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# BMP SIGNALING IN VASCULAR DEVELOPMENT AND DISEASE

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## Abstract

Genetic and functional studies indicate that common components of the Bone Morphogenetic Protein (BMP) signaling pathway play critical roles in regulating vascular development in the embryo, and in promoting vascular homeostasis and disease in the adult. However, discrepancies between *in vitro* and *in vivo* findings, and distinct functional properties of the BMP signaling pathway in different vascular beds have led to controversies in the field that have been difficult to reconcile. This review attempts to clarify some of these issues by providing an up to date overview of the biology and genetics of BMP signaling relevant to the intact vasculature.

### Keywords

Bone Morphogenetic Protein; BMP; signaling; vasculature; development; disease

## Introduction

Genetic studies in mice demonstrate that the BMP signaling pathway plays critical roles in regulating embryonic vascular development. Many of the same pathways that regulate vascular development are reactivated following vascular injury, suggesting that defective BMP signaling also plays a role in vascular homeostasis and disease in adults. Definitive evidence for this comes from genetic studies indicating that components of the BMP signaling pathway are mutated in patients with hereditary vascular diseases. These studies also suggest that defects in BMP signaling play a role in more common vascular diseases not associated with mutations in components of the BMP pathway. However, rapid advances in understanding the biology of this signaling pathway has made it difficult to grasp some of the complexity of these studies. The purpose of this review therefore is to provide an overview of the relevant biology of BMP signaling (Part 1) and to summarize current genetic and functional data linking abnormalities in BMP signaling with vascular development and disease (Part 2).

## PART I: BMP signaling

### BMP family of ligands

BMPs are secreted TGF-β superfamily ligands first identified in extracts from bone matrix that could induce ectopic bone formation when implanted subcutaneously in rats (1). It is now known that BMPs play essential roles in the development of nearly all vertebrate organs,

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including the embryonic vasculature (2–3). Within the TGF-β superfamily, BMP ligands (some of which are also referred to as growth and differentiation factors (GDFs)) can be organized into clades based on sequence similarity to conserved molecules in primitive organisms (Figure 1). On this basis, 15 *bona fide* mammalian BMP/GDF ligands have been identified that activate typical BMP-dependent responses. Some of these ligands, including GDF8, GDF11, GDF1 and GDF3 are misnamed since they activate pathways more typical of the TGF-β/Activin ligands (4–6), while others including BMP3 and Inhibin should be classified as BMP antagonists, since they act by sequestrating inactive BMP receptor signaling complexes (7–8).

Secretion of BMP ligands is regulated at a variety of levels. Transcription of *Bmp2*, *Bmp4*, *Bmp5* and *Gdf6* mRNA is regulated by long range, tissue specific *cis*-regulatory elements (9). Secretion of BMPs is also dependent on correct intracellular cleavage of pro-domains (10–11), which is regulated in a tissue specific manner (12). Unlike TGF-β, the cleaved pro-domain of most BMPs dissociates as the mature ligand is secreted, although BMP7 and BMP9 are secreted as stable complexes with their cleaved pro-domains (13–14). These regulatory mechanisms have implications for studying the role of BMP ligands in the intact vasculature, and reinforce the need to evaluate mature protein expression whenever possible.

## Extracellular regulation of BMP ligands

A number of BMP antagonists and other non-receptor binding proteins regulate long range effects of these ligands (15). These include BMP antagonists Noggin, Twisted gastrulation (TSG), Follistatin and members of the Chordin and DAN family of proteins. In addition, heparan sulphate proteoglycans and components of the extracellular matrix, including type IV collagen, fibrillin and matrix Gla-protein, interact with and modify specific BMP ligand activity in a context- and concentration-dependent manner. The mechanisms by which these factors regulate BMP signaling have been extensively investigated during embryonic development, where tight spatiotemporal control of BMP gradients plays a critical role in controlling developmental patterning (15). However, many of the same proteins are expressed post-natally, suggesting that the same mechanisms are likely to be operative in the adult.

These proteins inhibit BMP activity by sequestering ligands away from their receptors, reducing free movement in the extracellular space and/or promoting endocytic uptake and degradation of the ligands (15). Ligands bind with varying affinity to these inhibitors. For example, Noggin binds to BMP2, BMP4, BMP7 and GDF5 with high affinity but does not inhibit BMP9 or BMP10-activity at all (16–17). Chordin binds to BMP2, BMP4 and BMP7 but does not interact with other BMP family members (18). These effects are more complex in the intact organism where different binding proteins interact. For example, TSG forms a tri-molecular Chordin/BMP/TSG complex that inhibits BMP4 by stabilizing Chordin-BMP interactions. However, TSG also enhances BMP signaling by promoting cleavage of Chordin from the Chordin-BMP complex by the protease Tolloid (19). This sets the stage to establish a gradient of BMP activity where high concentrations of Chordin antagonize BMP activity, while relative excess of TSG releases BMP from the Chordin complex and activates of BMP signaling. Similarly, BMPER/CV2 inhibits BMP4 at high concentrations by promoting endocytic uptake and degradation of BMP4, but activates BMP signaling at low concentrations by facilitating ligand interaction with receptors (15). This phenomenon whereby BMP binding proteins can activate BMP signaling in a context and concentration-dependent fashion has implications for investigators exploring the mechanisms by which BMPs have different short and long range effects on the vasculature.

## BMP receptors

Signal transduction is initiated by binding to receptors and formation of heterotetrameric complexes comprising combinations of type 1 (Activin receptor-like kinases (ALK) 1/2/3 and 6) and type 2 BMP receptors (BMPR2, ACVR2A, and ACVR2B) (20–21). Proximity of the type 2 receptor kinase domain with the type 1 receptor occurs upon ligand-induced oligomerization and promotes phosphoryation of serine and threonine residues in the "GS box" N-terminal to the kinase domain of the type 1 receptor. This alters conformation of the type 1 receptor, activating the kinase domain and increasing affinity for its SMAD substrate. This promotes phosphorylation of a conserved SXS motif at the C-terminus of receptor activated SMADs (R-SMADs). In general, activated kinase domains of type 1 BMP receptors phosphorylate and activate SMAD1, SMAD5 and SMAD8, while TGF-β/Activin activated type 1 receptors (ALK4, ALK5 and ALK7) activate a different class of SMADs, SMAD2 and SMAD3. BMPR2 is unique in having a long C-terminal tail that may regulate BMP-dependent responses by promoting degradation the SMAD ubiquitin ligase, SMURF1 (22). Tail domain interactions with cGMP-dependent kinase 1 and LIM Kinase 1, may also enhance downstream responses to BMPs (23–24).

BMP type 1 receptors are divided into two groups based on sequence similarities: ALK3/6 and ALK1/2. These have distinct ligand binding properties (Table 1) but show promiscuity in binding to different members of the TGF-β superfamily (25). Moreover, over-expression studies indicate that BMP ligands can be secreted as hetero-dimers, presenting dual ligand-receptor binding sites that promote higher receptor binding affinities and/or recruit more complex assemblies of BMP receptors (26–28). It remains to be established whether BMPs are actually secreted as heteromeric ligands under physiological conditions. BMPs have different affinities for type 1 and type 2 receptors. BMP2 and 4 bind to ALK3 and 6 but only recruit type 2 receptors, BMPR2, ACVR2A and/or ACVR2B once bound to these type 1 receptors. In contrast, the BMP5-8 group of ligands, BMP5, BMP6, BMP7 and BMP8A/B, bind with high affinity to ACVR2A and ACVR2B and subsequently recruit type 1 receptors into active signaling complexes. Some BMPs such as GDF9, interact with BMPR2, but also recruit the TGF-β receptor ALK5 into active signaling complexes (29–30). Moreover, TGF-β can recruit ALK1, ALK2 and ALK3 into heteromeric complexes with the type 1 TGF-β receptor ALK5 (31–32). This accounts for the coincident activation of SMAD1/5/8 and SMAD2/3 by TGF-β in a variety of cell types (31,33). In addition, while BMPR2 only interacts with BMP family members, ACVR2A and ACVR2B interact with Activins, GDF8 and GDF11 which recruit TGF-β/Activin type 1 receptors into signaling complexes. This inhibits BMP responses by sequestrating type 2 receptors that also interact with BMP ligands (6,34). BMP3 and Inhibin also inhibit BMP signaling by sequestrating type 2 receptors without activating downstream signals (7–8).

## BMP co-receptors

A number of membrane-associated co-receptors have been identified that modify BMP signaling by altering BMP ligand-receptor interactions (Table 2). Of these, Betaglycan/TGFβR3 and Endoglin/CD105 play critical roles in vascular development and disease (35). Both have large extracellular domains and short cytoplasmic tails lacking kinase activity. Endoglin and Betaglycan undergo proteolytic cleavage, with the soluble extracellular domain being released into the extracellular space and antagonizing the effects of the membrane-associated co-receptors (36–37). Betaglycan is ubiquitously expressed and is the major cell surface TGF-β-binding protein, but also interacts directly with BMP2, BMP4, BMP7, GDF5 (38) and Inhibin (39). The classical mechanism by which Betaglycan enhances BMP signaling is by increasing BMP ligand binding to ALK3 and ALK6 (40). However, Betaglycan also enhances BMP signaling through its ability to regulate trafficking and cell localization of interacting receptors (41). In addition, Betaglycan has been shown to inhibit BMP2 and BMP7

signaling by sequestrating BMPR2 and ACVR2 into inactive complexes containing Inhibin (8). Endoglin is also a TGF-β interacting co-receptor that interacts with and modifies downstream signaling mediated by other TGF-β family ligands, including Activin A, BMP2, BMP7 and BMP9. Unlike Betaglycan which is ubiquitously expressed, Endoglin is dominantly expressed in ECs. In addition, most of the Endoglin-ligand interactions are dependent on the presence of the respective ligand binding TGF-β superfamily receptors (42), with the exception of BMP9, with which it can bind directly (43). Endoglin enhances BMP7 and BMP9-dependent SMAD1/5 responses (44–45), although the mechanism by which this occurs is largely unknown. Endoglin does not enhance (46) nor is it required (45) to generate type 1/type 2 receptor complexes. However, ALK5 phosphorylates the cytoplasmic tail of Endoglin and this is required for Endoglin to activate both TGF-β and BMP9-dependent ALK1 signaling in ECs (47). This suggests that the cytoplasmic tail of Endoglin plays a role in activating ALK1-signaling.

## SMAD signaling

Ligand-dependent activation of TGF-β family receptor signaling complexes initiates a series of events that mediate transcriptional and non-transcriptional responses in the cell. One of these events results from phosphoryation of R-SMADs by the activated type 1 receptor kinases. This increases their affinity for SMAD4, giving rise to heteromeric R-SMAD/SMAD4 complexes which have increased nuclear import rates, accumulate in the nucleus and activate a variety of transcriptional responses (48). Signaling specificity is determined by sequences in the L45 loop of the type 1 receptors (49). Invariant sequences in the L45 loop of ALK4/ALK5 and ALK7 interact with conserved sequences in the C-terminus of SMAD2/3, while distinct L45 loop sequences in ALK3/6, and ALK1/2, interact with conserved residues in SMAD1/5/8. However, recent studies also indicate that TGF-β-induced activation of SMAD1/5/8 can result from recruitment of SMAD1/5/8 to activated TGFβR2/ALK5 complexes (50–51). Conversely, both BMP2 and BMP9 treatment can induce phosphorylation and activation of SMAD1/5/8 and SMAD2 (52–53). This indicates that while the two pathway model of SMAD signaling is widely applicable, the BMP vs. TGF-β signaling paradigm is not as straightforward as originally thought.

The duration and intensity of SMAD signaling is regulated at a number of different levels. Caveolar-mediated endocytosis and association with inhibitory SMADs (Smad6 and Smad7) promotes proteosomal degradation of BMP and TGF-β receptor complexes and R-SMADs by SMAD ubiquitin ligases SMURF1 and SMURF2 (54). SMAD signaling is also regulated by type 1 receptor de-phosphorylation in the cytoplasm, and in the nucleus, the phosphatases Ppm1A and Scp1-3 de-phosphorylate activated R-SMADs (55). In addition, a number of kinases regulate SMAD activity by phosphorylating the linker and N-terminal domains of R-SMADs. These inhibit SMAD activity by preventing nuclear import and promoting SMURF1-dependent SMAD degradation. However some of these phosphorylation events activate SMAD signaling: SMAD2 phosphorylation induced by HGF and EGF stimulates SMAD activity (56–57), while serotonin activates BMP-dependent SMAD1/5 in vascular smooth muscle cells (VSMCs) (58).

Once in the nucleus, the R-SMAD/SMAD4 complexes activate transcriptional responses directly by binding to SMAD binding elements (SBE), or indirectly through interactions with DNA binding transcription factors and histone modifying factors (48). Given the diversity of transcriptional binding partners, this allows for cross-talk with a variety of other pathways. For example, gene expression by SMAD1/SMAD4 is activated by β-catenin when recruited to LEF1 DNA binding transcription factor (59). Similarly, SMAD1 interaction with Notch Intracellular Domain recruits BMP-SMADs to the RBP-Jk binding site in the *Herp2* promotor, synergistically activating this Notch target gene in ECs (60). In addition to regulating

transcriptional responses, BMP and TGF-β activated SMADs regulate microRNA processing by direct interaction with Drosha complexes through a SMAD4-independent mechanism (61). This is of importance since microRNA processing is required for their nuclear export and function, and there is evidence that a single mature microRNA can regulate expression of hundreds of proteins (62–63). This mechanism also plays an important role in regulating BMP-dependent VSMC differentiation (64).

### SMAD-independent signaling

Different limbs of the MAP kinase (ERK, p38 MAPK and JNK), PI3 kinase/AKT and PKC signaling pathways, and a variety of Rho-GTPases (direct regulators of cytoskeletal dynamics) can also be activated by BMPs in a cell-specific and context dependent fashion (65). Activation of SMAD vs. non-SMAD-signaling is dependent on the order of BMP receptor assembly. Over-expression studies indicate that unlike TGF-β receptors, BMP receptors form heteromeric complexes in the absence of ligand (66). These preformed receptor complexes (PRCs) activate SMAD-dependent responses while BMP-induced signaling complexes (BISCs) preferentially activate non-SMAD responses (p38 MAPK in these studies) (67–68). Deletion of the C-terminal tail of BMPR2 interferes with formation of PRCs, suggesting that the BMPR2 tail may play a role in promoting BMP-SMAD signaling (68). The mechanism by which these complexes promote selective SMAD vs. non-SMAD-dependent responses is unclear. Furthermore, given the paucity of reagents to study endogenous BMP receptor behavior, it is unknown whether PRCs occur endogenously, or whether they represent an artifact of protein over-expression.

Links between SMAD-independent responses and BMP receptors are poorly understood. Some are dependent on which type 1 and type 2 receptors are engaged. For example, BMP-dependent activation of the Rho GTPase CDC42 regulates cytoskeletal dynamics by phosphoryation of cofilin (promoting actin polymerization) (24). This is dependent on interaction of LIMK1 (a cofilin kinase) with the C-terminal tail of BMPR2. One of the better characterized SMAD-independent pathways is mediated by the MAPK activators TAK1 and TAB1. TAK1 and TAB1 are required to mediate TGF-β and BMP-dependent activation of p38 MAPK and JNK, and associate with ALK3 through an adaptor protein XIAP (69–71). However, TAK1 also interacts with BMP-SMADs and induces activating SXS phosphoryation of SMAD1/5/8 (but not TGF-β activated SMAD2/3) (72). This indicates that TAK1 has distinct functions in regulating both branches of the BMP signaling pathway, and suggests an alternative mechanism by which BMP signaling activates SMADs.

## PART 2: BMP signaling in vascular development and disease

BMP ligands, inhibitors, receptors and downstream signaling intermediates show distinct patterns of regulation during vascular development and disease. However, given the complex nature of this signaling pathway, definitive functional data in the intact vasculature is essential to understand the functional implication of these changes. The second part of this review outlines results of these studies from a molecular rather than a disease oriented perspective, focusing on selective molecular components of the BMP signaling pathway that have been properly evaluated *in vivo*. We will draw parallels between defects in different vascular beds, stages of development and adult disease resulting from mutations in different components of the BMP signaling pathway. We have summarized the relevant vascular phenotypes of humans and mice carrying defined mutations of BMP pathway components in Table 3.

## BMP ligands

A number of BMPs are upregulated at sites of vascular injury. This suggests that BMPs could play a role in regulating normal vascular homeostasis and disease-associated vascular pathology.

**BMP2 and BMP4—**BMP2 and BMP4 are upregulated in ECs at sites of systemic vascular injury and in atherosclerotic plaques (73–75). Moreover, BMP2 is upregulated in vessel walls in two different rat models of systemic hypertension (76). There are no data on the effects of genetic manipulation of BMP ligand expression on the systemic vasculature *in vivo*. However, over-expression of BMP2 induced using an adenoviral delivery system, reduces neointimal proliferation following carotid artery balloon injury in rats (77). This suggests that BMP2 may have protective effects in atherosclerotic plaque formation. Conversely, continuous subcutaneous infusion of BMP4 in mice promotes systemic hypertension, and is associated with impaired EC-dependent vasodilatation in aortic ring preparations (78). The latter effects are dependent of BMP4-induction of reactive oxygen species (ROS) in ECs, and are consistent with the observation that exogenous BMP2 induces ROS and inhibits EC-dependent vasodilatation in rat carotid artery rings (79). Moreover, both BMP2 and BMP4 increase EC adhesiveness to circulating inflammatory cells in a ROS-dependent fashion (75,80–81). These findings suggest that BMPs not only influence vascular cell proliferation and/or differentiation but also influence vascular reactivity and inflammatory responses. These effects may be dependent on the vascular bed, since BMP4 has been shown to inhibit EC-dependent vasodilatation in carotid and coronary but not pulmonary artery preparations (81).

Pulmonary BMP2 and BMP4 expression are also upregulated in a model of chronic, hypoxia-induced pulmonary hypertension (PH) (82). This model is characterized by sustained increases in pulmonary vascular resistance associated with increased peripheral pulmonary vascular remodeling (83). BMP4 expression is closely associated with the pulmonary vasculature but largely restricted to alveolar and bronchial epithelium (82). In contrast BMP2 is largely expressed in the peripheral resistance vessels of the pulmonary vasculature following exposure to hypoxia (84). *In vitro* studies provide conflicting reports about the effects of BMPs on pulmonary vascular cells. For example, BMP4 inhibits proliferation and promotes apoptosis in proximal pulmonary artery VSMCs (85–86), but increases proliferation and inhibits apoptosis in VSMCs derived from more peripheral vessels (87). Moreover, BMP4 promotes proliferation in early passage mouse proximal pulmonary VSMCs but has the opposite effect on late passage VSMCs (82). Given the complexity and heterogeneity of these *in vitro* responses, genetic approaches have been used to evaluate the functional role of these ligands in the intact vasculature.

Heterozygous null *Bmp2* and *Bmp4* mice show a blunted BMP2 and BMP4 response to hypoxia. However, while *Bmp2*-deficient mice have increased susceptibility to hypoxic PH (84), *Bmp4*-deficiency partially protects against the development of hypoxic PH (82). In addition, while *Bmp4*-deficient mice have reduced pulmonary VSMC proliferation and peripheral vessel remodeling, there is no change in VSMC proliferation or peripheral vessel remodeling in hypoxic *Bmp2*-deficient mice. This suggests that hypoxia-induced BMP4 expression has distinct effects on pulmonary VSMC proliferation and remodeling while BMP2 does not. Moreover, hypoxic *Bmp2* but not *Bmp4*-deficient mice have reduced pulmonary eNOS expression and activity (phospho-S239 VASP expression (84)), suggesting that hypoxia-induced BMP2 may reduce pulmonary vascular resistance by increasing EC production of nitric oxide. This is also consistent with the observation that mice carrying the heterozygous hypomorphic *Bmpr2* mutation, $Bmpr2^{\Delta Ex2/+}$, have abnormal pulmonary vascular reactivity associated with reduced eNOS expression (88). However, since both BMP2 and BMP4 induce eNOS expression in ECs (84,88), these findings suggest that the opposing effects of BMP2

and BMP4 in hypoxic PH result from their dominant effects on different cell types (BMP4 on VSMCs and BMP2 on ECs). Given their distinct spatial expression patterns, opposing effects of BMP2 and BMP4 on the pulmonary vasculature are likely to result from differences in access of these ligands to distinct vascular cells types.

**Circulating BMP ligands—**In addition to local tissue production of BMP ligands, high (nanogram/ml) concentrations of biologically active BMP4, BMP6, and BMP9 are detected in the circulation (89–90). These ligands would have direct access to the systemic and pulmonary vascular beds, suggesting they could play a role in maintaining normal vascular homeostasis. Unlike BMP4 and BMP6 which have pro-angiogenic effects on cultured ECs (91–92), exogenous BMP9 is a potent inhibitor of angiogenic responses at concentrations found in the circulation (43,90). BMP9 may therefore play a unique role as a circulating vascular quiescence factor. Interestingly, unlike BMP4, BMP9 is not inhibited by Noggin (16), suggesting that selective antagonism by Noggin could determine the net pro- vs. anti-angiogenic effects of circulating BMPs *in vivo.* Despite this, the functional role of circulating BMP9 (or other BMP ligands) *in vivo* is unknown.

## Extracellular regulators of BMP signaling

A number of studies have demonstrated changes in expression of extracellular modulators of BMP signaling associated with vascular injury and disease. These BMP regulators have been shown to modify vascular cell function *in vitro*, but there are limited data on their role in regulating vascular structure and/or function *in vivo*. The BMP antagonist Gremlin promotes aortic VSMC proliferation and migration (93), and is up-regulated in the systemic vasculature of patients with uremia (which is associated with extensive vascular calcification), in a carotid artery injury model and in alveolar hypoxia (associated with the development of PH) (93–95). However, the effects of BMP antagonists like Gremlin should be interpreted in the context of other components of the BMP signaling pathway. Gremlin expression in the vascular media is up-regulated in a rat model of vascular calcification, but this is also associated with increased expression of BMP2 (94). Similarly, BMP4 is co-expressed with BMP antagonists Noggin, Follistatin and Matrix Gla Protein at sites of oscillatory shear stress in the systemic vasculature (96). These studies underscore the importance of developing integrated approaches to evaluate gene/protein regulation and function in the BMP signaling pathway in vascular disease. BMPER/CV2 also regulates vascular development. Knockdown of BMPER/CV2 in zebrafish results in a defect in vascular patterning (97). In mammalian cells, BMPER/CV2 is expressed ECs and modulates BMP4-dependent angiogenic responses (91). Low level BMPER/CV2 expression promotes EC sprouting and migration *in vitro*, while higher level BMPER/CV2 repress these responses (91). However, functional evidence that BMPER/CV2 regulates mammalian vascular homeostasis *in vivo* is lacking. Matrix Gla-protein (MGP) is an extracellular matrix component produced by VSMCs that inhibits or activates BMP2 and BMP4 in a concentration-dependent fashion (98–99). *MGP* deficient mice have widespread vascular calcification (100) and stenosis of peripheral pulmonary vessels is seen in patients with Keutel syndrome who inherit homozygous germ line MGP mutations (101). There are no data on the effects of these mutations on BMP signaling. However, over-expression of MGP in the lung (MGP BAC transgenic mice) is associated with reduced BMP-SMAD signaling and reduced pulmonary vascular branching (102). This suggests that vascular effects of MGP may be mediated through inhibition of BMP signaling.

## BMP receptors and co-receptors

Definitive evidence that the BMP receptors play a role in maintaining normal vascular homeostasis and promote vascular disease in man has come from genetic studies in patients with rare hereditary vascular diseases. Genetic studies of BMP receptor mutations in mice provide insight into the mechanism by which these receptors regulate vascular development

and homeostasis in the adult. Moreover, there is increasing evidence from these studies that defects in BMP receptor signaling not only regulate vascular structure but also have potent effects on vascular tone. This section will summarize data on the dominant receptors and co-receptors that have been identified and studied genetically in mice and man (summarized in Table 3)

**BMPR2—**More than 70% of patients with rare, autosomal dominant inherited Hereditary Pulmonary Arterial Hypertension (HPAH) inherit mutations at the *BMPR2* locus (103–104). In addition, ~20% of patients with sporadic (non hereditary) PAH also carry *BMPR2* mutations. These mutations occur throughout the *BMPR2* open reading frame indicating that they are likely to cause loss of function and/or dominant negative effects. Given that the pathology of HPAH is identical to other forms of PAH (105), these findings suggest that defects in BMPR2 signaling may also participate in pathogenesis of other forms of PH not associated with *BMPR2* mutations. This is supported by the observation that components of the BMP signaling pathway are down-regulated in the lungs of patients with non-hereditary forms of PAH (106–107). Despite this, there is no clear consensus as to how BMPR2 signaling affects the pulmonary vasculature. Genetic manipulation of BMPR2 expression in mice has provided insight into the mechanisms by which *BMPR2* mutations gives rise to pulmonary vascular disease in HPAH. Using RNAi in the germline to reduce BMPR2 expression to ~10% of normal leads to intestinal hemorrhage, vascular dysplasia, and impaired VSMC recruitment to pulmonary vessels (108). This indicates that BMPR2 plays a role in maintaining normal systemic and pulmonary vascular stability and structure. Moreover, ~30% of mice with conditional deletion of *Bmpr2* in ECs develop spontaneous PH (109). This is associated with focal infiltration of inflammatory cells around distal pulmonary arteries, indicating that loss of BMPR2 expression in ECs gives rise to local vascular inflammation. Similar effects have been described in another mouse model in which a mutant form of BMPR2 is over-expressed in VSMCs (110). Since these mice also develop spontaneous PH, it is possible that BMPR2-dependent inflammation within the pulmonary vasculature plays a role in the development of PH. This is consistent with the observation that pulmonary vascular lesions in the patients with PAH contain infiltrating mononuclear cells and lymphocytes (111). However, the effect of mononuclear cell depletion on the development of PH has yet to be determined. Furthermore, these findings are hard to reconcile with the fact that BMP2 and BMP4 have pro-inflammatory effects on the systemic vasculature. This suggests that BMPR2-signaling may have opposite, anti vs. pro-inflammatory effects on the pulmonary vs. systemic vasculature, respectively. Careful analysis of the systemic vasculature in mice with disruption of BMPR2 signaling in ECs would address this question.

Studies in mice carrying heterozygous germ line mutations at the *Bmpr2* locus also provide insight into the pathobiology of HPAH. Heterozygous germ line null and hypomorphic *Bmpr2* mutant mice (*Bmpr2ΔEx2*, in which there is an in-frame deletion of Exon 2) do not develop spontaneous PH, but have increased susceptibility to PH in response to inflammatory mediators and serotonin (112–114), or chronic hypoxia (88), respectively. However, none of these models develop obliterative vascular remodeling that is more typical of human HPAH. In fact, if anything, heterozygous null *Bmpr2* mutant mice have reduced pulmonary vascular remodeling in response to hypoxia (115), suggesting that increased susceptibility to PH does not result from structural alterations in the pulmonary vasculature. It has been argued that this failure to recapitulate the severity of human PAH is a significant limitation of mouse models of PH (116). However, another possibility is that changes observed in the pulmonary vasculature of mice carrying different *Bmpr2* mutations are indicative of early, pre-clinical events that occur in the pulmonary vasculature of patients with HPAH. For this reason, the observation that heterozygous null and hypomorphic *Bmpr2* mutant mouse pulmonary vasculature show increased pulmonary vasoconstriction in response to different agonists (88, 114), is of particular interest. Moreover, hypomorphic *Bmpr2* mutant mice have a marked

defect in EC-dependent pulmonary vasodilatation associated with reduced pulmonary vascular eNOS expression (88). This suggests that *BMPR2* mutations may have a dominant effect on EC function in patients with HPAH. This is consistent with the observation that BMPR2 is dominantly expressed in EC vs. VSMC compartments of the pulmonary vasculature (88, 107). Taken together, these findings suggest that aberrant BMP signaling associated with *BMPR2* mutations in patients with HPAH not only regulate vascular cell proliferation, remodeling and inflammation, but also promotes abnormal pulmonary vascular reactivity in the patients.

**ALK1—**Inactivating mutations at the *ALK1* locus have been identified in patients with Hereditary Hemorrhagic Telangiectasia type 2 (HHT2). HHT2 is an autosomal dominant heritable disease characterized by vascular dysplasia that results in abnormal, dilated blood vessels and arterio-venous malformations (117). A functional role for ALK1 in this disease has been confirmed by gene targeting in mice. Homozygous *Alk1* null mice die embryonically with widespread arteriovenous malformations resulting from defective pericyte recruitment to the primitive vasculature (118–119). Similar vascular defects are seen in *Bmpr2* deficient (germ line RNAi knockdown) and *Endoglin* null mice (108,120), suggesting that all three receptors play a role in inducing or maintaining embryonic vascular stability. Ageing adult heterozygous *Alk1* mutant mice also develop systemic vascular malformations that phenocopy the lesions seen in human HHT (121). Deletion of *Alk1* in ECs alone also phenocopies human HHT (122). Furthermore, a subset of patients with HHT2 carrying *ALK1* mutations develop PAH (123), suggesting that *ALK1* and *BMPR2* mutations have common effects on the pulmonary vasculature. Taken together, these findings suggest that ALK1 participates with BMPR2 and Endoglin in regulating systemic and pulmonary vascular homeostasis in the adult.

**ALK3—***ALK3* has not been genetically linked to human disease but has been functionally implicated in vascular development and homeostasis from genetic studies in mice. Conditional *Alk3* deletion in embryonic vascular progenitor cells results in widespread vascular abnormalities in vessel maturation and remodeling (124). Moreover, patchy conditional deletion of *Alk3* in VSMCs reduces the ability of pulmonary VSMCs to remodel in response to chronic hypoxia (125). These findings suggest that ALK3 expression in VSMCs regulates BMP-dependent vascular remodeling. Moreover, since *Bmp4* deficient mice have defective pulmonary vascular remodeling and VSMC proliferation in response to chronic hypoxia (82), these findings suggest that BMP4 may be signaling through ALK3 in the lung vasculature to promote VSMC proliferation and vascular remodeling.

**Endoglin—**Genetic studies show that patients with HHT1 inherit heterozygous mutations at the *ENDOGLIN (ENG)* locus (126). The vascular defects in patients with HHT1 are indistinguishable from those seen in patients with HHT2. Moreover, there are families with HHT1 and PAH that have *ENG* mutations (104,123), suggesting that like ALK1, Endoglin plays a common role in regulating systemic and pulmonary vascular homeostasis. Heterozygous *Eng* mutant mice phenocopy human HHT (127). These mice also develop spontaneous PH (128). In addition to structural changes in the vasculature, *Eng* heterozygous mutant mice have abnormalities in systemic and pulmonary vascular tone mediated by ROS-dependent uncoupling of eNOS in ECs (128–129). These findings are consistent with the observation that heterozygous hypomorphic *Bmpr2* mutant mice have abnormalities in pulmonary vascular tone associated with decreased activity and/or expression of eNOS (88), and suggest that Endoglin-dependent regulation of eNOS may be mediated by BMPR2.

Defects in Endoglin signaling are also reported in patients with pregnancy-associated hypertension resulting from pre-eclampsia, a condition resulting from destabilization and leakage of maternal vascular ECs (130). Increased circulating levels of placental derived soluble Endoglin correlate with severity of pre-eclampsia, and adenoviral over-expression

studies indicate that over-expression of soluble Endoglin induces pre-eclampsia in pregnant rats (37). Endoglin may also play a role in the development of aging associated hypertension. Two splice variants of Endoglin long (L-) and short (S-) Endoglin, which differ in their intracellular tails, have been identified in humans and mice (131–132). The ratio of S/L Endoglin increases with aging (133), while transgenic over-expression of S-Endoglin in ECs leads to systemic hypertension with decreased eNOS activity in mice (133). Since L-Endoglin enhances ALK1 and S-Endoglin enhances ALK5 signaling in ECs (134), alterations in the ratio of the two isoforms may modify vascular function by influencing the balance of ALK1/5 signaling in the vasculature.

## Downstream signaling

BMP signaling activates both SMAD-dependent and SMAD-independent responses in a variety of cultured vascular cell types. While the functional roles of some of these responses have been determined from genetic studies in mice, many of these effects are poorly defined and their roles in mediating BMP-dependent effects in vascular disease are largely unknown.

**BMP-activated SMADs—**Biochemical studies tend to lumped together BMP activated SMAD1, SMAD5 and SMAD8 as a single response. However, genetic studies indicate that different SMADs have non-redundant functions at different stages of embryonic development (135). *Smad5* null mice die early in gestation with defects in vascular organization and pericyte recruitment to the primitive vasculature (136–137). These defects are reminiscent of the angiogenesis defects seen in *Bmpr2* knockdown and *Eng* and *Alk1* null embryos, suggesting SMAD5 may be functioning in the same pathway. However, mice in which *Smad5* is conditionally deleted in ECs slightly later in development are viable and do not have defects in the embryonic or adult vasculature (138). This indicates that there is functional compensation for loss of SMAD5 expression later in development. In contrast to the *Smad5* null mouse, germ line *Smad1* null mutants have prominent defects in chorioallantoic membrane fusion, but only minor defects (possibly secondary) in the yolk sack vasculature (139). Moreover, *Smad8* mutant mice are viable (135,140), indicating that SMAD1 and SMAD8 play redundant roles in early and late embryonic vascular development, respectively. Interesting, aging *Smad8* null mice develop spontaneous remodeling of the intrapulmonary vasculature reminiscent of the obliterative pulmonary vasculopathy seen in patients with HPAH (140). Furthermore, SMAD8 is highly expressed in the pulmonary vasculature, and an inactivating, nonsense mutation in *SMAD8* has recently been reported in one idiopathic PAH patient (141). These findings indicate that SMAD8 plays a selective role in regulating adult pulmonary vascular homeostasis, and suggest that defective SMAD8 signaling could mediate BMPR2-dependent effects in HPAH.

**SMAD4—**In humans, *SMAD4* mutations have been linked to patients with combined HHT and juvenile polyposis (JV/HHT) (142). Moreover, while *Smad4* deficient mice display early embryonic lethality due to defective gastrulation (143–144), conditional deletion of *Smad4* in ECs leads to defects in the embryonic vasculature that resemble those seen in *Alk1* and *Eng* knockout mice (124,145–146). Since SMAD4 is a common mediator of BMP and TGF-β activated SMAD signaling, the relative contribution of SMAD4 in mediating BMP-dependent effects in the vasculature is unknown.

**SMAD6—**SMAD6 is widely expressed in the embryonic cardiovascular system, and in the adult SMAD6 expression is largely restricted to the vascular ECs (147). While a significant proportion of *Smad6* mutant mice die due to cardiac septation defects, those that survive develop systemic hypertension, enhanced vascular contractility and impaired NO-dependent vasodilatation of aortic rings (147). These findings are reminiscent of the effects of exogenous BMP4 on the systemic vasculature, but are difficult to reconcile with the known functional properties of SMAD6 as a selective inhibitor of BMP-dependent SMAD signaling. This is

consistent with the observation that these BMP4-dependent effects are mediated through SMAD-independent ROS-mediated mechanisms, and that the effects of SMAD6 on the systemic vasculature may be independent of its effects on BMP activated SMAD signaling.

**SMAD-independent signaling—**BMP ligands activate a range of different SMAD-independent transcriptional and non-transcriptional pathways in cultured vascular cells. However, the functional role of these responses in mediating BMP-dependent effects in the intact vasculature is largely unknown. An exception to this is the downstream mediator of BMP-dependent MAPK signaling, TAK1. *Tak1* deficient mice have abnormal extra-embryonic and embryonic vasculature resulting from defective VSMC recruitment to the remodeling vessels (148). This phenotype is similar to that seen in *Alk1* and *Eng* null mice, suggesting that TAK1 is mediating signaling downstream of the ALK1/ENG signaling complex. Moreover, over-expression of TAK1 rescues the vascular phenotype resulting from *Alk1* deficiency in zebrafish embryos (148). However, it is unclear whether these effects result from TAK1-dependent activation of p38 MAPK and JNK, or result from the effects of TAK1-dependent activation of BMP activated SMADs. This illustrates the complexity of understanding the true nature of BMP signaling in the intact organism, and of the challenges ahead.

## Concluding Remarks

The functional role and mechanisms by which of BMP signaling defects promote human vascular diseases remain unclear. However genetic studies in mice indicate that components of the BMP signaling pathway stabilize EC/VSMC interactions and play critical roles in regulating vascular reactivity, EC function and vascular inflammation. Further insight into these mechanisms will be provided as cell-specific conditional deletion of different BMP pathway components is performed in the vasculature. Moreover identification of small molecule inhibitors (and potentially activators) of BMP signaling using high content screening (149), raises new possibilities that modulation of BMP signaling *in vivo* that will assist in future discovery and therapeutics.

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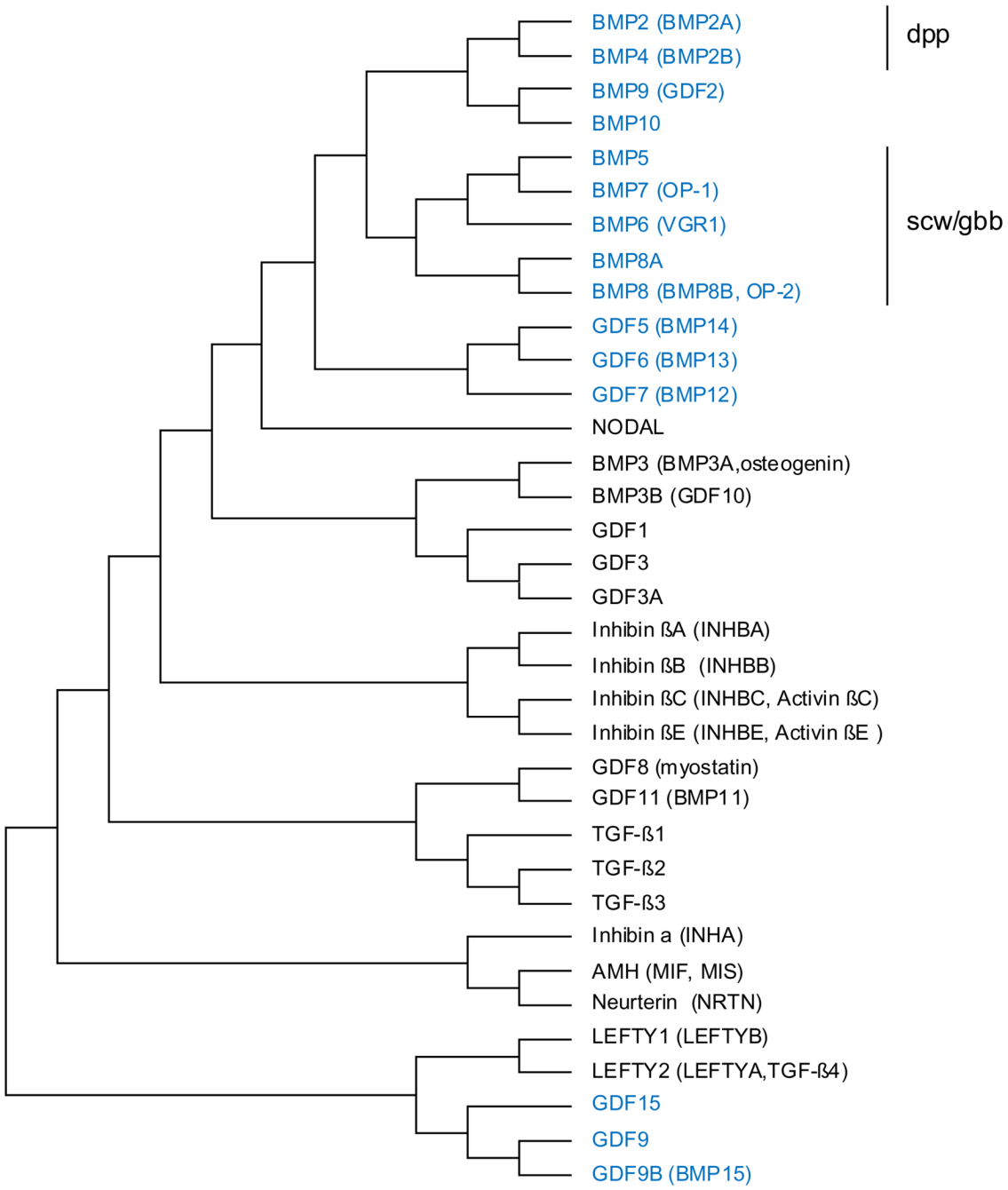


**Figure 1.**
Phylogenic comparison of TGF-β superfamily ligands based upon full length human protein sequences. Sequence similarity to *Drosophila* BMP orthologues is indicated. Alternative names are listed in parentheses. Ligands in blue are *bona fide* BMPs that have been shown to activate BMP SMADs. Phylogenetic analyses were conducted in MEGA4 (200) using the Neighbor-Joining method (201).

## Table 1

Demonstrated type I and type II receptor interactions for *bona fide* BMP ligands. Alternative names are listed in parentheses.

| | Receptor | Ligand | References |
|---|---|---|---|
| **Type I** | ALK1 (ACVRL1) | BMP9 (GDF2) | (43,150–153) |
| | | BMP10 | (152–153) |
| | ALK2 (ACVR1) | BMP6 (VGR1) | (154) |
| | | BMP7 (OP-1) | (155–157) |
| | | BMP9 (GDF2) | (43) |
| | ALK3 (BMPR1A) | BMP2 (BMP2A) | (157–164) |
| | | BMP4 (BMP2B) | (155,163,165) |
| | | BMP5 | (166) |
| | | BMP6 (VGR1) | (154) |
| | | BMP7 (OP-1) | (155) |
| | | BMP10 | (167) |
| | | GDF5 (BMP14) | (168) |
| | | GDF6 (BMP13) | (168) |
| | | GDF9 | (30) |
| | ALK6 (BMPR1B) | BMP2 (BMP2A) | (169) |
| | | BMP4 (BMP2B) | (155) |
| | | BMP6 (VGR1) | (154) |
| | | BMP7 (OP-1) | (155,157) |
| | | BMP10 | (167) |
| | | GDF5 (BMP14) | (168,170) |
| | | GDF6 (BMP13) | (168) |
| | | GDF9 | (30) |
| | | GDF9B (BMP15) | (171) |
| **Type II** | BMPR2 | BMP2 (BMP2A) | (172–173) |
| | | BMP4 (BMP2B) | (174) |
| | | BMP6 (VGR1) | (154) |
| | | BMP7 (OP-1) | (173–174) |
| | | BMP9 (GDF2) | (43,150) |
| | | BMP10 | (167) |
| | | GDF5 (BMP14) | (168,170) |
| | | GDF6 (BMP13) | (168) |
| | | GDF9 | (30) |
| | | GDF9B (BMP15) | (171) |
| | ACVR2A | BMP2 (BMP2A) | (164,175) |
| | | BMP4 (BMP2B) | (175) |
| | | BMP6 (VGR1) | (154) |

| | Receptor | Ligand | References |
|---|---|---|---|
| | | BMP7 (OP-1) | (156,176) |
| | | BMP9 (GDF2) | (43) |
| | | GDF9 | (30) |
| | ACVR2B | BMP2 (BMP2A) | (172) |
| | | BMP4 (BMP2B) | (156) |
| | | BMP6 (VGR1) | (154) |
| | | BMP9 (GDF2) | (43) |

## Table 2

Co-receptors of BMP signaling. Alternative names are listed in parentheses.

| Co-receptor | Partner | References |
|---|---|---|
| Endoglin (CD105) | BMP2 (BMP2A) | (42) |
| | BMP7 (OP-1) | (42) |
| | BMP9 (GDF2) | (152) |
| TGFβR3 (Betaglycan) | BMP2 (BMP2A) | (40) |
| | BMP4 (BMP2B) | (40) |
| | BMP7 (OP-1) | (40) |
| | GDF5 (BMP14) | (40) |
| RGMa | BMP2 (BMP2A) | (177–178) |
| | BMP4 (BMP2B) | (177) |
| | GDF7 (BMP12) | (178) |
| RGMb (DRAGON) | BMP2 (BMP2A) | (178–179) |
| | BMP4 (BMP2B) | (179) |
| | GDF7 (BMP12) | (178) |
| RGMc (Hemojuvelin) | BMP2 (BMP2A) | (178,180–183) |
| | BMP4 (BMP2B) | (182) |
| | BMP6 (VGR1) | (182,184) |
| | GDF7 (BMP12) | (178) |
| Bambi (Nma) | ALK1 (ACVRL1) | (185) |
| | ALK3 (BMPR1A) | (185) |
| | ALK6 (BMPR1B) | (185) |
| | ACVR2A | (185) |
| TRKC | BMPR2 | (186) |
| ROR2 | ALK6 (BMPR1B) | (187–188) |

## Table 3

Vascular phenotypes of BMP mutations in humans (all capital letters) and mice. All mutations are global unless noted otherwise. Embryonic lethality is indicated in parenthesis as day post coitum.

| Genetic Modification | Het/Homozygous | Vascular Phenotype | Survival | References |
|---|---|---|---|---|
| *Bmp2* null | Heterozygous | Susceptible to hypoxic PH | Viable | (84) |
| *Bmp4* null-LacZ | Heterozygous | Less severe hypoxic PH, impaired vascular remodeling | Viable | (82) |
| *Bmpr2* null | Heterozygous | Susceptible to PH, defective vascular remodeling, abnormal vascular tone | Viable | (113–115) |
| *Bmpr2* hypomorph ( Ex2) | Heterozygous | Susceptible to hypoxic PH; endothelial dysfunction and abnormal vascular tone | Viable | (88) |
| *Bmpr2* knockdown | 90% knockdown by RNAi | Hemorrhage; vascular dysplasia; impaired VSMC recruitment | Viable | (108) |
| *Bmpr2* conditional | Pulmonary EC | Spontaneous PH, vascular inflammation | Viable | (109) |
| *BMPR2* (mis-sense, non-sense) | Heterozygous | HPAH, HPAH with HHT | Viable | (103–104,189) |
| *Alk1* null | Homozygous | Angiogenesis defects, impaired VSMC recruitment | Lethal (E10.5) | (118–119) |
| | Heterozygous | Models HHT | Viable | (121) |
| *Alk1* conditional | EC-specific | Vascular dysplasia; susceptibility to arteriovenous malformations | Viable | (122,190) |
| *ALK1*(mis-sense, non-sense) | Heterozygous | HHT2, HPAH with and without HHT2 | Viable | (104,191–192) |
| *Alk3* conditional | Mesoderm deletion | Hemorrhage, cardiac defects, impaired VSMC recruitment | Lethal (E10.5) | (124) |
| | SMC (embryo) | Cardiac defects, hemorrhage, impaired vascular remodeling | Lethal (E11.5) | (193) |
| | SMC (adult) | Impaired vascular remodeling | Viable | (125) |
| *Endoglin* null | Homozygous | Angiogenesis defects | Lethal (E10.5) | (120) |
| | Heterozygous | Models HHT, spontaneous PH, Abnormal vascular tone | Viable | (194) |
| *Endoglin* conditional | EC-specific | Arteriovenous malformations | Viable | (195) |
| *ENDOGLIN* (mis-sense, non-sense) | Heterozygous | HHT1, HPAH with HHT1 | Viable | (104,123,126) |
| *Smad1* null | Homozygous | Defects in fusion of the chorioallantoic membrane and (minor) yolk sac angiogenesis | Lethal (E9.5) | (139) |
| *SMAD1* (mis-sense) | Heterozygous | HHT | Viable | (196) |
| *Smad4* conditional | EC | Cardiac and angiogenesis defects , VSMC recruitment | Lethal (E10.5) | (146) |

| Genetic Modification | Het/Homozygous | Vascular Phenotype | Survival | References |
|---|---|---|---|---|
| *SMAD4* (mis-sense, non-sense) | Heterozygous | HHT (with and without JP) | Viable | (197–198) |
| *Smad5* null | Homozygous | Cardiac and angiogenesis defects | Lethal (E9.5) | ((136–137,199) |
| *Smad6* null | Homozygous | Cardiac defects, vascular calcification, systemic hypertension | Some viable | (147) |
| *Smad8* null-LacZ | Homozygous | Spontaneous pulmonary vascular remodeling | Viable | (140) |
| *SMAD8* (non-sense) | Heterozygous | HPAH | Viable | (141) |
| *Tak1* null | Homozygous | Angiogenesis/cardiac defects | Lethal (E10.5) | (148) |

PH: pulmonary hypertension, VSMC: vascular smooth muscle cell, EC: endothelial cell, HPAH: Heritable Pulmonary Arterial Hypertension, HHT: Hereditary Hemorrhagic Telangiectasia, VSMC: vascular smooth muscle cell, JP: Juvenile Polyposis.