (Fig. 1) (Hill 2016; Xu et al. 2016). These complexes translocate to the nucleus where they regulate target gene transcription by binding to Smad-binding elements (SBEs) and interacting with other coactivators or corepressors (Ross and Hill 2008). The specific type I receptor in the ligand–receptor complex determines which R-Smads are activated. Most BMPs induce phosphorylation of Smad1, Smad5, and Smad8 (also named Smad9), although, in pulmonary ECs, BMP-9 can also activate Smad2, which is normally associated with TGF-β signaling (Upton et al. 2009). The underlying mechanism for this Smad2 response remains to be determined. The Smad-dependent pathway is directly activated by the type I receptor, although Smads may also be activated by alternative mechanisms that must be further explored. Indeed, TGF-β-activated kinase 1 (TAK1), which is generally thought to be involved in Smad-independent pathways, has been reported to phosphorylate Smad1 at the carboxyl terminus (Shim et al. 2009).

On ligand binding, BMP receptors can also activate non-Smad pathways that are important for correct vascular system establishment (Zhang 2017). Such non-Smad pathways include signaling by mitogen-activated proteins kinases (MAPKs), such as p38, Erk, and JNK (Gallea et al. 2001; Guicheux et al. 2003), the phosphatidylinositol 3-kinase (PI3K)—Akt pathway and small Rho-like GTPases (Gamell

et al. 2008). Cross-talk between VEGF and BMP-6 can enhance p38 MAPK activation and inhibit Akt activation, but does not impact the carboxy-terminal phosphorylation of Smad1, Smad5 and Smad8 or Erk MAPK activation (Li et al. 2015a). BMP-9 inhibits JNK activation in ECs (Long et al. 2015). Endoglin and Gα-interacting protein carboxyl terminus interacting protein (GIPC)-mediated trafficking of PI3K regulates endothelial signaling and function (Lee et al. 2008, 2012). The BMPRII carboxy-terminal extension also interacts with effectors that can be involved in non-Smad signaling (Hassel et al. 2004; Chan et al. 2007). BMP signaling pathways interact with other growth factor pathways that are essential for vascular development and homeostasis, including the FGF, Notch, and WNT pathways, and possibly also the Hippo pathway (Guo and Wang 2009; Garcia de Vinuesa et al. 2016). BMPs, together with other vascular signaling pathways, also interact with vascular endothelial (VE) cadherin, an essential component of endothelial adherent junctions, which plays a crucial role in vascular permeability (Weis and Cheresh 2005; Lagendijk and Hogan 2015). It has been shown that VE-cadherin can associate with ALK-2 and BMPRII in a BMP-6-dependent manner (Benn et al. 2016). BMP-6 induces c-Src phosphorylation leading to VE-cadherin internalization and an increase in vessel permeabilization.

BMP Signal Termination

As the duration of BMP signaling will determine its effects in vascular development and disease, it has to be tightly controlled (Fig. 1). BMP signaling can be terminated in many ways. Inhibitory Smads (I-Smads, Smad6, and Smad7) inhibit BMP signaling through various mechanisms, including interfering with the interactions between R-Smads and type I receptors, down-regulation of cell surface type I receptors, prevention of complex formation by R-Smads and co-Smads, and transcriptional regulation in the nucleus (Miyazawa and Miyazono 2016). For example, inhibitory Smads can recruit the Smad E3 ubiquitin ligases Smurf1 or Smurf2 to the type I receptors, thus promoting receptor degradation (Kavsak et al. 2000; Ebisawa et al. 2001; Murakami et al. 2003). Smad6 and Smad7 can also recruit protein phosphatases, such as protein phosphatase 1 α (PPA1α), to dephosphorylate ALK-1 (Valdimarsdottir et al. 2006). Interestingly, Smad6 and Smad7 were originally identified as vascular Smads because their expression is induced in ECs by laminar shear stress (Topper et al. 1997). The expression of both inhibitory Smads is induced by BMPs, thus creating a negative feedback loop (Ishisaki et al. 1999). Smad7 inhibits both BMP and TGF-β signaling by competing with recruitment of phosphorylated R-Smads and by promoting the degradation of the type I receptors, whereas Smad6 more selectively inhibits BMP signaling by recruiting Smurf1 to BMP type I receptors or by competing with R-Smad for binding to Smad4 (Hata et al. 1998; Murakami et al. 2003).

BMP SIGNALING MUTATIONS IN VASCULAR DISEASES

Two human genetic vascular disorders have been linked to mutations in BMP signaling components showing that BMPs are key players in vascular biology. Hereditary hemorraghic telangiectasia (HHT) or Osler–Weber–Rendu syndrome has been linked mostly to mutations in *ENG*, the gene encoding endoglin, and in *ACVRL1*, which encodes the ALK-1 receptor. The second vascular disease caused by mutations in a BMP signaling component is hereditary pulmonary arterial hypertension (hPAH). Mutations in *BMPR2*, and occasionally in *ACVRL1* or *ENG*, have been linked to hPAH. Fibrodysplasia ossificans progressiva (FOP) is a rare autosomal-dominant disorder that is mainly characterized by episodic heterotopic ossification (HO) of muscle, fascia, ligaments, and tendons. FOP is driven by activating mutations in *ACVR1* in the coding region of the ALK-2 intracellular domain. It is under debate whether this disease categorizes as a vascular disease.

Hereditary Hemorrhagic Telangiectasia

The first report describing that disrupting BMP signaling can cause a vascular disorder was the

identification of mutations in *ENG* that led to the haploinsufficient vascular disease HHT1 (Fig. 2) (McAllister et al. 1994). Soon after, mutations in *ACVRL1*, which encodes ALK-1, were identified in HHT2 (Johnson et al. 1996). Several years later, *SMAD4* mutations in combined juvenile polyposis-HHT syndrome (JP-HHT) were reported (Gallione et al. 2004). Mutations in *GDF2*, which encodes BMP-9, have now also been described in a related form of HHT, HHT5 (Wooderchak-Donahue et al. 2013; Hernandez et al. 2015). HHT is a rare autosomal-dominant vascular disorder (incidence of 1/8000) (McDonald et al. 2015), which is characterized by frequent epistaxis, skin and mucosa telangiectasia, arteriovenous malformations (AVMs) in the lungs, liver or brain, and hemorrhages associated with these vascular lesions (Dupuis-Girod et al. 2010; Shovlin 2010). HHT1 patients more commonly show pulmonary and cerebral AVMs, whereas HHT2 patients show higher incidences of hepatic AVMs and gastrointestinal telangiectasia (Letteboer et al. 2006; Lesca et al. 2007). These differences may be partly explained by the differential distribution and expression levels of BMP signaling components in these tissues, suggesting overlapping but nonidentical functions for endoglin and ALK-1. AVMs are thought to arise from enlargement of capillary vessels, creating a shunt between an artery and a vein, although the molecular mechanism is still not completely understood (Fig. 2) (Park et al. 2009). It has been proposed that idiopathic AVM and Notch-induced AVMs are distinct from those of HHT (Peacock et al. 2016).

Pulmonary Arterial Hypertension

The second vascular disease reported with mutations in BMP signaling is pulmonary arterial hypertension (PAH), which is mainly due to mutation in *BMPR2*, which encodes the BMPRII receptor (Deng et al. 2000; Lane et al. 2000). PAH is a rare vascular disorder with an incidence of 1/25,000, and is characterized by an elevated mean resting pulmonary arterial pressure of >25 mmHg, with normal left atrial pressure and high pulmonary vascular resistance (Simonneau et al. 2013). The increased pulmonary vascular resistance in PAH is attributed to small pulmonary artery constrictions caused by profound vascular remodeling (Guignabert et al. 2015). ECs, VSMCs, and fibroblasts show aberrant proliferation and resistance to apoptosis, resulting in reduced luminal diameter, increased blood pressure and, ultimately, lethality from right ventricular failure (Fig. 3).

Three forms of hypertension have been defined: idiopathic PAH (iPAH), familial or heritable PAH (hPAH), and environmental PAH. Approximately 70% of hPAH patients show mutations in the *BMPR2* gene, and ~25% of sporadic iPAH cases show de novo *BMPR2* mutations. Mutations in *BMPR2* have been identified in nearly all exons of the coding sequence—including exons encoding parts of the ligand binding domain, kinase domain, and cytoplasmic tail. The vast majority of *BMPR2* mutations are frame-shift mutations leading to a premature stop codon and resulting in haploinsufficiency owing to nonsense-mediated decay of mutant BMPRII mRNA transcripts (Fessel et al. 2011). The remaining mutations lead to loss of function via a variety of mechanisms, including loss of kinase activity and impairments of receptor folding and trafficking (Rudarakanchana et al. 2002). A meta-analysis of data of 1550 patients with PAH revealed that PAH patients harboring a *BMPR2* mutation present at a younger age a more severe disease and carry an increased risk of death (Evans et al. 2016). There is no full penetrance of the disease onset, and women carrying a *BMPR2* mutation show a greater incidence of PAH (Shapiro et al. 2012), likely caused by estrogen-induced inhibition of BMPRII signaling in VSMCs (Mair et al. 2015).

Although mutations in *ACVRL1* usually cause HHT2, cases of severe PAH that are indistinguishable from iPAH have been documented in families segregating with HHT (Harrison et al. 2003; Girerd et al. 2010). Of note, mutations in the genes encoding endoglin (*ENG*), Smad8 (*SMAD9*), and BMP-9 (*GDF2*) have also been described in PAH (Machado et al. 2015; Wang et al. 2016a). As in HHT,

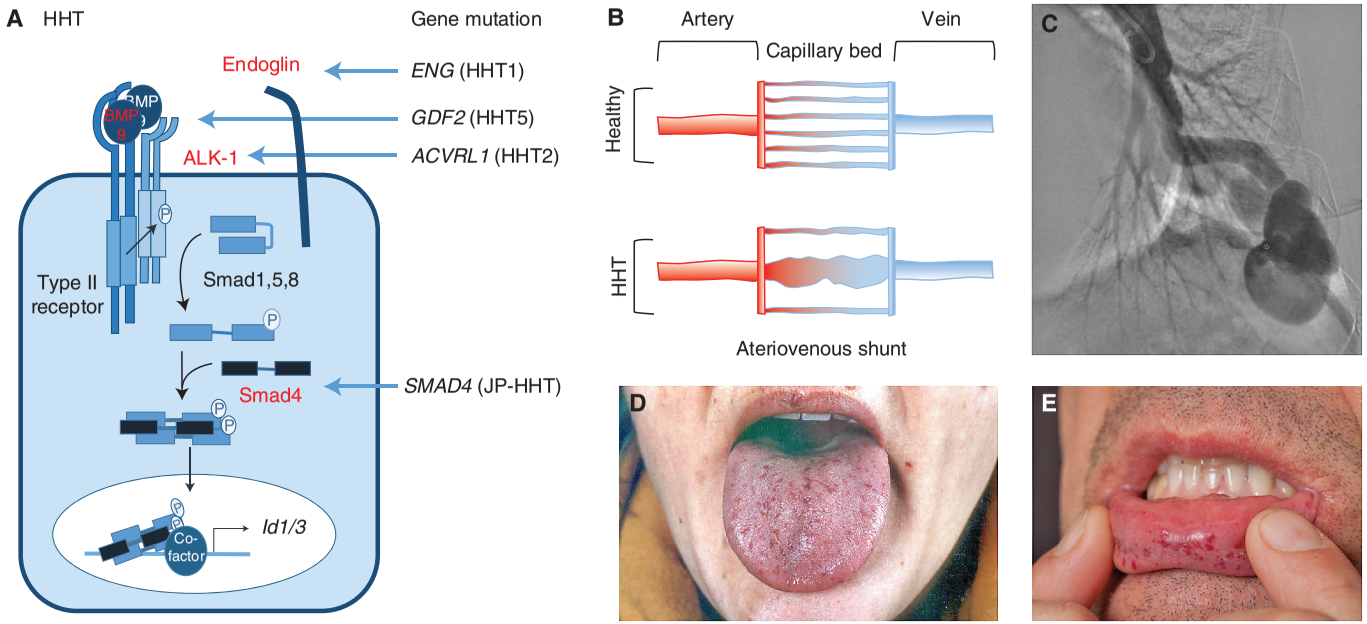

Figure 2. Mutations in bone morphogenetic protein (BMP) signaling components result in hereditary hemorrhagic telangiectasia (HHT). (*A*) The genetic vascular disorder HHT, also known as the Osler–Weber–Rendu syndrome, is linked to mutations (shown in red) in the *ENG* gene, which encodes the coreceptor endoglin (HHT1), *ACVRL1*, which encodes the activin receptor-like kinase (ALK)-1 type I receptor (HHT2), *GDF2*, which encodes BMP-9 (HHT5), and *SMAD4* (juvenile polyposis [JP]-HHT). (*B*) Mutations in these genes result in disturbed endothelial cell (EC) growth and specification, the development of tortuous fragile vessels, and loss of arterial venous specification that lead to arteriovenous shunts (AVMs). (*C*,*D*) Typical features of the disease are pulmonary AVMs (*C*) and telangiectasia visible as red dots on lips and tongue (*D*).

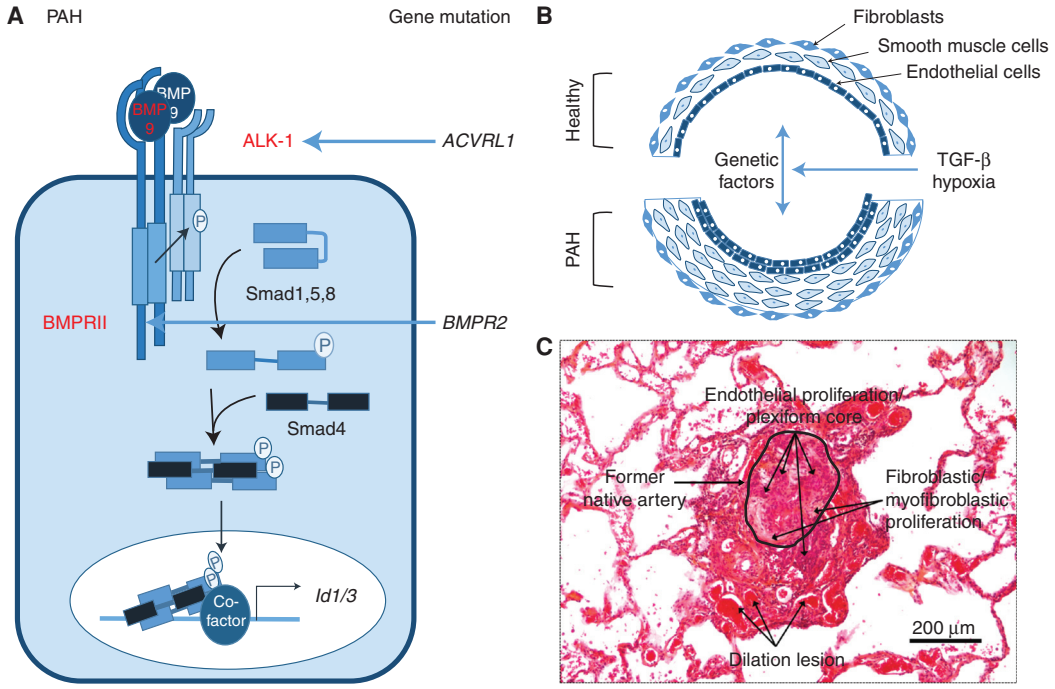

Figure 3. Mutations in bone morphogenetic protein (BMP) signaling cascade results in pulmonary arterial hypertension (PAH). (*A*) The genetic vascular disorder PAH has been linked to mutations mainly in the *BMPR2* gene for the BMPRII receptor, as well as the *ACVRL1* gene, encoding the activin receptor-like kinase (ALK)-1 receptor (mutations indicated in red). Recently, a mutation in *GDF2*, encoding BMP-9, was described in a PAH patient. Rare cases of mutation in *ENG* or *SMAD8* have also been described but are not shown here. (*B*) Mutations in these genes increase endothelial cell (EC) and vascular smooth muscle cell (VSMC) proliferation, resulting in a multilayered vessel that will lead to vessel obstruction. (*C*) Plexiform lesion distal to a pulmonary artery in the lung of a patient with idiopathic PAH, which is characteristic for plexogenic pulmonary arteriopathy. The section is stained with hematoxylin and eosin.

BMP-9 and BMP-10 signaling via BMPRII and ALK-1 likely plays a central role in PAH pathobiology, implicating ECs as an important cell type in the initiation of both diseases (Morrell et al. 2016).

Despite advances in our understanding of the pathogenesis of these genetic diseases in humans, many important questions remain unanswered. For example, it is unknown how loss-of-function mutations in *BMPR2*, *ACVRL1*, or *ENG* can cause a disease that seems confined to pulmonary, liver, or brain vascular beds. We also lack an explanation for the variable penetrance of these diseases—with ~100% penetrance for carriers of HHT mutations compared with only 20% for carriers of PAH mutations. Genetic studies in mice have clarified some of these issues and guide our design of improved targeted therapies for patients. Indeed, for most of the genes encoding BMP signaling mediators that are found to be mutated in vascular diseases, genetic deletion results in strong and often lethal vascular phenotypes in mice, further supporting the view that this pathway plays a role in vascular development. Further insights into the disease mechanisms have been obtained using heterozygote animal models of tissue-specific deletions, which mimic a human patient's phenotype. These analyses have characterized different (non-)cell-autonomous players involved in disease development. In the following section, we will discuss the mouse models that have helped elucidate the role of BMPs in the human pathologies.

LOSS-OF-FUNCTION ANIMAL MODELS IN BMP SIGNALING LINKED TO VASCULAR DISEASES

Defects in Blood Vascular Remodeling

In mice, genetic deletion of *Eng, Acvrl1*, or *Smad5* causes embryonic lethality because of cardiovascular system defects (Goumans and Mummery 2000), suggesting that these genes function in a common pathway. Embryos deficient for *Eng* (Li et al. 1999; Arthur et al. 2000; Bourdeau et al. 2000), *Acvrl1* (Oh et al. 2000; Urness et al. 2000), or *Smad5* (Chang et al. 1999; Yang et al. 1999) each show impaired yolk sac and embryonic vascular development, resulting in embryonic lethality around embryonic day (E) 11.5. The phenotypes look remarkably similar, but there are subtle differences. Endoglin-deficient embryos do not have a layer of VSMCs covering their major vessels at E9.5 (Li et al. 1999), and this VSMC developmental deformity precedes the vascular remodeling defects. $Eng^{-/-}$ ECs produce less TGF-β1, hampering the recruitment of mesenchymal cells and their differentiation into pericytes and VSMCs, and thus preventing formation of the VSMC layer (Carvalho et al. 2004). In addition to this vascular phenotype, $Eng^{-/-}$ embryos also show an enlarged ventricle and outflow tract, abnormal cardiac looping, and truncal cushions with a disorganized endothelial layer—which are all signs of abnormal cardiac development (Arthur et al. 2000). $Acvrl1^{-/-}$ embryos also show large vessel dilation and delayed VSMC differentiation and migration. Unlike $Eng^{-/-}$ embryos, $Acvrl1^{-/-}$ embryos show excessive fusion of capillary networks in the embryo proper. Furthermore, they form AVMs because of fusion of major arteries and veins (Oh et al. 2000; Urness et al. 2000; Park et al. 2008).

Both BMP-9 and BMP-10 are physiological ligands of ALK-1. This finding, coupled to the fact that $Acvrl1^{-/-}$ mice show a strong vascular phenotype and $Bmp9^{-/-}$ mice show normal blood vascular development, has led to a reevaluation of $Bmp10^{-/-}$ mice that were described to have a cardiac phenotype (Chen et al. 2004). *Bmp10*-deficiency leads to defects in the embryo proper, including absent vitelline vessels, a stalled vascular development at the primary capillary plexus stage, and presence of AVMs (Chen et al. 2013), which are reminiscent of the vascular phenotype observed in $Acvrl1^{-/-}$ embryos (Oh et al. 2000; Urness et al. 2000). The same research group generated a *Bmp9* knockin mouse line, in which the *Bmp10* coding region was replaced with that of *Bmp9*. These mice showed normal vascular development up to E16.5, supporting the view that BMP-9 and BMP-10 are functionally interchangeable during vascular development. However, at E17.5, the heart was enlarged with pronounced ventricular septal defects consistent with previous reports showing that BMP-10 is critical for heart development (Chen et al. 2004). Interestingly, ALK-1 and BMP-10 are first expressed at E8.5 (Seki et al. 2003; Chen et al. 2013), whereas BMP-9 expression begins 2 days later, at E10.5 (Chen et al. 2013). Collectively, these data show that BMP-10 plays a crucial role in early vascular development. Moreover, at the onset of BMP-9 synthesis, both BMPs are interchangeably involved in blood vascular development, and in ensuring vascular quiescence. In contrast, BMP-10 plays a specific role in heart development, likely because of the different affinities of BMP-9 and BMP-10 for their receptors (Townson et al. 2012).

The BMP pathway is controlled by extracellular BMP modulators, such as BMPER and twisted gastrulation (TSG), which finely tune the BMP-induced proangiogenic events and maintain vascular homeostasis (Heinke et al. 2013). In the embryonic heart, BMPER expression is restricted to the ECs and the endothelium-derived cushions. $Bmper^{-/-}$ mice die at birth because of skeletal and kidney defects (Ikeya et al. 2006), and they have coronary heart anomalies because of impaired migration of ventricular ECs (Dyer et al. 2015). Furthermore, these hearts develop mitral valve prolapse (Willis et al. 2013) because of dysregulation of endothelial-to-mesenchymal transition (EndMT) in the cardiac cushions (Dyer et al. 2015). Finally, BMPER was shown to coregulate the BMP-mediated inflammation associated with laminar and oscillatory shear stress (Pi et al. 2012) and may regulate the osteoblast-like differenti-

ation of human coronary artery VSMCs (Satomi-Kobayashi et al. 2012). Interestingly, *Mgp*$^{-/-}$ mice develop AVMs of the lung and kidney, which are characteristic of vascular dysplasia HHT (Yao et al. 2013).

Another modulator of BMP signaling is TAK1, which is activated in response to both BMP (Shibuya et al. 1998) and TGF-β (Yamaguchi et al. 1995) stimulation, as well as interleukin-1 (IL-1) and several other unrelated cytokines. Among other defects, *Tak1*$^{-/-}$ embryos show dilated blood vessels associated with the loss of smooth muscle differentiation at mid-gestation (Jadrich et al. 2006). Endothelial-specific *Tak1* deletion reveals the importance of TAK1 for embryonic EC survival and migration (Morioka et al. 2012). The phenotype of *Tak1* deletion in mice is strikingly similar to that of *Acvrl1* and *Eng* deletion, which is in line with the notion that TAK1 is an effector of this pathway. Consistently, TAK1 overexpression can rescue the vascular defect elicited by silencing *Acvrl1* expression using morpholino oligonucleotides in zebrafish (Jadrich et al. 2006).

Defects in Lymphatic Vascular Remodeling

Embryonic lymphatic ECs (LECs) transdifferentiate from embryonic venous ECs through the cooperation of the SOX18 and COUP-TFII transcription factors to promote PROX1 expression (Francois et al. 2008; Yang et al. 2012; Aranguren et al. 2013; Srinivasan et al. 2014). Migration of PROX1-expressing cells from embryonic veins and the subsequent podoplanin-mediated lymphaticovenous separation require VEGF-C (Zheng et al. 2014). Following establishment of the primary lymphatic vasculature, lymphatic vessels undergo further remodeling to form a functional and hierarchical lymphatic vessel network comprising lymphatic capillaries, precollectors, and collecting vessels. Fluid is taken up by lymphatic capillaries via discontinuous VE-cadherin-positive button junctions. The fluid passes into precollectors that contain intraluminal valves to prevent retrograde flow, ensuring unidirectional flow of lymph to the blood circulation (Coso et al. 2014; Vittet 2014; Yang and Oliver 2014).

The essential role of BMP signaling in the lymphatic development of mice was first shown by the disrupted lymphatic development observed in the intestine and tail of newborn mice following injection of the extracellular domain of ALK-1 fused to the Fc fragment of human IgG1 (ALK1-FC) or of a blocking anti-ALK-1 antibody (Niessen et al. 2010). Mice with an induced genetic deletion of *Acvrl1* have enlarged lymphatic vessels within the intestine, cornea, and diaphragm, consistent with the antiproliferative role of ALK-1 in postnatal lymphatic development (Yoshimatsu et al. 2013). *Bmp9*$^{-/-}$ mice also show enlarged lymphatic capillaries and collecting lymphatic vessels in mesentery, ear skin, and back skin, suggesting that BMP-9 could be a major ALK-1 ligand required for lymphatic vascular development (Levet et al. 2013; Yoshimatsu et al. 2013). Furthermore, *Bmp9*$^{-/-}$ mice show reduced numbers of lymphatic valves and decreased drainage efficiency (Levet et al. 2013), whereas adenovirus-mediated BMP-9 overexpression diminishes inflammatory lymphangiogenesis in mice (Yoshimatsu et al. 2013). No lymphatic defects have been described so far in *Bmp10*$^{-/-}$ mice, suggesting a specific role for BMP-9 signaling through ALK-1 in lymphatic development. Other studies report that BMP-2 signaling through Alk-3, Alk-3b, BMPRIIa, BMPRIIb, and Smad5 can decrease the number of LECs in zebrafish (Dunworth et al. 2014; Kim and Kim 2014). However, zebrafish and mice may show different context-dependent molecular and cellular roles for BMP signaling in vascular remodeling.

GENETICALLY MODIFIED MOUSE MODELS FOR HUMAN VASCULAR DISEASES AND TREATMENTS

Mice homozygous for null mutations in *Eng* or *Acvrl1* are embryonic lethal at mid-gestation because of cardiovascular defects. Although these models are very helpful for determining the function of endoglin or ALK-1, they do not phenocopy the human disease. Therefore, heterozygous or inducible homozygous null mice, which have grown normally to adulthood before deletion, may be very useful models of BMP-

related genetic vascular diseases. These models will also aid the design of new treatments.

Hereditary Hemorrhagic Telangiectasia

Mice that are heterozygous for mutations in *Acvrl1* or *Eng* develop vascular abnormalities that resemble the clinical pathology of HHT (Bourdeau et al. 1999; Srinivasan et al. 2003; Torsney et al. 2003). These phenotypes are, like in patients, quite variable and mild, occur with a late onset, and depend on the genetic background (observed in 129/Ola background only), suggesting that additional factors are required for HHT development, such as genetic modifiers or environmental triggers (Bourdeau et al. 1999; Torsney et al. 2003). Angiogenesis has been proposed to act as an additional trigger or secondary hit, because $Eng^{+/-}$ and $Acvrl1^{+/-}$ mice injected with VEGF-expressing adenoviral particles show increased vascular dysplasia (Xu et al. 2004; Hao et al. 2010). Homozygous deletion of *Eng* or *Acvrl1* using cell type-specific or time-dependent Cre drivers results in consistent and robust AVMs in a pro-angiogenic and inflammatory environment (e.g., VEGF administration, normal angiogenesis in early postnatal life, inflammation, wounding) (Park et al. 2009; Walker et al. 2011; Choi et al. 2012, 2014). The use of endothelium-specific expression of Cre recombinase clearly shows that ECs are the prime cell type affected by the mutation, because no AVMs are detected when the loss of *Eng* or *Acvrl1* occurs in VSMCs, pericytes or macrophages (Choi et al. 2014; Garrido-Martin et al. 2014).

The neonatal retina is initially avascular, and the vascular plexus develops during the first week of life in a stereotypical fashion (Fruttiger 2007). The neonatal mouse retina is a widely used model for studying angiogenesis because it allows a detailed study of the different stages of vascular development. This retinal angiogenesis model was used to show the roles of ALK-1 and endoglin in retinal vascular differentiation (Mahmoud et al. 2010; Tual-Chalot et al. 2014). Induced endothelium-specific *Eng* or *Acvrl1* inactivation result in delayed vascular remodeling of the retina capillary plexus. Administration of ALK1-FC as a ligand trap in wild-type mice (Larrivee et al. 2012), or anti-BMP-10 antibody in $Bmp9^{-/-}$ mice, or neutralizing anti-BMP9 and anti-BMP10 in wild-type mice further revealed that both BMP-9 and BMP-10 are involved in retinal angiogenesis and can modulate the Notch pathway (Ricard et al. 2012; Chen et al. 2013; Baeyens et al. 2016; Ola et al. 2016; Ruiz et al. 2016). It has been shown that Smad1 and Smad5 are required for modulation of the Notch pathway during angiogenesis (Moya et al. 2012).

Together, these mouse models led to the identification of several key events in HHT development: (1) ECs are the critical cell type involved in HHT; (2) loss of *Acvrl1* or *Eng* is not required in every EC of an AVM, because mosaicism can account for the phenotype (Tual-Chalot et al. 2014); and (3) genetic modifiers may influence the susceptibility to HHT disease (Bourdeau et al. 2001). Studies in these mouse models also led to the proposal that AVM formation requires three events: (1) heterozygosity of *Eng* or *Acvrl1* mutations; (2) loss of heterozygosity by somatic mutation, which has not yet been described in patients, or alternatively by protein shedding of endoglin, or potentially also by ALK-1; and (3) an additional proangiogenic or inflammatory trigger.

Heterozygous and homozygous *Eng* and *Acvrl1* mice are also used in experiments to test potential new HHT treatments. Thalidomide treatment can restore the reduced VSMC coverage of vessels in $Eng^{+/-}$ mice (Lebrin et al. 2010). Anti-VEGF treatment of inducible $Acvrl1^{-/-}$ mice has led to attenuation of brain AVMs (Walker et al. 2012), and topical application of a VEGF-neutralizing antibody shortly after wounding can prevent AVM formation, block the progression of established AVMs, and even induce regression of early AVMs in mice with induced endothelial *Acvrl1* inactivation (Han et al. 2014). For an extensive discussion on this topic we refer to the review by Tual-Chalot et al. (2015). In agreement with the results obtained in animal models, therapeutic approaches to block angiogenesis, such as anti-VEGF antibodies, thalidomide, or PI3-kinase inhibition, have been developed and show ben-

eficial effects in HHT patients (Lebrin et al. 2010; Dupuis-Girod et al. 2012; Ola et al. 2016). Taken together, these results support the hypothesis that HHT pathogenesis is caused by an enhanced response to angiogenic cues when ALK-1 or endoglin expression are diminished (Choi et al. 2013).

Pulmonary Arterial Hypertension

Mice heterozygous for *Bmpr2* do not develop or show only minimal signs of PAH without additional stimuli (Beppu et al. 2004; Song et al. 2005; Long et al. 2006). Expression of a dominant-negative form of BMPRII represses BMPRII signaling in smooth muscle cells and causes elevated right ventricle systolic pressure (RVSP) (West et al. 2004). Interestingly, $Bmpr2^{-/-}$ deficiency in pulmonary ECs (pECs) elicits PAH in some mice (Hong et al. 2008), providing in vivo evidence that absence of BMPRII signaling in ECs is sufficient to cause a predisposition to PAH. Interference with BMP signaling at the level of the Smads has shown that EC- or VSMC-specific *Smad1* deletion yields mutant mice with elevated pulmonary pressure, right ventricular hypertrophy, and thickened pulmonary arterioles (Han et al. 2013). $Smad8^{-/-}$ mice show a defective pulmonary vasculature (Huang et al. 2009). Additionally, VSMC-specific homozygous replacement of the wild-type *Bmpr2* sequence with $Bmpr2^{R899X}$, corresponding to the $BMPR2^{R899X}$ mutation in a PAH patient that results in a truncated BMPRII, induces an increase of RVSP in approximately one-third of the transgenic mice (West et al. 2008). Additionally, heterozygous $Bmpr2^{+/R899X}$ mice developed RVSP by six months of age (Long et al. 2015). Signs of pulmonary hypertension have also been described in heterozygote $Acvrl1^{+/-}$ or $Eng^{+/-}$ mice (Toporsian et al. 2010; Jerkic et al. 2011).

Because mice rarely develop pronounced pulmonary hypertension and their vessels are difficult to image and catheterize, rat models are often preferred for studies of PAH. Several rat models of PAH have been developed using environmental induction (e.g., chronic hypoxia, monocrotaline injection, or chronic hypoxia combined with VEGF receptor inhibition using the SU-5416 kinase inhibitor). These models show pronounced pulmonary hypertension with plexiform lesions and right ventricular hypertrophy (Gomez-Arroyo et al. 2012; Nicolls et al. 2012; Dickinson et al. 2013). Strain-specific differences in the SU-5416 rat model of PAH have been reported, supporting the notion that genetic modifiers play an important role in the PAH phenotype (Jiang et al. 2016a). A *Bmpr2* mutant rat model has been generated that carries a heterozygous 140-bp deletion in the first *Bmpr2* exon ($Bmpr2^{\Delta 140Ex1/+}$), as identified in a PAH patient (Ranchoux et al. 2015). Although this rat does not spontaneously develop the hemodynamic features of pulmonary hypertension, by three months of age, it displays intense pulmonary vascular remodeling and neomuscularization of intraparenchymal distal arterioles (Ranchoux et al. 2015). Further analyses will reveal whether this genetic rat model will be a useful model for PAH. No existing single model can recapitulate the many diverse forms of PAH; however, they do show endothelial dysfunction, an imbalance of proliferation and apoptosis, and a glycolytic metabolic profile (Maarman et al. 2013; Tuder et al. 2013; Lawrie 2014; Rabinovitch et al. 2014).

Experimental PAH animal models have already been successfully used to test new therapeutic approaches. To date, two approaches to enhance BMP signaling in animal models are showing great promise; however, several questions remain unanswered and further work is needed (Guignabert et al. 2016). In animals with experimentally induced pulmonary hypertension, treatment with either FK506/tacrolimus (Spiekerkoetter et al. 2013) or BMP-9 (Long et al. 2015) reverse the established PAH. Tacrolimus treatment has also shown some benefits in end-stage PAH patients (Spiekerkoetter et al. 2015).

Fibrodysplasia Ossificans Progressiva

FOP is a rare autosomal-dominant disorder that is mainly characterized by episodic heterotopic ossification (HO) of muscle, fascia, ligaments, and tendons. FOP is driven by *ACVR1* mutations in the coding region of the ALK-2

intracellular domain (Fig. 4). The most common mutation, accounting for ~97% of cases, is a gain-of-function Arg206His (R206H) amino acid change in the ALK-2 intracellular juxtamembrane GS subdomain (Shore et al. 2006). A mouse model carrying the human R206H mutation in the *Acvr1* gene confirmed that this mutation can lead to hyperactivation of Smad signaling, thus identifying an underlying molecular mechanism for the development of this disease. Normally, ALK-2, together with the type II receptors, bind activin but the resulting complex does not stimulate Smad1/5/8 phosphorylation, thus activin acts as an inhibitor of canonical BMP-mediated signaling through ALK-2. However, the R206H variant of ALK-2 responds to activin, inducing signaling via Smad1 and Smad5 (Hatsell et al. 2015). This has led to a new therapeutic approach using a neutralizing activin A antibody that prevents heterotopic ossification in $Acvr1^{R206H}$ mice (Hatsell et al. 2015). Interestingly, ECs are incorporated into the heterotopic cartilage and bone of FOP patients, which was not observed in the bone of healthy controls (Medici et al. 2010). Furthermore, the *ACVR1* mutation and resulting activation of ALK-2 signaling in ECs leads to EndMT, and the newly generated mesenchymal-like cells can further differentiate into osteoblast-like cells (Fig. 4). This effect is mediated by TGF-β2 and BMP-4, but not BMP-7, in a manner dependent on ALK-2 and ALK-5. These data support the idea that BMP-dependent vascular dysfunction could be important in the development of FOP.

BMP-RELATED VASCULAR DISEASES WITHOUT MUTATIONS IN THE BMP SIGNALING CASCADE

Vascular Calcification

Vascular calcification is a tightly regulated process that results from the phenotypic plasticity of vascular cells. It has been observed in several pathologies, including atherosclerosis, chronic kidney disease, diabetes, and hypertension. ECs can undergo EndMT to form myofibroblast-like cells or they can differentiate into chondrocyte- and osteoblast-like cells. When this happens, the surrounding extracellular matrix can become mineralized. When subjected to high shear stress, ECs disassemble their primary cilium, which renders them more sensitive to shear-induced EndMT (Egorova et al. 2011; Tkachenko et al. 2013) and BMP-induced, Slug-dependent osteogenic differentiation (Sanchez-Duffhues et al. 2015). Increased BMP-2 and BMP-4 expression at atherosclerotic sites potentiates such calcification (Boström et al. 1993; Dhore et al. 2001), whereas pharmacological inhibitors of BMP signaling or the BMP antagonist MGP can reduce vascular inflammation and calcification (Cai et al. 2012). Indeed, MGP overexpression attenuates vascular calcification in $ApoE^{-/-}$ mice (Boström et al. 2001; Yao et al. 2010), and treatment with the extracellular domain of ALK-3 ($ALK3_{ECD}$) or LDN-193189, a small BMPRI kinase inhibitor, reduces vascular inflammation and calcification in $Ldlr^{-/-}$ mice (Derwall et al. 2012). Furthermore, oxidized-LDL (oxLDL) and BMP-6 synergistically promote osteogenic differentiation in ECs, providing a potential mechanism for the interactions between BMP signaling, oxidative stress, and inflammation in the induction of atherosclerosis-associated vascular calcification (Yung et al. 2015).

The vascular calcification process also requires an inflammatory response and a phenotypic transformation of VSMCs into osteogenic cells under chronic inflammatory conditions (New and Aikawa 2011). Activation of the BMP-2 pathway increases both valvular and vascular calcification (Nakagawa et al. 2010; Song et al. 2015). Tumor necrosis factor (TNF)-α can induce vascular calcification both indirectly (Buendia et al. 2015) and directly. In a paracrine manner, TNF-α induces BMP-2 expression in ECs and stimulates the release of BMP-2-containing endothelial microparticles (EMPs) (Li et al. 2010). Vascular injury may facilitate fusion of the EMPs with VSMCs and favor osteogenic transformation of VSMCs, thus promoting vascular calcification (Buendia et al. 2015). Furthermore, $Smad6^{-/-}$ mice develop vascular and valvular calcification, suggesting that Smad6 plays a protective role in this process

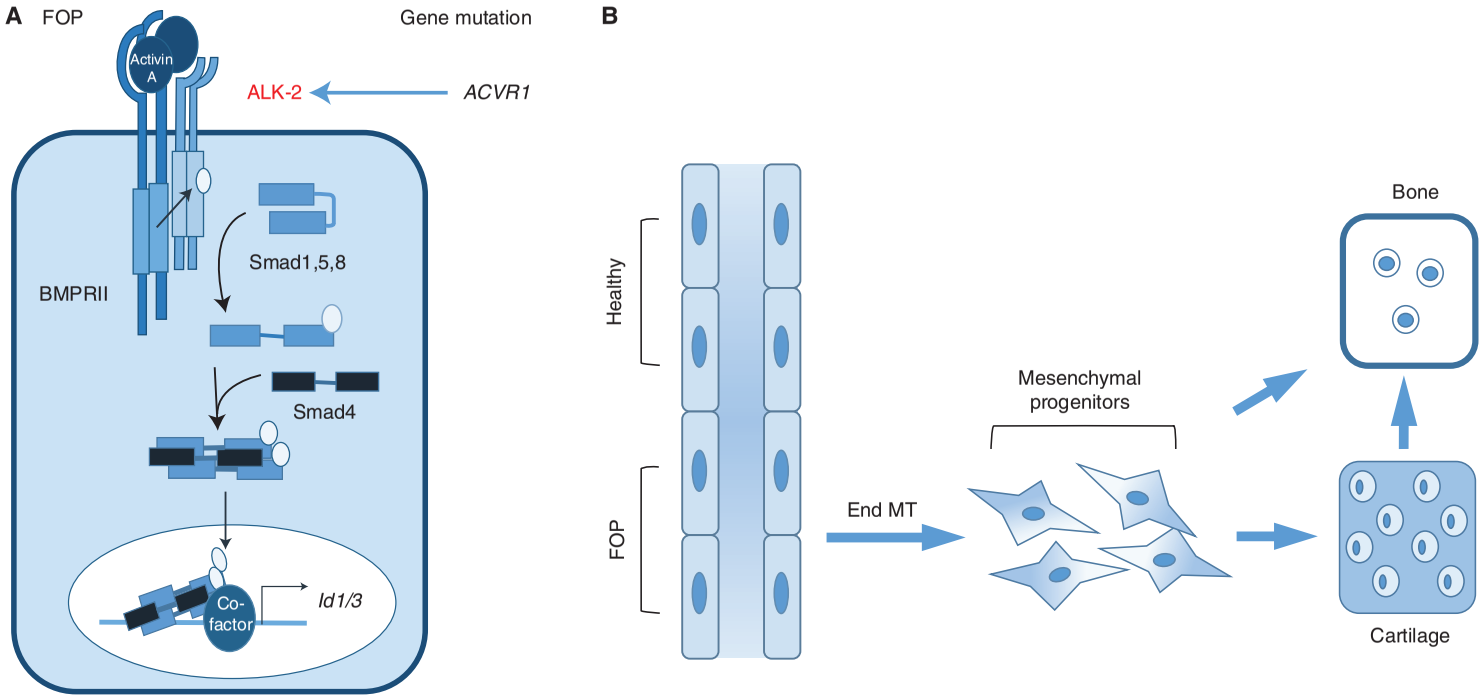

Figure 4. Mutations in the bone morphogenetic protein (BMP) type I receptor activin receptor-like kinase (ALK)-2 results in fibrodysplasia ossificans progressiva (FOP). (*A*) The genetic vascular disorder FOP is linked to activating mutations in *ACVR1* encoding the receptor ALK-2 (indicated in red). (*B*) These mutations enhance activin and BMP signaling and heterotopic endochondral ossification. Cells of endothelial origin have been found in bone, which could result from endothelial-to-mesenchymal transition (EndMT). These mesenchymal precursors will then differentiate into chondrocytes and osteoblasts.

(Galvin et al. 2000). TNF-α also reduces Smad6 expression, highlighting a third potential mechanism for enhancing BMP-2 signaling levels in vascular cells (Li et al. 2015b).

Atherosclerosis

Atherosclerosis is a chronic inflammatory response in the arterial wall, which is characterized by vascular plaque formation (Libby et al. 2011). Stable plaques contain more collagen and VSMCs, whereas unstable plaques possess a large lipid core covered with a thin fibrous cap containing macrophages. An unstable plaque is more likely to rupture, which can cause myocardial infarction or cerebrovascular accidents. Within atherosclerotic lesions, TGF-β signaling components are detectable in ECs, VSMCs, myofibroblasts, dendritic cells, T cells, monocytes, and macrophages, and their expression is rapidly enhanced during vascular injury (Bobik et al. 1999; Frostegard et al. 1999; Kalinina et al. 2004; Bot et al. 2009). Atherosclerosis, plaque instability, and vascular calcification are also linked to altered BMP signaling, although no mutations in BMP signaling components have as yet been identified (Yao et al. 2010; Simoes Sato et al. 2014; Grgurevic et al. 2016).

BMPs are expressed in atherosclerotic plaques (Dhore et al. 2001) and colocalize with the valvular fibrosa (i.e., the calcified surface of the valve leaflets) (Mohler et al. 2001). Among patients with type 2 diabetes mellitus, plasma BMP-2 levels correlate positively with plaque burden and calcification (Zhang et al. 2015). Furthermore, increased BMP-2 levels exert pro-inflammatory and pro-atherogenic effects by stimulating oxidative stress and endothelial dysfunction as well as promoting plaque calcification by inducing an osteogenic phenotype in VSMCs (Li et al. 2008). Unexpectedly, BMPRII expression was found to be reduced in advanced human coronary atherosclerotic lesions, and *Bmpr2* heterozygosity in *ApoE3*$^{-/-}$ mice leads to robust inflammation and atherosclerosis (Kim et al. 2013). *Bmpr2* deficiency increases monocyte adhesion owing to elevated ICAM-1 and VCAM-1 levels, whereas silencing of *Acvrl1* decreases endothelial inflammation (Kim et al. 2013). *ApoE*$^{-/-}$;*Ldlr*$^{-/-}$ mice that were fed a cholesterol diet showed significantly higher levels of soluble endoglin compared with *ApoE*$^{-/-}$ C57BL/6J mice and *ApoE*$^{-/-}$;*Ldlr*$^{-/-}$ mice that were fed a chow diet (Strasky et al. 2011). Furthermore, soluble endoglin interferes with rolling and adhesion of leukocytes to ECs in culture (Walshe et al. 2009). Finally, BMP-7 combined with a partial left carotid artery ligation inhibits plaque formation in vivo in *ApoE*$^{-/-}$ mice (Singla et al. 2016).

Cerebral Cavernous Malformation

Cerebral cavernous malformation (CCM) is a vascular dysplasia that is characterized by enlarged and irregular capillaries, mainly localized within the brain (Fischer et al. 2013). The affected capillaries have abnormally thin and fragile walls that make them prone to leakage, resulting in cerebral hemorrhages. CCM occurs in both sporadic and familial forms. Loss-of-function mutations in *KRIT1* (also known as *CCM1*), which encodes the cerebral cavernous malformations 1 protein CCM1, *CCM2*, or *CCM3* account for ∼90% of all cases of familial CCM, resulting in weakened cell-to-cell junctions and increased leakage from vessels (Stahl et al. 2008). EndMT occurs in ECs of the lesions in both the familial (Akers et al. 2009) and sporadic forms (Bravi et al. 2016) of CCM, and EndMT in *Ccm1*$^{-/-}$ ECs is mediated by KLF-4-dependent induction of BMP-6 expression (Maddaluno et al. 2013; Cuttano et al. 2016). Inhibitors of the TGF-β or BMP pathways can partially reduce both the number and the size of vascular lesions in *Ccm1*$^{-/-}$ mice (Maddaluno et al. 2013; Cuttano et al. 2016).

Preeclampsia

Preeclampsia (PE) is characterized by hypertension and proteinuria and is associated with maternal endothelial dysfunction. Soluble endoglin is increased in PE, and the level of soluble endoglin has been proposed as a biomarker for PE that may reflect the extent of endothelial dysfunction (Levine et al. 2006; Venkatesha et al. 2006; Rathouska et al. 2015). Injection of

soluble endoglin acts in concert with the soluble Flt1 ectodomain to induce severe PE (Venkatesha et al. 2006). Furthermore, a transgenic mouse model overexpressing human soluble endoglin developed a PE-like phenotype, including increased arterial pressure (Valbuena-Diez et al. 2012). When the levels of membranous endoglin are suppressed and replaced by soluble endoglin, adhesion between vascular ECs and mural cells is inhibited (Rossi et al. 2016), suggesting that endoglin plays a critical role in mural cell adhesion and differentiation.

Arteriogenesis and Ischemic Neovascularization

Vasculogenesis and angiogenesis also play pivotal roles in various ischemic disorders in adults. Researchers are exploring several therapeutic approaches that use endothelial progenitor cells (EPCs) to promote reendothelialization of damaged vessels, and enhance neovascularization following ischemic diseases, such as heart and limb ischemia. BMP-9 administration enhances blood flow recovery in vivo in a mouse model of hindlimb ischemia (Kim et al. 2015). This is corroborated by laser Doppler perfusion imaging results showing a significant decrease in blood flow recovery in $Eng^{+/-}$ and $Acvrl1^{+/-}$ mice with hindlimb ischemia. Interestingly, compared with controls, collateral artery size is significantly reduced in $Eng^{+/-}$ mice, but not in $Acvrl1^{+/-}$ mice (Seghers et al. 2012). This suggests that endoglin contributes to both shear-induced collateral artery growth and ischemia-induced angiogenesis, whereas ALK-1 may only be involved in ischemia-induced angiogenesis.

SECOND HIT IN HHT AND PAH DISEASE PROGRESSION

Accumulating evidence suggests that haploinsufficiency of *BMPRII*, *ENG*, or *ACVRL1* are insufficient for HHT or PAH development. Furthermore, although all cells carry the same mutations, the pathology is only seen at specific locations and in specific vascular beds. Finally, different patients with the same mutations, show different timing of onset and severity of the disease. Therefore, a second hit is required for disease onset and progression. Analyses of different animal models suggest that the second hit that triggers disease onset and progression may include genetic modifiers, angiogenesis (discussed above), inflammation, metabolic stress, and/or hypoxia. Here, we discuss these putative additional triggers in the context of BMP signaling.

Genetic Modifiers

As discussed above, the genetic background of an animal model determines the disease onset and severity of both HHT and PAH, highlighting the importance of (epi)genetic modifiers. Accordingly, the *PTPN14* gene—encoding the protein tyrosine phosphatase, nonreceptor type 14 (PTPN14)—is identified as a modifier of pulmonary AVM incidence in HHT (Benzinou et al. 2012). Other weaker genetic associations have also been identified between *ENG* and *EMILIN2*, which encodes the extracellular matrix protein elastin microfibril interfacer 2 (Pawlikowska et al. 2005; Letteboer et al. 2015).

BMPs and Inflammation in Vascular Disease

Inflammation plays a fundamental role in the initiation and progression of many vascular disorders, including HHT and PAH. During inflammation, monocytes migrate to and infiltrate the injured tissue, where they differentiate into macrophages. Macrophages can be divided in two main types, pro-inflammatory M1 macrophages that enhance the inflammatory response by secreting factors that stimulate disease progression, such as TNF-α and monocyte chemoattractant protein (MCP)-1, and anti-inflammatory M2 macrophages that promote wound healing and tissue repair (Dutta and Nahrendorf 2014; Dingenouts et al. 2015).

BMPs play roles in macrophage differentiation and modulate the inflammatory response. BMP-2 and BMP-4, which can be produced by atherosclerotic VSMCs, promote BMPRII-dependent monocyte attraction leading to inflammation of the atherosclerotic lesion (Simoes Sato et al. 2014). BMP-6 can activate M1 mac-

rophages, and stimulates IL-6 expression dependent on ALK-2, Smad1, and p38 MAPK (Lee et al. 2010). On the other hand, BMP-7 induces macrophage differentiation toward an M2 phenotype and stimulates the production of anti-inflammatory cytokines, such as IL-10, while reducing IL-6 expression in a Smad- and PI3K-dependent manner (Rocher and Singla 2013; Rocher et al. 2012). BMP-7 treatment of $ApoE^{-/-}$ mice inhibits monocyte infiltration and reduces the production of pro-inflammatory cytokines (Singla et al. 2016).

Monocytes express low endoglin levels, which are up-regulated during differentiation into macrophages (Lastres et al. 1992; O'Connell et al. 1992). Endoglin is a key determinant for both monocyte migration and differentiation (van Laake et al. 2006). Indeed, *Eng* heterozygosity hampers the ability of monocytes both to respond to stromal cell-derived factor-1 (SDF1) and to migrate toward and invade damaged tissue (Post et al. 2010). Moreover, monocytes with perturbed endoglin levels show impaired macrophage differentiation (van Laake et al. 2006; Aristorena et al. 2014; Dingenouts et al. 2015; Ojeda-Fernandez et al. 2016). It is unknown which ligand causes this effect and how it influences the development of HHT.

Ubiquitous expression of a cytoplasmically truncated BMPRII receptor ($BMPRII^{delx4/+}$), which acts as dominant-negative inhibitor of endogenous BMPRII, results in mild hypertension but a substantial increase in pulmonary inflammation, in mice at 8 weeks of age, suggesting that BMPRII has anti-inflammatory effects (Talati et al. 2014). This effect appears to be unique to BMPRII, as silencing the expression of ActRIIA and ActRIIB does not induce an inflammatory phenotype (Kim et al. 2013). Furthermore, $BMPRII^{delx4}$-expressing macrophages show an M1 phenotype, with increased transcription factor NF-κB activation and enhanced IL-6 secretion (Talati et al. 2014). Conditioned medium of the activated macrophages stimulates SMC migration, which is dependent on the BMP type I receptor. Finally, chronic lipopolysaccharide administration to $Bmpr2^{+/-}$ mice induces an exacerbated inflammatory response and pulmonary hypertension development with elevated IL-6 levels in the lungs and circulation (Soon et al. 2015). Inflammation has also been proposed to be a trigger for HHT, because $Eng^{+/-}$ mice with blepharitis have more pronounced bleeding lesions (Torsney et al. 2003). Lipopolysaccharide was also shown to trigger the formation of skin AVMs (Han et al. 2014).

BMPs and Metabolism

Even though ECs are in close proximity to oxygenated blood, they primarily rely on glycolysis rather than oxidative metabolism for the production of ATP (Goveia et al. 2014). Under physiological conditions, ECs produce >80% of their ATP by converting glucose into lactate, and <1% of glucose-derived pyruvate enters the mitochondria for oxidative metabolism through the tricarboxylic acid (TCA) cycle. The switch from a quiescent to an angiogenic phenotype is mediated by alterations of EC energy metabolism. Although such adaptations were first described in tumor angiogenesis, emerging evidence suggest that similar metabolic abnormalities characterize PAH. Metabolic features of ECs from PAH patients include high aerobic glycolysis, low oxidative metabolism, up-regulation of the pentose phosphate pathway, and increases in the nucleotide salvage and polyamine biosynthesis pathways (Fessel et al. 2012). ECs isolated from mice with EC-specific *Bmpr2* inactivation show similarly increased expression of phosphoglycerate kinase (PGK) 1 (Majka et al. 2011). The similarities between the metabolic adaptations in PAH and the metabolic profile of angiogenic ECs could represent an opportunity for therapeutically targeting of EC metabolism in PAH by pharmacological inhibition of the glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase isoform 3 (PFKFB3), for example, by using 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) (Clem et al. 2008).

BMP and Hypoxia

Hypoxia causes oxidative stress by increasing mitochondrial superoxide production. Both

BMPRII and endoglin levels are affected by hypoxia indicating that oxidative stress and metabolic dysfunction play direct pathogenic roles in HHT and PAH. Hypoxia increases endoglin expression in ECs in cell culture and infarcted murine hearts in vivo (van Laake et al. 2006). In hypoxic ECs, increased endoglin expression promotes expression of ALK-1 but not ALK-5 (Tian et al. 2010). Pulmonary endothelial expression of BMP-2 or BMP-4, but not BMP-5, BMP-6 or BMP-7, is increased following exposure to hypoxia (Frank et al. 2005). In mice heterozygous for *Bmp4* (*Bmp4*$^{LacZ/+}$), loss of hypoxia-induced BMP-4 expression is associated with decreased pulmonary vascular remodeling and VSMC proliferation, and reduced PH (Anderson et al. 2010). BMP-9 signaling through BMPRII, ALK-1, and the transcription factor Id1 protects ECs from hypoxia-induced apoptosis partly by inducing expression of the anti-apoptotic protein crystallin-αB (CRYAB) (Ciumas et al. 2013). Hypoxia induces BMPRII down-regulation, and concomitantly reduces Smad1 and Smad5 phosphorylation and Id1 expression in pulmonary artery SMCs (Takahashi et al. 2006; Maruyama et al. 2016). Hypoxia-induced HIF-1α activates the expression of miR-322, which in turn inhibits expression of *ALK3* and *SMAD5* in pulmonary artery smooth muscle cells (PASMCs,) promoting hypoxia-induced PASMC proliferation and migration (Zeng et al. 2015). Moreover, hypoxia represses the expression of BMPER, resulting in enhanced BMP signaling and revascularization of the hypoxic retina (Moreno-Miralles et al. 2011). Together, these findings suggest that hypoxia modulates BMP signaling and may, therefore, be an interesting target for HHT and PAH treatment.

BMP Signaling Goes Vascular

Unbalanced BMP signaling is involved in several human vascular diseases, caused directly by mutations in BMP signaling components or indirectly by alteration of signaling levels. Together with evidence from numerous genetic animal models, these genetic correlations provide clear support for the view that BMP signaling plays a crucial role in vascular remodeling. BMPs exert potent effects on all blood and lymphatic cell lineages, affecting the capacity of ECs to migrate, proliferate, and form basic tubular structures. BMPs also help determine the fates of the surrounding pericytes, VSMCs, and adventitial cells that contribute to vessel structural integrity and function. BMP inhibition may be useful in the treatment of vascular disorders, such as CCM, vascular calcification and atherosclerosis, whereas BMP stimulation could be an effective treatment for HHT, PE, and PAH. Current knowledge suggests that BMP-9 and BMP-10, which share 65% amino acid sequence identity, are the most important BMP ligands in vascular development. This is supported by their binding with high affinity to BMPRII, ALK-1, and endoglin, which are expressed at relatively high levels by ECs and VSMCs, and their presence in blood (David et al. 2008). BMP-4 and BMP-6 also exist in serum, supporting their potential role in vascular development (Herrera and Inman 2009; Vukicevic and Grgurevic 2009), which is also supported by their involvement in vascular dysfunction like shear-mediated vascular remodeling and CCM. Furthermore, BMP-2, in particular in zebrafish (Wakayama et al. 2015), is gaining importance in vascular biology, including also in flow-mediated vascular calcification.

BMP-9 is produced by the liver (Miller et al. 2000; Bidart et al. 2012) and BMP-10 is produced in the heart, chiefly by the right atrium in adults (Neuhaus et al. 1999). The physiological levels of BMP-9 and BMP-10 are similar (0.5–15 ng/ml) in both human and mouse serum (David et al. 2007, 2008; Souza et al. 2008; Chen et al. 2013; Kienast et al. 2016). BMP-9 is considered as the main bioactive form in adult circulation, and indeed most BMP-Smad reporter activity is inhibited by a neutralizing anti-BMP-9 antibody (David et al. 2007; Ricard et al. 2012; Chen et al. 2013), and plasma from *Bmp9*$^{-/-}$ mice cannot activate this reporter (Ricard et al. 2012).

In vivo data clearly indicate that BMP-9 and BMP-10 signaling play major roles in vascular remodeling through BMPRII and ALK-1 (Ricard et al. 2012; Chen et al. 2013). However,

the precise cellular mechanisms and molecular responses induced by these ligands in ECs and VSMCs remain incompletely understood. One possible explanation for contradictory findings is that context-specific mechanisms may be at play (David et al. 2009; Suzuki et al. 2010; Tillet and Bailly 2014).

One major difference between HHT and PAH is that they involve mutations in different types of receptors. Although HHT is linked to mutations in genes encoding the type I receptor, *ACVRL1*, or the coreceptor, *ENG*, PAH correlates with mutations of the type II receptor *BMPR2*, although in few cases mutations in *ACVRL1* have also been found. This raises the question as to whether the etiologies of these diseases can be explained by differences in ligand binding or downstream Smad-dependent or -independent signaling pathways. Another interesting point is that the type II receptor ActRIIA or ActRIIB cannot rescue BMPRII deficiency. Also, unlike ALK-1 and endoglin, which are primarily expressed in ECs, BMPRII is highly expressed in both ECs and VSMCs and, thus, is likely to directly influence EC and VSMC function.

It remains unclear as to how and why a disease that is caused by a mutation in a gene expressed widely throughout different vascular beds manifests primarily in only a few organs (e.g., the lung, brain, gastrointestinal tract, and liver). Vascular bed-specific endothelial effects of BMP-4 have been observed. BMP-4 exerts pro-oxidant, prohypertensive, and pro-inflammatory effects only in the systemic circulation, whereas pulmonary arteries are protected from these adverse effects of BMP-4 (Csiszar et al. 2008). Moreover, differences in shear stress and responses in different vascular beds likely contribute in the selective development of defects. The expression levels of various BMP receptors and attenuators at the cell surface, as well as the ligand availability in various vascular beds, likely contribute to the greater sensitivity of some vascular beds to mutations and phenotypic outcomes. A change in receptor availability may also alter the composition of preformed receptor complexes, such that BMPs might bind to different type I and type II receptors, thereby influencing the ratio between Smad-dependent and Smad-independent cascades (Hassel et al. 2003). In addition, some mutant receptors may become activated by uncommon ligands, as shown for activin binding to the mutant ALK-2 in FOP (Hatsell et al. 2015). Improving our understanding of how receptor complex localization and composition are disturbed in diseased ECs and VSMCs in different vascular beds may help explain why some tissues are more prone to develop vascular lesions than others.

The specific endothelial expression of ALK-1 and endoglin and their roles in vessel network formation has led to the hypothesis that targeting ALK-1 or endoglin could be a new therapeutic approach to regulate tumor angiogenesis and lymphangiogenesis. Thus, recent strategies for cancer treatment have focused on simultaneous inhibition of VEGF and ALK-1 or endoglin (Bhatt and Atkins 2014; Gupta et al. 2015). A neutralizing ALK-1 antibody (Necchi et al. 2014), or the ALK1-Fc (Wang et al. 2016b) or endoglin-Fc ligand traps (Gordon et al. 2014), combined with a VEGF inhibitor or chemotherapy have been reported to inhibit tumor growth. Although these trials are encouraging, the highly context-dependent effects of ALK-1 and endoglin in controlling vascular function warrant further research to prevent unwanted side-effects of these approaches.

CONCLUSION

Genetic vascular diseases have revealed major roles of BMPs in vascular development and dysfunction. Unraveling their functions has enabled the development of new potential therapeutics for several vascular-related pathologies such as PAH and FOP. In the future, the development of selective small molecules modifiers of BMP signaling will be an important challenge and should lead to new therapeutic opportunities.

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