Regulation of the ALK1 ligands, BMP9 and BMP10

Wei Li, Richard M Salmon, He Jiang and Nicholas W Morrell

The Department of Medicine, University of Cambridge School of Clinical Medicine, Cambridge, United Kingdom

KEY WORDS:
BMP9, BMP10, ALK1, BMPR-II, endothelial cell, signal transduction

Corresponding Author:
Wei Li
University of Cambridge, Department of Medicine
Level 5, Addenbrooke's Hospital
Box 157, Hills Road, Cambridge, CB2 2QQ
Email: wl225@cam.ac.uk
Tel.: +44 (0) 1223 761304
Fax: +44 (0) 1223 336846

ABBREVIATIONS:
BMP: Bone morphogenetic protein; ALK1: activin receptor-like kinase 1; PAH: pulmonary arterial hypertension; HHT: hereditary haemorrhagic telangiectasia; BMPR-II: BMP receptor type II; TGFβ: transforming growth factor β; BRE: BMP-responsive element; AVM: arteriovenous malformation; ECs: endothelial cells; D-form (M-form): dimeric (monomeric) form on the non-reducing SDS-PAGE; CV2: crossveinless 2.

FUNDING:
This work was supported by the British Heart Foundation grants PG/12/54/29734 (to W.L. and N.W.M), PG/15/39/31519 (to W.L. and N.W.M.) and CH/09/001/25945 (to N.W.M.).

Abstract

Bone morphogenetic protein (BMP)9 and BMP10 are high affinity ligands for activin receptor-like kinase 1 (ALK1), a type I BMP receptor mainly expressed on vascular endothelial cells. ALK1-mediated BMP9/BMP10 signalling pathways have emerged as essential in endothelial cell biology and in angiogenesis. Several genetic mutations in the genes encoding the ligands and receptors of this pathway have been reported in two cardiovascular diseases, pulmonary arterial hypertension (PAH) and hereditary haemorrhagic telangiectasia (HHT). Administration of recombinant BMP9 reverses experimental PAH in preclinical rodent models. Dalantercept, an Fc-fusion protein of the extracellular domain of ALK1 and a ligand trap for BMP9 and BMP10, is in phase II clinical trials for anti-tumour angiogenesis. Understanding the regulation of BMP9 and BMP10, at both gene and protein levels, under physiological and pathological conditions, will reveal essential information and potential novel prognostic markers for the BMP9/BMP10-targeted therapies.

1. Overview of the endothelial BMP signalling pathway

The endothelial BMP signalling pathway is one of the major pathways in angiogenesis and in maintaining vascular homeostasis. The pathway is tightly regulated at multiple levels. The interplay between endothelial BMP signalling with VEGF, Notch, WNT and Hippo pathways confers endothelial cell identity and heterogeneity, and has been reviewed recently (1).

BMP9 and BMP10 are cysteine-knot homodimeric growth factors. They are the major BMP ligands acting on endothelial cells (2), which initiate the signalling cascade by forming a complex with two copies of type I receptor ALK1 and two copies of the type II receptor, including BMP receptor type II (BMPR-II), activin receptor type IIa and/or IIb. BMP receptors are serine-threonine kinases, which mediate signals through the phosphorylation of Smad1/5/8 and Smad-independent pathways, inducing the expression of a number of target genes, including *ID1-3*, *BMPR2* and Notch target genes *Hey1, Hes1*, etc (Figure 1). It has been shown that cell surface endothelial BMPR-II protein undergoes rapid turnover through lysosomal degradation pathways (3) and the induction of *BMPR2* expression by circulating BMP9 and BMP10 contribute to the basal levels of the endothelial cell surface BMPR-II expression (Figure 1) (2,4). Endoglin, a co-receptor of the transforming growth factor β (TGFβ) family, binds to BMP9 and BMP10 with very high affinity (5), and is required for BMP9 signalling (6).

Over the past decade, several observations support an important role for BMP9/BMP10 signalling pathways in diseases and as drug targets. However, reports on the regulation of BMP9 and BMP10 at the gene or protein levels are limited. Understanding the regulation of BMP9 and BMP10 in both physiological and pathological conditions will provide essential baseline information on how to manipulate BMP9/BMP10 pathways for therapeutic purposes. In this review, we will summarise the role of BMP9 and BMP10 in health and diseases, the therapeutic potential of targeting the BMP9/BMP10 pathways, followed by a review and discussion on the regulation of BMP9 and BMP10.

2. The physiology of BMP9 and BMP10

Bmp9-/- mice are viable and grossly normal, with no circulating BMP9 detected by either ELISA or using a BMP-response element (BRE)-luciferase assay in ALK1-transfected NIH-3T3 cells (7). Injection of ALK1 ECD (ALK1-Fc) or anti-BMP10 antibody into *Bmp9*-/- pups led to increased postnatal retinal vascularisation (7), supporting a role of BMP10 in compensating for BMP9 signalling in the *Bmp9*-/- mice. Detailed examination revealed that *Bmp9*-/- pups had

imperfect closure of the ductus arteriosus, and injection of anti-BMP10 antibody further exacerbated the defect (8), indicating that both BMP9 and BMP10 play roles in the closure of the ductus. Furthermore, *Bmp9*-/- mice have lymphatic defects, including decreased lymphatic draining efficiency as well as a significant reduction in number and maturation of the lymphatic valves (9).

Bmp10-/- mice, however, die at E10.0-E10.5, due to severely impaired cardiac development and function (10). Despite both BMP9 and BMP10 participating in many overlapping functions in postnatal retinal angiogenesis, the role of BMP10 in cardiac development cannot be replaced by BMP9, even when BMP9 expression was under the control of the endogenous BMP10 promoter (11).

BMP9 expression is predominantly detected in the liver (11,12), and at lower levels, also in the lung (11) and the brain (13,14). Intrahepatic biliary epithelial cells express the highest levels of BMP9, although BMP9 expression was also detected in other liver cells, including hepatocytes, liver sinusoidal endothelial cells, as well as hepatic stellate cells (12,14).

BMP10 expression is under tight temporal-spatial regulation during development. In mouse embryos, high levels of BMP10 expression can be detected from around E8.5 (11), mostly restricted to the trabeculated myocardium of atria and ventricles, playing an important role in the trabeculation of the embryonic heart (15,16). In adults, BMP10 is still highly expressed in the heart, but its expression is restricted to only the right atrium (10). Low levels of BMP10 mRNA were also detected in the lungs and livers of adult mice (15).

3. The BMP9/BMP10 pathways in disease and their therapeutic potential

3.1 Targeting BMP9/BMP10 pathways in cardiovascular diseases

Genetic mutations in genes involved in the BMP9/BMP10 signalling pathways have been directly linked to two vascular disorders, PAH and HHT. PAH is characterised by the narrowing of the pulmonary arteries, leading to the increased right ventricular systolic pressure and right heart failure (17). Loss-of-function *BMPR2* mutations are the major genetic cause for PAH, and of the additional seven mutated genes identified in PAH patients to date, six are involved in the endothelial ALK1/BMPR-II pathway, of which BMP9 and BMP10 are the cognate ligands (18). In fact, a BMP9 homozygous nonsense mutation was recently reported in a 5-year old paediatric PAH patient (19), which would be predicted to lead to absent BMP9. HHT typically presents with arteriovenous malformations (AVMs) and small vascular malformations called the telangiectases. AVMs most commonly affect lung, brain and liver, and telangiectases affect

the gastrointestinal tract and nasal mucosa. Untreated brain AVMs are a common cause of haemorrhagic stroke in HHT families. Heterozygous loss-of-function mutations in ALK1 or endoglin are the major genetic causes for HHT, and rare BMP9 variants have also been reported in patients with HHT-like syndrome (17).

Loss of endothelial BMPR-II expression leads to increased susceptibility to vascular permeability and PAH (20). BMP9, an endothelial- and ALK1-specific ligand, can protect pulmonary artery endothelial cells (ECs) from early apoptosis *in vitro*, and lipopolysaccharide-induced lung vascular leakage *in vivo* (21). Targeted gene delivery of *BMPR2* to the endothelium via adenovirus transduction prevents and attenuates PAH in hypoxia and monocrotaline rat models (22). Direct augmentation of endothelial BMP signalling by administering recombinant BMP9 can prevent and reverse PAH in several genetic and non-genetic preclinical rodent models (21). Since heterozygous ALK1 mutations in the HHT patients resulted in compromised BMP9 signalling (23), administration of recombinant BMP9 to further enhance the signalling through the remaining wild type ALK1 might be expected to have similar beneficial effects as seen in PAH.

3.2 Targeting BMP9/BMP10 signalling pathways in tumour angiogenesis

3.2.1 BMP9/BMP10 in angiogenesis

The role of BMP9 and BMP10 in angiogenesis is still under debate. The anti-angiogenic functions of BMP9 have been demonstrated in a number of reports using bovine aortic ECs, human umbilical vein ECs and human pulmonary artery ECs in assays including scratch assay, metatarsal outgrowth assay and tube formation assay (21,24,25). However, BMP9 was also shown to be pro-angiogenic using allantoic explants (26) and matrigel tubule formation assays (27).

3.2.2 Targeting BMP9/BMP10 signalling pathways in cancer angiogenesis

The role of BMP9 and BMP10 in cancer and tumorigenesis seems to be context dependent. BMP9 expression is increased in the tumours in the RIP-Tag2 mouse model (28), ovarian cancer cells (29) and in 15 out of 35 human hepatocellular carcinoma tissues that were investigated (30). However, in a survey of 97 breast cancer specimens, BMP10 expression was decreased measured by both immunohistochemical staining and qPCR, and the decreased BMP10 expression was positively correlated with disease progression, bone metastasis and

poor prognosis (31). Interestingly, another independent study reported decreased BMP9 expression in 23 breast cancer tissues compared with adjacent tissues by qPCR, and when overexpressing BMP9 in MDA-MB-231 cells, BMP9 inhibited tumour formation and induced apoptosis in the xenogenic mouse model (32). Nevertheless, the anti-BMP9/BMP10 strategy has been extensively explored in anti-cancer therapy. Dalantercept (ALK1-Fc), an extracellular ligand trap for BMP9 and BMP10, has demonstrated promising anti-tumour angiogenesis activity in preclinical rodent models (28). Clinical trials revealed that it was well tolerated and the adverse effects were different from anti-VEGF therapy, indicating that it acts through a different mechanism from anti-VEGF therapy, but efficacy was not obvious when used as a single agent (33). Multiple phase II trials using Dalantercept as a combination with anti-VEGF therapies are currently ongoing.

The therapeutic potential of targeting BMP9/BMP10 pathways clearly justifies a need for better understanding the regulation of these two important ligands and their interactions with cognate and non-cognate receptors, as well as with extracellular ligand traps. Such understanding will provide important factors to consider when applying BMP9/BMP10 agonist therapies, as well as improving specificity and reducing side effects with anti-BMP9/BMP10 treatments.

4. Regulation of BMP9

BMP9 has been shown to be a vascular quiescence factor, inhibiting endothelial cell migration and proliferation. BMP9 signalling on endothelial cells is mediated by the high affinity receptor ALK1 with an EC_{50} of around 50 pg/ml (2). On the other hand, BMP9 can also initiate signalling in non-endothelial cells, such as in mesenchymal stem cells to induce ectopic bone formation (34) and in multiple ovarian cancer cell lines to promote proliferation (29). Such signalling in non-endothelial cells is mediated through low affinity receptors (29) and requires much higher BMP9 concentration (RMS and WL, unpublished observation).

Optimum circulating BMP9 levels are essential for the endothelial-specific activity. Circulating BMP9 levels are reported to be 2-10 ng/ml measured by activity (25) and ~300 pg/ml by ELISA (35). Since BMP9 is constitutively secreted from the liver into the circulation (12,14), this raises the question as to how circulating levels of BMP9 are maintained at such low levels to maintain endothelial-specific signalling.

BMPs are synthesized as the pre-pro-form and processed into the mature ligand by furin-like proprotein convertase in the Golgi apparatus. The prodomain remains bound to the mature

ligands in circulation (14) (Figure 2A). When BMPs expressed in mammalian cells were subjected to non-reducing SDS-PAGE, a monomeric form (M-form) is present alongside the canonical disulphide-linked dimeric form (D-form) (36-38). In the case of BMP9, two high-resolution crystal structures, both crystallised from mammalian cell expressed proteins and purified under native conditions, have shown that the M-form of BMP9 is in fact a non-covalently linked dimer, almost identical to the D-form (Figure 2B and 2C) (37,39). Further characterisation and mutagenesis studies have shown that although the M-form can bind to the prodomain and ALK1 indistinguishably from the D-form, it is very sensitive to the presence of the redox buffer. At the physiological redox potential, the M-form is readily converted to the fully reduced M*-form and degraded by proteolysis. Such redox-dependent proteolysis provides at least one mechanism to maintain circulating BMP9 at constantly low levels under physiological conditions (37). To date, this redox-dependent regulation was only observed for BMP9, not for BMP6 (37) or BMP10 (RMS and WL, unpublished observation).

5. Regulation of BMP10

BMP10 has the highest sequence similarity to BMP9 and mediates flow-dependent arterial quiescence in zebrafish (40). Whether BMP10 plays a similar role in man is yet to be shown. Reports on circulating BMP10 levels and activity have been conflicting. Although BMP10 protein was detected by ELISA and proteomic approaches (7,41), its activity has been difficult to detect in human serum or plasma (25,42). In addition, the prodomain of BMP10 has been shown to inhibit BMP10 growth factor domain activity potently in C2C12 mouse myoblast cells, hence prodomain bound BMP10 was thought to be latent, like prodomain bound TGFβ and myostatin (43). Using mammalian cells expressed full-length human recombinant BMP10 and *ex vivo*-cultured mouse right atria, we have recently demonstrated that prodomain bound BMP10 is not latent but fully active on multiple ECs. Circulating BMP10 activity can be detected, albeit at much lower levels than BMP9 (38).

The findings that right atrium-secreted, prodomain-bound BMP10 is not latent provides new hypotheses for a role of BMP10 in the endocardium and pulmonary endothelium, since they are directly downstream of the right atrium and expected to be exposed to the highest concentrations of active BMP10. Interestingly, although BMPR-II is ubiquitously expressed in the human body, genetic loss-of-function mutations in *BMPR2* only cause disease in the pulmonary circulation. It would be interesting to test whether there is a compromised

BMP10/BMPR-II signalling due to *BMPR2* mutations and whether this plays a role in the pathogenesis of PAH.

6. Extracellular BMP antagonists

Extracellular BMP antagonists regulate the activities of many BMPs *in vivo*. For example, during the developmental process, the localised expression of the BMP inhibitors, such as chordin and Noggin, confers a gradient of BMP4 activity, essential for dorsal-ventral patterning in *Xenopus* gastrulae (44). Noggin is a broad spectrum BMP inhibitor, inhibiting the activities of BMP2, 4, 6, 7 as well as GDF5 (45-47). However, BMP9 and BMP10 are naturally insensitive to Noggin (25,46). To date, the only extracellular BMP antagonist reported to inhibit BMP9 activity is crossveinless 2 (CV2, also called BMPER). It has been shown that CV2 preferentially binds and inhibits BMP9 activity and it was proposed that CV2 disrupts complex formation involving ALK2, ALK1, BMP4 and BMP9 (48). How these molecules interact is not known, nor do we know whether CV2 is present in the circulation to inhibit BMP9 activity. A direct BMP10 antagonist has not been reported, although the prodomain of BMP10 has been shown to inhibit BMP10 signalling in C2C12 cells, probably due to competition for the type II receptor binding (38,43). Endoglin is a co-receptor in the TGFβ superfamily. Endoglin-Fc binds to BMP9 and BMP10 tightly, competing for the type II receptor binding and inhibiting BMP9 and BMP10 signalling (5). Soluble endoglin, without the Fc fusion and at large molar excess, was also shown to inhibit BMP9-induced Smad1/5 phosphorylation in mouse embryonic endothelial cells (49). Very recently, it was shown that TGFβ family ligands could function as antagonists by competing with the type II receptors and activin A has been shown to antagonize BMP9 and BMP10 signalling *in vitro* (50).

7. Discussion

BMPs are powerful cytokines, and their activities need to be tightly controlled. The temporal and spatial expression of the BMPs and their extracellular antagonists are essential for dorsal-ventral specification during development. In adults, BMP9 and BMP10 expression is limited to specific organs. Given that the liver is the largest internal organ in humans and probably the most powerful protein-producing organ, constitutively produced BMP9 needs to be tightly controlled. A redox- and proteolytic-dependent equilibrium provides one effective way to achieve this.

Are there BMP9/BMP10 gradients *in vivo* in adults? Given the specific production sites of BMP9 and BMP10, and no confirmed ligand traps for these two BMPs present in the circulation, it is conceivable that the vascular endothelium proximal to the production site will be exposed to higher concentrations of BMP9/BMP10 than the distal sites. If we map the BMP9 and BMP10 production sites along the blood flow path, the hepatic vein endothelium, endocardium and pulmonary endothelium are clearly the sites that encounter the highest concentrations of BMP9 and/or BMP10 (Figure 3). Measurement of BMP9/BMP10 levels from different locations across the circulation will be required to confirm this. In addition, BMP9 and BMP10 activate ALK1 much more effectively than other type I receptors (2), hence type I receptor specificity will help to achieve the specific function of BMP9/BMP10 on the ALK1-enriched endothelium. On the other hand, BMP family ligands and receptors share the same three-dimensional structural folds and similar receptor-ligand binding interfaces. When the concentration of the ligand is above a certain threshold, non-cognate receptor-ligand interaction can occur. For example, BMP9 has been shown to activate ALK2 at higher concentrations in signalling assays *in vitro*. Whether the BMP9/ALK2 axis plays a role in normal physiological process remain to be shown *in vivo*.

Most of the cell biology studies published to date utilised BMP9 and BMP10 mature growth factor domains. The circulating prodomain-bound forms of BMP9 or BMP10 are not yet commercially available. *In vitro* binding experiments have shown different affinities of BMP9 and prodomain bound BMP9 for the same type II receptors (51). Given that the prodomain binding site overlaps with the type II receptor binding site, it would be logical for the prodomain to be able to regulate the type II receptor selectivity, hence signalling specificity, although experimental evidence supporting this hypothesis is still to be shown.

A number of immediate questions relating to the physiological roles and regulation of BMP9 and BMP10 signalling remain to be investigated. For example, how are BMP9 and BMP10 regulated under pathological conditions, such as in PAH/HHT patients, and under inflammatory conditions, the second hit that might contribute to the pathogenesis of PAH? Does the heart require BMP10 activity to maintain its normal function in adults? In other words, will conditional knock out of BMP10 in adults cause heart disease, such as PAH or heart failure? Answers to these questions will provide significant insight into the functions of BMP9 and BMP10 in human physiology and facilitate the clinical translation of therapies targeting the BMP9/BMP10 signalling pathways.

Figure 1. Overview of BMP9/BMP10 signalling pathways. BMP9 and BMP10 are the major BMP ligands acting on endothelial cells. They form complexes with ALK1 and BMPR-II, and signal either through the phosphorylation of Smad1/5/8, or through Smad-independent pathways, activating downstream target genes. Endothelial surface BMPR-II undergoes a rapid turnover via a lysosomal-degradation pathway and BMP9/BMP10 signalling induces *BMPR2* gene and protein expression on endothelial cells.

Figure 2. Schematic and structures of BMP9. A. Schematic diagram of BMP9 production and processing. **B&C.** High-resolution crystal structures of BMP9 showing the presence of non-covalently linked BMP9 dimer. **B.** 2.5 Å BMP9 structure from Brown *et al* (39)(PDB code: 1ZKZ). The –SH groups from the two monomers are 4.8 Å apart and do not form a disulphide bond. **C**. 1.9 Å BMP9 structure from Wei *et al* (37)(PDB code: 4PML). The –SH groups from the two monomers exist in two conformations, one is 2.03 Å apart, which is the S-S bond distance, the other is 4.49 Å apart, too far to form a disulphide bond.

Figure 3. Model of BMP9 and BMP10 distribution in adults. In adults, BMP9 is predominantly produced in the liver and BMP10 from the right atrium. Liver-produced BMP9 and right heart-produced BMP10 will be carried to different vascular beds with the blood flow. Vascular endothelia that are proximal to the BMP9/BMP10 production sites will be likely to encounter higher concentrations of active BMP9 and BMP10 than those distal to the production sites. Extracellular BMP9 and BMP10 antagonists are not captured here since the expression and regulation of CV2 and soluble endoglin have not been reported to be restricted to specific organs and tissues.

References

1. Garcia de Vinuesa, A., Abdelilah-Seyfried, S., Knaus, P., Zwijsen, A., and Bailly, S. (2016) BMP signaling in vascular biology and dysfunction. *Cytokine Growth Factor Rev.* **27**, 65-79
2. David, L., Mallet, C., Mazerbourg, S., Feige, J. J., and Bailly, S. (2007) Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. *Blood* **109**, 1953-1961
3. Dunmore, B. J., Drake, K. M., Upton, P. D., Toshner, M. R., Aldred, M. A., and Morrell, N. W. (2013) The lysosomal inhibitor, chloroquine, increases cell surface BMPR-II levels and restores BMP9 signalling in endothelial cells harbouring BMPR-II mutations. *Hum. Mol. Genet.* **22**, 3667-3679

4. Upton, P. D., Davies, R. J., Trembath, R. C., and Morrell, N. W. (2009) Bone morphogenetic protein (BMP) and activin type II receptors balance BMP9 signals mediated by activin receptor-like kinase-1 in human pulmonary artery endothelial cells. *J. Biol. Chem.* **284**, 15794-15804
5. Castonguay, R., Werner, E. D., Matthews, R. G., Presman, E., Mulivor, A. W., Solban, N., Sako, D., Pearsall, R. S., Underwood, K. W., Seehra, J., Kumar, R., and Grinberg, A. V. (2011) Soluble endoglin specifically binds bone morphogenetic proteins 9 and 10 via its orphan domain, inhibits blood vessel formation, and suppresses tumor growth. *J. Biol. Chem.* **286**, 30034-30046
6. Nolan-Stevaux, O., Zhong, W., Culp, S., Shaffer, K., Hoover, J., Wickramasinghe, D., and Ruefli-Brasse, A. (2012) Endoglin requirement for BMP9 signaling in endothelial cells reveals new mechanism of action for selective anti-endoglin antibodies. *PLoS One* **7**, e50920
7. Ricard, N., Ciais, D., Levet, S., Subileau, M., Mallet, C., Zimmers, T. A., Lee, S. J., Bidart, M., Feige, J. J., and Bailly, S. (2012) BMP9 and BMP10 are critical for postnatal retinal vascular remodeling. *Blood* **119**, 6162-6171
8. Levet, S., Ouarne, M., Ciais, D., Coutton, C., Subileau, M., Mallet, C., Ricard, N., Bidart, M., Debillon, T., Faravelli, F., Rooryck, C., Feige, J. J., Tillet, E., and Bailly, S. (2015) BMP9 and BMP10 are necessary for proper closure of the ductus arteriosus. *Proc. Natl. Acad. Sci. U. S. A.* **112**, E3207-3215
9. Levet, S., Ciais, D., Merdzhanova, G., Mallet, C., Zimmers, T. A., Lee, S. J., Navarro, F. P., Texier, I., Feige, J. J., Bailly, S., and Vittet, D. (2013) Bone morphogenetic protein 9 (BMP9) controls lymphatic vessel maturation and valve formation. *Blood* **122**, 598-607
10. Chen, H., Shi, S., Acosta, L., Li, W., Lu, J., Bao, S., Chen, Z., Yang, Z., Schneider, M. D., Chien, K. R., Conway, S. J., Yoder, M. C., Haneline, L. S., Franco, D., and Shou, W. (2004) BMP10 is essential for maintaining cardiac growth during murine cardiogenesis. *Development* **131**, 2219-2231
11. Chen, H., Brady Ridgway, J., Sai, T., Lai, J., Warming, S., Chen, H., Roose-Girma, M., Zhang, G., Shou, W., and Yan, M. (2013) Context-dependent signaling defines roles of BMP9 and BMP10 in embryonic and postnatal development. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 11887-11892
12. Miller, A. F., Harvey, S. A., Thies, R. S., and Olson, M. S. (2000) Bone morphogenetic protein-9. An autocrine/paracrine cytokine in the liver. *J. Biol. Chem.* **275**, 17937-17945
13. Lopez-Coviella, I., Berse, B., Krauss, R., Thies, R. S., and Blusztajn, J. K. (2000) Induction and maintenance of the neuronal cholinergic phenotype in the central nervous system by BMP-9. *Science* **289**, 313-316
14. Bidart, M., Ricard, N., Levet, S., Samson, M., Mallet, C., David, L., Subileau, M., Tillet, E., Feige, J. J., and Bailly, S. (2012) BMP9 is produced by hepatocytes and circulates mainly in an active mature form complexed to its prodomain. *Cell. Mol. Life Sci.* **69**, 313-324
15. Neuhaus, H., Rosen, V., and Thies, R. S. (1999) Heart specific expression of mouse BMP-10 a novel member of the TGF-beta superfamily. *Mech. Dev.* **80**, 181-184
16. Somi, S., Buffing, A. A., Moorman, A. F., and Van Den Hoff, M. J. (2004) Expression of bone morphogenetic protein-10 mRNA during chicken heart development. *Anat. Rec. A Discov. Mol. Cell. Evol. Biol.* **279**, 579-582
17. Morrell, N. W., Bloch, D. B., Ten Dijke, P., Goumans, M. J., Hata, A., Smith, J., Yu, P. B., and Bloch, K. D. (2016) Targeting BMP signalling in cardiovascular disease and anaemia. *Nat. Rev. Cardiol.* **13**, 106-120

18. Ormiston, M. L., Upton, P. D., Li, W., and Morrell, N. W. (2015) The promise of recombinant BMP ligands and other approaches targeting BMPR-II in the treatment of pulmonary arterial hypertension. *Glob Cardiol Sci Pract* **2015**, 47
19. Wang, G., Fan, R., Ji, R., Zou, W., Penny, D. J., Varghese, N. P., and Fan, Y. (2016) Novel homozygous BMP9 nonsense mutation causes pulmonary arterial hypertension: a case report. *BMC Pulm. Med.* **16**, 17
20. Burton, V. J., Ciuclan, L. I., Holmes, A. M., Rodman, D. M., Walker, C., and Budd, D. C. (2011) Bone morphogenetic protein receptor II regulates pulmonary artery endothelial cell barrier function. *Blood* **117**, 333-341
21. Long, L., Ormiston, M. L., Yang, X., Southwood, M., Graf, S., Machado, R. D., Mueller, M., Kinzel, B., Yung, L. M., Wilkinson, J. M., Moore, S. D., Drake, K. M., Aldred, M. A., Yu, P. B., Upton, P. D., and Morrell, N. W. (2015) Selective enhancement of endothelial BMPR-II with BMP9 reverses pulmonary arterial hypertension. *Nat. Med.* **21**, 777-785
22. Reynolds, A. M., Holmes, M. D., Danilov, S. M., and Reynolds, P. N. (2012) Targeted gene delivery of BMPR2 attenuates pulmonary hypertension. *Eur. Respir. J.* **39**, 329-343
23. Ricard, N., Bidart, M., Mallet, C., Lesca, G., Giraud, S., Prudent, R., Feige, J. J., and Bailly, S. (2010) Functional analysis of the BMP9 response of ALK1 mutants from HHT2 patients: a diagnostic tool for novel ACVRL1 mutations. *Blood* **116**, 1604-1612
24. Scharpfenecker, M., van Dinther, M., Liu, Z., van Bezooijen, R. L., Zhao, Q., Pukac, L., Lowik, C. W., and ten Dijke, P. (2007) BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. *J. Cell Sci.* **120**, 964-972
25. David, L., Mallet, C., Keramidas, M., Lamande, N., Gasc, J. M., Dupuis-Girod, S., Plauchu, H., Feige, J. J., and Bailly, S. (2008) Bone morphogenetic protein-9 is a circulating vascular quiescence factor. *Circ. Res.* **102**, 914-922
26. Suzuki, Y., Ohga, N., Morishita, Y., Hida, K., Miyazono, K., and Watabe, T. (2010) BMP-9 induces proliferation of multiple types of endothelial cells in vitro and in vivo. *J. Cell Sci.* **123**, 1684-1692
27. Park, J. E., Shao, D., Upton, P. D., Desouza, P., Adcock, I. M., Davies, R. J., Morrell, N. W., Griffiths, M. J., and Wort, S. J. (2012) BMP-9 induced endothelial cell tubule formation and inhibition of migration involves Smad1 driven endothelin-1 production. *PLoS One* **7**, e30075
28. Cunha, S. I., Pardali, E., Thorikay, M., Anderberg, C., Hawinkels, L., Goumans, M. J., Seehra, J., Heldin, C. H., ten Dijke, P., and Pietras, K. (2010) Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis. *J. Exp. Med.* **207**, 85-100
29. Herrera, B., van Dinther, M., Ten Dijke, P., and Inman, G. J. (2009) Autocrine bone morphogenetic protein-9 signals through activin receptor-like kinase-2/Smad1/Smad4 to promote ovarian cancer cell proliferation. *Cancer Res.* **69**, 9254-9262
30. Herrera, B., Garcia-Alvaro, M., Cruz, S., Walsh, P., Fernandez, M., Roncero, C., Fabregat, I., Sanchez, A., and Inman, G. J. (2013) BMP9 is a proliferative and survival factor for human hepatocellular carcinoma cells. *PLoS One* **8**, e69535
31. Ye, L., Bokobza, S., Li, J., Moazzam, M., Chen, J., Mansel, R. E., and Jiang, W. G. (2010) Bone morphogenetic protein-10 (BMP-10) inhibits aggressiveness of breast cancer cells and correlates with poor prognosis in breast cancer. *Cancer Sci.* **101**, 2137-2144
32. Wang, K., Feng, H., Ren, W., Sun, X., Luo, J., Tang, M., Zhou, L., Weng, Y., He, T. C., and Zhang, Y. (2011) BMP9 inhibits the proliferation and invasiveness of breast cancer cells MDA-MB-231. *J. Cancer Res. Clin. Oncol.* **137**, 1687-1696
33. Gupta, S., Gill, D., Pal, S. K., and Agarwal, N. (2015) Activin receptor inhibitors--dalantercept. *Curr. Oncol. Rep.* **17**, 14

34. Kang, Q., Sun, M. H., Cheng, H., Peng, Y., Montag, A. G., Deyrup, A. T., Jiang, W., Luu, H. H., Luo, J., Szatkowski, J. P., Vanichakarn, P., Park, J. Y., Li, Y., Haydon, R. C., and He, T. C. (2004) Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery. *Gene Ther.* **11**, 1312-1320
35. van Baardewijk, L. J., van der Ende, J., Lissenberg-Thunnissen, S., Romijn, L. M., Hawinkels, L. J., Sier, C. F., and Schipper, I. B. (2013) Circulating bone morphogenetic protein levels and delayed fracture healing. *Int. Orthop.* **37**, 523-527
36. Jones, W. K., Richmond, E. A., White, K., Sasak, H., Kusmik, W., Smart, J., Oppermann, H., Rueger, D. C., and Tucker, R. F. (1994) Osteogenic protein-1 (OP-1) expression and processing in Chinese hamster ovary cells: isolation of a soluble complex containing the mature and pro-domains of OP-1. *Growth Factors* **11**, 215-225
37. Wei, Z., Salmon, R. M., Upton, P. D., Morrell, N. W., and Li, W. (2014) Regulation of Bone Morphogenetic Protein 9 (BMP9) by Redox-dependent Proteolysis. *J. Biol. Chem.* **289**, 31150-31159
38. Jiang, H., Salmon, R. M., Upton, P. D., Wei, Z., Lawera, A., Davenport, A. P., Morrell, N. W., and Li, W. (2016) The Prodomain-bound Form of Bone Morphogenetic Protein 10 Is Biologically Active on Endothelial Cells. *J. Biol. Chem.* **291**, 2954-2966
39. Brown, M. A., Zhao, Q., Baker, K. A., Naik, C., Chen, C., Pukac, L., Singh, M., Tsareva, T., Parice, Y., Mahoney, A., Roschke, V., Sanyal, I., and Choe, S. (2005) Crystal structure of BMP-9 and functional interactions with pro-region and receptors. *J. Biol. Chem.* **280**, 25111-25118
40. Laux, D. W., Young, S., Donovan, J. P., Mansfield, C. J., Upton, P. D., and Roman, B. L. (2013) Circulating Bmp10 acts through endothelial Alk1 to mediate flow-dependent arterial quiescence. *Development* **140**, 3403-3412
41. Souza, T. A., Chen, X., Guo, Y., Sava, P., Zhang, J., Hill, J. J., Yaworsky, P. J., and Qiu, Y. (2008) Proteomic identification and functional validation of activins and bone morphogenetic protein 11 as candidate novel muscle mass regulators. *Mol. Endocrinol.* **22**, 2689-2702
42. Herrera, B., and Inman, G. J. (2009) A rapid and sensitive bioassay for the simultaneous measurement of multiple bone morphogenetic proteins. Identification and quantification of BMP4, BMP6 and BMP9 in bovine and human serum. *BMC Cell Biol.* **10**, 20
43. Sengle, G., Ono, R. N., Sasaki, T., and Sakai, L. Y. (2011) Prodomains of transforming growth factor beta (TGFbeta) superfamily members specify different functions: extracellular matrix interactions and growth factor bioavailability. *J. Biol. Chem.* **286**, 5087-5099
44. Dale, L., and Wardle, F. C. (1999) A gradient of BMP activity specifies dorsal-ventral fates in early Xenopus embryos. *Semin. Cell Dev. Biol.* **10**, 319-326
45. Gazzerro, E., and Canalis, E. (2006) Bone morphogenetic proteins and their antagonists. *Rev. Endocr. Metab. Disord.* **7**, 51-65
46. Seemann, P., Brehm, A., Konig, J., Reissner, C., Stricker, S., Kuss, P., Haupt, J., Renninger, S., Nickel, J., Sebald, W., Groppe, J. C., Ploger, F., Pohl, J., Schmidt-von Kegler, M., Walther, M., Gassner, I., Rusu, C., Janecke, A. R., Dathe, K., and Mundlos, S. (2009) Mutations in GDF5 reveal a key residue mediating BMP inhibition by NOGGIN. *PLoS genetics* **5**, e1000747
47. Wang, Y., Hong, S., Li, M., Zhang, J., Bi, Y., He, Y., Liu, X., Nan, G., Su, Y., Zhu, G., Li, R., Zhang, W., Wang, J., Zhang, H., Kong, Y., Shui, W., Wu, N., He, Y., Chen, X., Luu, H. H., Haydon, R. C., Shi, L. L., He, T. C., and Qin, J. (2013) Noggin resistance contributes to the

potent osteogenic capability of BMP9 in mesenchymal stem cells. *J. Orthop. Res.* **31**, 1796-1803
48. Yao, Y., Jumabay, M., Ly, A., Radparvar, M., Wang, A. H., Abdmaulen, R., and Bostrom, K. I. (2012) Crossveinless 2 regulates bone morphogenetic protein 9 in human and mouse vascular endothelium. *Blood* **119**, 5037-5047
49. Gregory, A. L., Xu, G., Sotov, V., and Letarte, M. (2014) Review: the enigmatic role of endoglin in the placenta. *Placenta* **35 Suppl**, S93-99
50. Aykul, S., and Martinez-Hackert, E. (2016) Transforming Growth Factor-beta family ligands can function as antagonists by competing for type II receptor binding. *J. Biol. Chem.* pii: jbc.M115.713487. [Epub ahead of print]
51. Mi, L. Z., Brown, C. T., Gao, Y., Tian, Y., Le, V. Q., Walz, T., and Springer, T. A. (2015) Structure of bone morphogenetic protein 9 procomplex. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 3710-3715

Figure 1

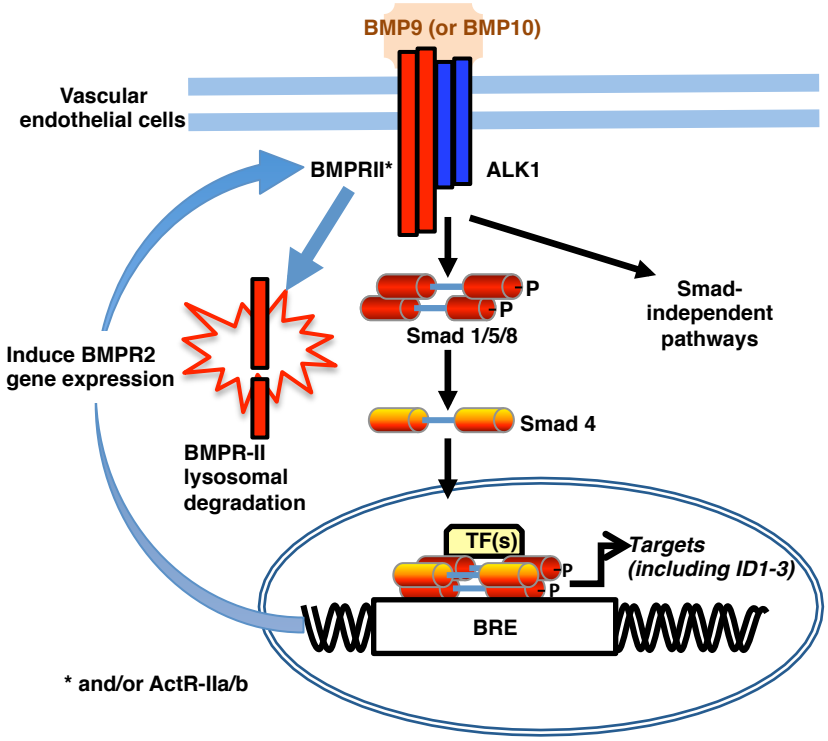

BMP9 (or BMP10)
Vascular endothelial cells
BMPRII*
ALK1
Smad-independent pathways
P
P
Smad 1/5/8
Induce BMPR2 gene expression
Smad 4
BMPR-II lysosomal degradation
TF(s)
Targets (including ID1-3)
P
P
BRE
* and/or ActR-IIa/b

Figure 2

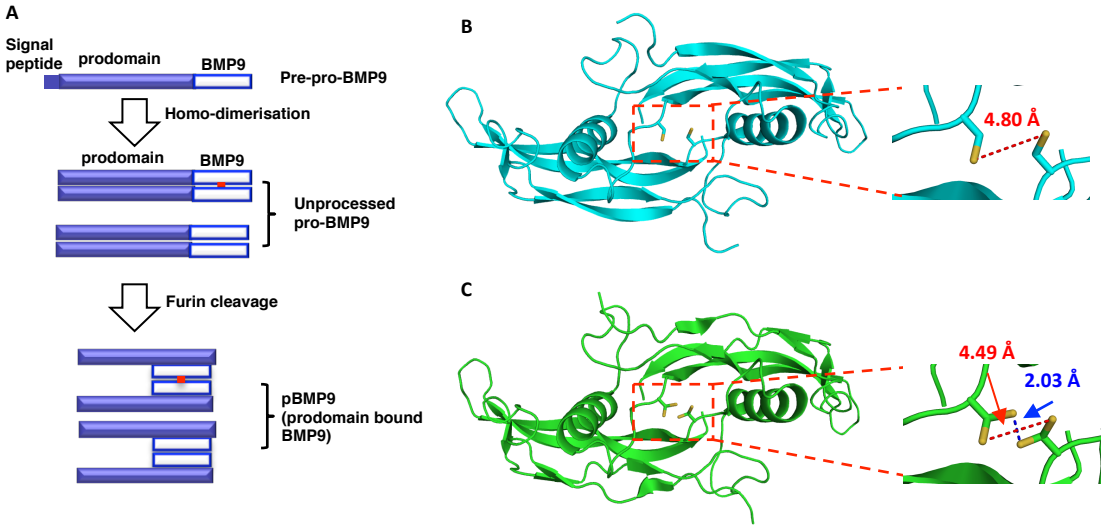
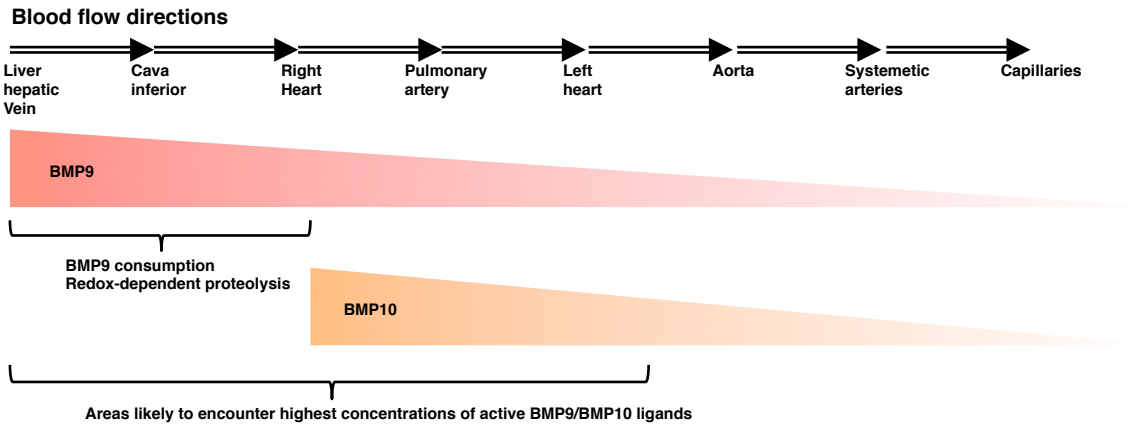

A
Signal peptide
prodomain
BMP9
Pre-pro-BMP9
Homo-dimerisation
prodomain
BMP9
Unprocessed pro-BMP9
Furin cleavage
pBMP9 (prodomain bound BMP9)
B
4.80 Å
C
4.49 Å
2.03 Å

Figure 3


Blood flow directions
Liver hepatic Vein
Cava inferior
Right Heart
Pulmonary artery
Left heart
Aorta
Systemetic arteries
Capillaries
BMP9
BMP9 consumption
Redox-dependent proteolysis
BMP10
Areas likely to encounter highest concentrations of active BMP9/BMP10 ligands