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Mechanisms of disease

The promise of recombinant BMP ligands and other approaches targeting BMPR-II in the treatment of pulmonary arterial hypertension

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ABSTRACT

Human genetic discoveries offer a powerful method to implicate pathways of major importance to disease pathobiology and hence provide targets for pharmacological intervention. The genetics of pulmonary arterial hypertension (PAH) strongly implicates loss-of-function of the bone morphogenetic protein type II receptor (BMPR-II) signalling pathway and moreover implicates the endothelial cell as a central cell type involved in disease initiation. We and others have described several approaches to restore BMPR-II function in genetic and non-genetic forms of PAH. Of these, supplementation of endothelial BMP9/10 signalling with exogenous recombinant ligand has been shown to hold considerable promise as a novel large molecule biopharmaceutical therapy. Here, we describe the mechanism of action and discuss potential additional effects of BMP ligand therapy.

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INTRODUCTION

Pulmonary arterial hypertension (PAH) is a disease of pathological vascular remodeling, characterized by medial thickening and the formation of occlusive vascular lesions that obstruct the pulmonary circulation, predominantly at the level of the pre-capillary arterioles. This loss of the pulmonary distal circulation, and the resultant increase in pulmonary vascular resistance (PVR), leads to an elevation of pulmonary arterial pressure and right ventricular hypertrophy. PAH is a rare disease, with a prevalence of 15 to 26 patients per million individuals and an incidence of 2.4 to 7.6 cases per million annually.^{1,2} It can arise as a primary disease, either in an idiopathic (IPAH) or heritable (HPAH) form, or as a condition associated with immune disorders such as HIV, connective tissues diseases, or exposure to particular drugs or toxins. In its idiopathic and heritable forms, PAH presents in a relatively young patient population, aged 30–50 years, and preferentially in women at a rate of roughly 2.3:1. If left untreated, PAH can lead to death from right-sided heart failure within 3–5 years of diagnosis.

Existing treatments for PAH were developed for this indication because of their effects on vascular tone. The regulation of vascular tone by endothelial cells and vascular smooth muscle cells (SMCs) is mediated by a balance of vasodilators, such as prostacyclin and nitric oxide (NO), and vasoconstrictive agents, including endothelin-1. Established PAH is associated with a shift in this balance towards excessive pulmonary vasoconstriction. Recognition of this imbalance was the driving force behind the development and approval of a range of vasodilatory therapies for PAH.³ These therapies can be divided into three main classes: (i) prostanoids, including epoprostenol and more stable prostacyclin analogues such as iloprost, beraprost and treprostinil; (ii) endothelin receptor antagonists, including bosentan, ambrisentan and macitentan; and (iii) phosphodiesterase 5 inhibitors, such as sildenafil and tadalafil. While these treatments have been successful in improving the haemodynamic parameters and functional status of patients, the three-year survival rate for PAH patients remains poor.⁴ Although these therapies may also have modest effects on vascular SMC proliferation, available evidence suggests a minimal impact on the process of vascular remodeling in the lungs of patients with PAH.⁵ Here we propose that more effective therapies for PAH will derive from a greater understanding of the molecular basis of pathological pulmonary vascular remodeling, particularly by targeting pathways identified by human genetics in PAH patients.

THE GENETIC BASIS OF PAH

Heritable PAH (HPAH) is an autosomal dominant disease, marked by a low penetrance (average 20–30%) in at-risk individuals.⁶ While the existence of this familial form of PAH has been recognized since the first description of the disease, it was only 15 years ago that mutations in *BMPR2*, the gene encoding the bone morphogenetic protein (BMP) type II receptor (BMPR-II), was identified as the cause of approximately 75% of HPAH cases.^{7,8} *BMPR2* mutations also account for 15–26% of seemingly idiopathic or sporadic cases of PAH, including cases of *de novo* mutations and parental transmission with no record of a previous family history of disease. As a result of these findings, the definition of HPAH was recently updated to include not only patients in a family with two or more documented cases of PAH, but also includes any PAH patient possessing a mutation in *BMPR2*.⁹

A range of mutations in *BMPR2* have been reported in PAH patients. The majority of these mutations lead to a state of haploinsufficiency,¹⁰ where the mutant allele leads to no production of a protein product. The protein expression from the wild type allele is normal but overall protein expression is reduced by at least 50%. Patients bearing *BMPR2* mutations develop PAH earlier, have more severe disease and die sooner than those without mutations.⁶ Interestingly, PAH patients with *BMPR2* mutations exhibit reductions in BMPR-II protein levels of greater than 75% when compared to control subjects, suggesting that the development of PAH can suppress receptor levels to a greater extent than what can be accounted for by haploinsufficiency alone.¹¹ Reduced BMPR-II protein levels and impaired downstream signaling have also been identified in idiopathic PAH patients lacking mutations in *BMPR2*, as well as in common, non-genetic rodent models of disease,¹² further supporting a central role for reduced BMPR-II signaling in most forms of the disease, independent of etiology.

When taken together, these factors make the BMPR-II signaling pathway an extremely attractive target for next-generation therapeutic intervention. However, the translation of this approach into *in vivo* pre-clinical studies has been limited by uncertainty regarding which cell type or types are critically affected by the loss of BMPR-II signaling, and the complexity of the BMP signalling family. Fortunately, genetic studies describing disease phenotypes associated with mutations in other components of the BMP signaling pathway, coupled with knowledge of the tissue-specific distribution

of these proteins, can be used to inform decisions on which cell types, and which BMPs, play important roles in the initiation of PAH.

BMPs are members of the transforming growth factor- β (TGF β) superfamily, a highly complex family of proteins including over 30 ligands that signal through heteromeric complexes of type I and type II receptors. As a type II receptor of this superfamily, BMPR-II can form complexes with several type I receptors, including the activin like receptor kinases ALK1, ALK2, ALK3 or ALK6, with each receptor complex recognizing a specific subset of BMP ligands.¹³ Canonical signaling generally involves phosphorylation of the Smad-1, 5 or -8 transcription factors, which complex with the co-smad, Smad-4, and translocate to the nucleus to induce gene expression. The tissue-specific nature of BMP responses is highly dependent upon the components making up the ligand-receptor complex. Numerous accessory receptors, such as endoglin (*ENG*), also modify signaling, further contributing to the tissue-specificity of BMP responses.

Following the initial identification of *BMPR2* mutations in PAH, much of the work investigating the role of deficiency of this receptor in disease pathogenesis focused on the impact of these mutations on pulmonary arterial SMC (PASMC) proliferation and migration.¹⁴ In smooth muscle cells, BMP ligands, including BMP2 and BMP4, block serum-induced proliferation via complexes of BMPR-II with ALK3 or ALK6.¹⁴ However, mutations in *ALK3* and *ALK6* do not cause pulmonary vascular disease, but are instead associated with juvenile gastrointestinal polyposis¹⁵ and hereditary brachydactyly,¹⁶ respectively. This fact calls into question the importance of these receptors, and the signaling complexes that they form, in the pathogenesis of PAH.

More recently, several studies have demonstrated a role for impaired endothelial BMP signaling in the pathogenesis of PAH. In addition to increased endothelial cell proliferation and enhanced susceptibility to apoptosis,^{17,18} loss of BMPR-II has also been shown to influence endothelial cell barrier integrity and pulmonary vascular permeability.¹⁹ Genetic evidence also supports a role for the pulmonary endothelium as the critical initiating cell type in PAH. Mutations in Endoglin and ALK1 (gene symbol *ACVRL1*), two proteins found almost exclusively on the endothelium, are primarily associated with hereditary haemorrhagic telangiectasia (HHT), a disease that causes vascular abnormalities, including telangiectases in the skin and mucosal regions, and arteriovenous malformations in the lung, liver, gastrointestinal tract and brain. In addition to HHT, *ACVRL1* mutations are occasionally associated with PAH, demonstrating a role for these molecules, and the receptor complexes they form, in the maintenance of pulmonary vascular homeostasis.²⁰ To date, mutations in eight genes have been found to play a causal role in idiopathic and heritable PAH (Fig. 1). Notably, of all the known mutations identified to date, six out of eight of these directly or indirectly implicate the BMP signalling pathway, and particularly endothelial BMP signaling, as central to pathobiology. Taken together, these findings strongly support the development of therapeutic strategies that restore expression or function of BMPR-II, particularly in pulmonary vascular endothelial cells.

TARGETING BMPR-II DEFICIENCY FOR THE TREATMENT OF PAH

Considering the genetic evidence indicating an important role for BMPR-II in the pathobiology of PAH, several groups have begun to use this information to test the potential of next generation therapies that directly target BMPR-II deficiency. Although not limited to the endothelium, a number of these studies directly target pulmonary endothelial BMPR-II for their mode of action. These therapies are directed towards various stages of BMPR-II signaling, including gene expression, translation, surface expression and receptor degradation, as well as enhancing receptor activity through the delivery of BMPR-II agonists. The various strategies can be grouped into three main approaches: (i) rescue of mutant receptor function, (ii) enhancing the expression or longevity of the wild-type receptor or (iii) enhancing BMP signaling through small molecules or recombinant BMP ligands. The published studies examining these three approaches are summarized below and in Table 1.

Rescuing mutant BMPR-II receptor function: Over 400 different PAH associated *BMPR2* mutations have been identified in all functional domains of the BMPR-II protein.⁶ These include missense mutations within the extracellular domain, transmembrane region, and kinase region, as well as nonsense mutations that encode premature termination codons (PTCs). Strategies designed to enhance BMP signaling by rescuing the mutated copy of the *BMPR2* gene can take one of two forms, depending on the type of mutation present.

Chemical or pharmacological chaperones: Missense mutations can lead to the production of misfolded BMPR-II through the replacement of critical cysteine residues that are essential to the folding

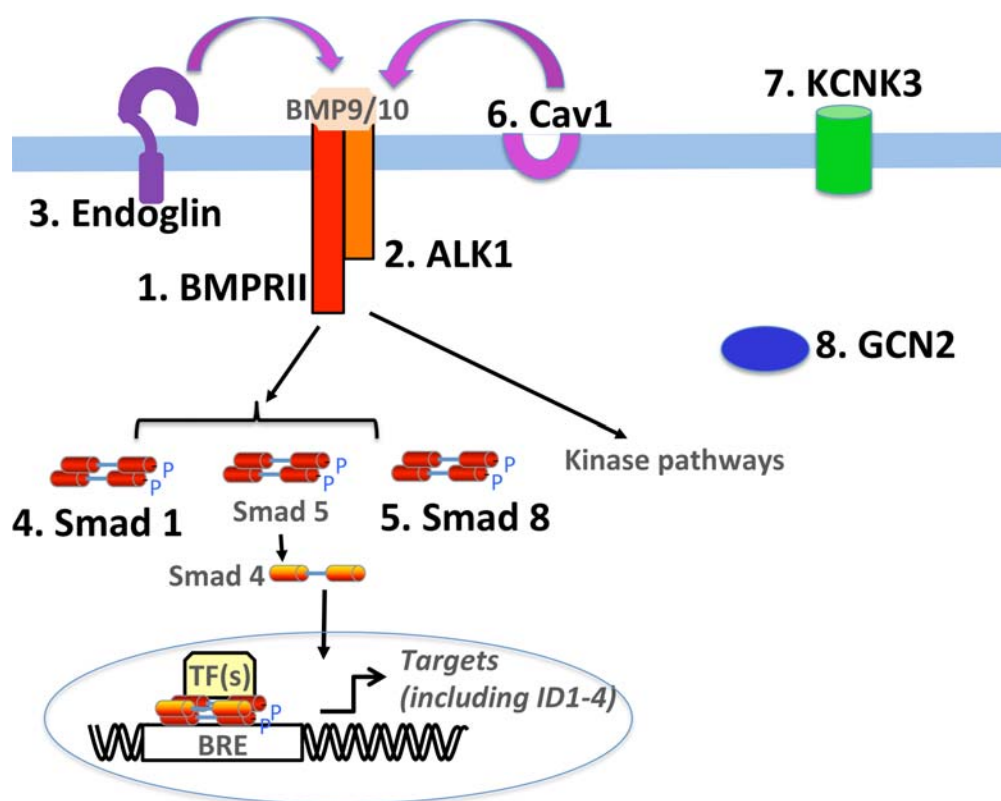


Figure 1. Simplified schematic summarizing the BMP signalling pathway and genes that have to date been shown to be mutated in PAH and demonstrating that many of these mutations encode protein involved in BMP signalling. Known mutations are numbered and in bold.

of the extracellular domain within the endoplasmic reticulum (ER). These mutants can be targeted with chemical chaperones that enhance the transport of the mutant protein from the ER to the cell surface. Some chemical chaperones, such as glycerol, non-selectively stabilize the tertiary structure of the mutant proteins and facilitate folding. Other compounds, including thapsigargin and sodium 4-phenylbutyrate (4-PBA), inhibit the interactions of the mutant protein with the chaperone proteins responsible for their recognition, retention and degradation. In PAH, a panel of chemical chaperones, including 4-PBA, glycerol and thapsigargin, were shown to promote folding and trafficking of mutant BMPRII protein to the cell surface. Since, in this case, the mutant receptor was still capable of normal signalling, downstream Smad signalling was restored.²¹ While these results are promising, there are several factors limiting the clinical application of these compounds for PAH. The PAH patient population contains a large variety of missense mutations, only a subset of which encode mutations that lead to the misfolding a receptor that is otherwise functional. Nevertheless, 4-PBA has been used for other indications in man and may be a personalized approach in patients with these specific mutations.

Suppression of nonsense mutations: In contrast to missense mutations, where the mutant protein is produced and can be targeted for rescue, the mRNA transcripts produced by alleles bearing nonsense mutations are typically subject to nonsense-mediated decay (NMD), resulting in reduced *BMPRII* mRNA and decreased protein levels. The rescue of these mutations requires the use of compounds that suppress or silence PTCs, allowing for the translation of mutant mRNA into full-length, functional protein.²² A number of compounds belonging to the aminoglycoside family of antibiotics, including gentamicin, have been shown to suppress disease-causing PTCs and partially restore protein function in nonsense mutation-bearing cell culture models of multiple diseases, including cystic fibrosis, Duchenne muscular dystrophy and PAH.²³ However, the high doses required to achieve these beneficial effects *in vivo* are also associated with renal toxicity and hearing loss, thus preventing the widespread use of aminoglycosides in clinical applications.

More recently, high throughput screens have identified other compounds distinct from aminoglycosides that possess the ability to effectively suppress PTCs without affecting the recognition

Table 1. Summary of experimental PAH therapies targeting BMPR-II

Therapeutic Strategy	Agent	Model	Citation
<i>Rescue of Mutant BMPR-II Receptor</i>			
Chemical Chaperones	4-PBA, glycerol and thapsigargin	In vitro: HeLa cells	[21]
Suppression of Nonsense Mutations	Gentamycin Ataluren (PTC124)	In vitro: cell-based reporter assay In vitro: PAH endothelial and smooth muscle cells	[23] [24]
<i>Enhancement of BMPR-II Expression and Longevity</i>			
Inhibition of lysosomal degradation	Chloroquine, hydroxychloroquine	In vitro: PAH endothelial cells	[26, 28]
BMPR2 Gene Therapy	Endothelial-targeted adenoviral vector	In vivo: chronic hypoxia and monocrotaline rat models	[29, 30]
<i>Enhancement of BMPR-II-mediated Signaling</i>			
Small molecule BMPR-II agonists	Iloprost Sildenafil FK506	In vitro: PAH smooth muscle cells In vivo: Monocrotaline rat model In vitro: PAH smooth muscle cells In vivo: Monocrotaline rat model	[32] [33] [34]
Recombinant BMP ligand	Recombinant human BMP9	In vitro: PAH endothelial cells In vivo: <i>bmpr2-R899X</i> mouse, Monocrotaline rat model, Sugen-Hypoxia rat and mouse models	[41]

of normal stop signals. One such compound, the small molecule Ataluren (PTC124), is currently in phase III clinical trials for Duchenne muscular dystrophy. In PAH, Ataluren was shown to increase BMPR-II protein levels and downstream signaling in primary cells from patients with nonsense mutations in *BMPR2* and *SMAD9*.²⁴ These effects were accompanied by a restoration of normal proliferation rates in hyperproliferative, patient-derived PAECs and PSMCs.

Enhancing BMPR-II surface expression

Inhibiting BMPR-II degradation: An alternative approach that has been explored for enhancing BMP signalling involves extending the longevity of BMPR-II at the cell surface. BMPR-II is subject to constitutive endocytosis by both clathrin and caveolae mediated pathways²⁵ and is rapidly turned over in endothelial cells. Notable protein loss is observed in these cells as early as 1 hour following the blockade of new protein synthesis.²⁶ BMPR-II can also be targeted for ubiquitination by Kaposi's sarcoma herpes virus K5 E3 ligase, and directed for lysosomal degradation.²⁷ Each of these findings highlights the lysosome as the primary route for BMPR-II turnover and points to lysosomal inhibition as a promising target for preserving BMPR-II longevity and enhancing receptor-mediated signaling. The 4-aminoquinolone, chloroquine, achieves lysosomal inactivation through the prevention of vesicular acidification. Used for decades as a prophylactic antimalarial drug, chloroquine, and its less toxic derivative hydroxychloroquine, have recently been repurposed for use in the treatment of rheumatoid arthritis and cancer. In vitro, chloroquine was shown to enhance BMPR-II protein levels, surface expression and downstream signaling in *BMPR2* mutation-bearing endothelial cells.²⁶ In vivo, both chloroquine and hydroxychloroquine inhibited the development of pulmonary hypertension and blocked the progression of established disease in the monocrotaline rat model.²⁸ Although this therapeutic effect was associated with restoration of BMPR-II protein levels, which are decreased in monocrotaline-treated rats, it is likely that the beneficial effect of chloroquine administration may also be linked to the inhibition of excessive autophagy, which has been linked to the aberrant proliferation of vascular cells in PAH. Translation of hydroxychloroquine into a clinical therapy is facilitated by the fact that the compound is inexpensive and presents acceptable toxicity or side effects at therapeutically effective doses. Long-term treatment with hydroxychloroquine has been associated with retinopathy in 0.5–1% of patients. However, this can be avoided with proper monitoring of patients undergoing treatment.

BMPR2 gene therapy: Gene therapy techniques have also been explored in animal models of PAH as a means to restore BMPR-II receptor levels. The targeted delivery of an adenoviral vector containing the *BMPR2* gene to the pulmonary vascular endothelium of rats substantially reduced the severity of pulmonary hypertension in the chronic hypoxia and monocrotaline models of PAH.^{29,30} For these studies, targeting of the adenoviral vectors to the pulmonary endothelium appeared to be a key feature of therapeutic efficacy, as similar studies using aerosol vector delivery and a non-specific promoter failed to demonstrate any measurable benefit in the monocrotaline rat model.³¹

Enhancement of BMPR-II downstream signalling: One alternative to enhancing BMPR-II receptor expression or longevity involves enhancing signaling through the action of small molecules. Existing PAH therapies, including prostanoids and sildenafil, have been shown to partially restore BMP signaling in PSMCs bearing *BMPR2* mutations and prevent the development of PAH in animal models of disease.^{32,33} More recently, a high throughput screening approach demonstrated that the calcineurin inhibitor, tacrolimus (FK506), potentiates BMPR-II-mediated signaling in endothelial cells, rescues dysfunctional BMP signaling in endothelial cells from PAH patients with *BMPR2* mutations and reverses established disease in multiple rodent models of PAH.³⁴ Early clinical trials are currently underway to establish the viability of this strategy as a treatment for PAH. However, this approach is likely to lead to widespread and non-specific activation of BMP receptors, as well as having off target effects independent of BMP signalling.

BMP9 AND BMP10 AS POTENTIAL THERAPIES FOR PAH

One of the most direct strategies for targeting BMPR-II deficiency in PAH involves the delivery of exogenous BMP ligand to enhance signaling via the remaining functional receptor. Proof-of-concept studies using patient-derived pulmonary arterial smooth muscle cells (PSMCs) have shown that the addition of increasing concentrations of BMP ligand can overcome the functional defects associated with *BMPR2* mutation *in vitro*.¹⁴ However, identifying the appropriate BMP ligand or ligands to selectively target the endothelium *in vivo* presents a significant challenge. The selective expression of

ALK1 on the endothelium makes the ALK1 /BMPR-II complex an ideal target for exogenous ligand therapy. Although ALK1 was originally thought to be an orphan receptor, two studies in 2007 identified BMP9 and BMP10 as ligands that signal via complexes of ALK1 with either BMPR-II or type II activin receptors (ActRII).^{35,36}

Further examination of the receptor selectivity of these ligands has primarily focused on BMP9. Endogenously, BMP9 activates ALK1 with high affinity ($EC_{50} = 50$ pg/ml). BMP9 also activates ALK2 but with much a lower affinity in the ng/ml range.^{35–39} Biochemical analysis has also revealed discrete differences in the type II receptor selectivity of BMP9 and BMP10. Although BMP9 and BMP10 both bind to BMPRII, ActRIIA and ActRIIB, BMP9 exhibits a preference towards ActRIIB compared to BMPRII and ActRIIA while BMP-10 binds to all type II receptors with similar affinities.⁴⁰ In endothelial cells, type II receptor redundancy means that loss of either BMPR-II or ActRIIA has a minor impact on the stimulation of Smad1/5 phosphorylation due to compensation by the remaining type II receptor.³⁷ Loss of both receptors, however, abolished the Smad phosphorylation responses. Of note, some transcriptional responses to BMP9, such as E-selectin and IL8 induction are more reliant on BMPR-II signaling as ActRIIA does not compensate.³⁷ This implies that there are BMPR-II-specific signals that may contribute more than others to the pathology of PAH. Another feature of the BMPR-II selectivity of BMP9 relates to the transcriptional regulation of the receptors themselves. BMP9 induces the expression of BMPR-II, but not ALK1 or ActRIIA, suggesting that this ligand switches the balance of signaling in the endothelial cell towards BMPR-II.^{35,37} Furthermore, this induction is ALK1-dependent and Smad1-dependent, suggesting a feed forward signaling mechanism.^{37,41} It was recently shown that the therapeutic effects of BMP9 include restoration of BMPR-II expression and signaling of BMPR-II in animal models of PAH.⁴¹ Therefore, BMP9 represents a therapy that promotes its key downstream signalling pathways partly via rescue of the deficient BMPR-II expression that underlies the pathogenesis of PAH. A proposed mechanism for this upregulation is shown in Fig. 2.

Based on our studies of BMPR-II dysfunction in PAH and the emerging role of BMP9 as a key circulating regulator of endothelial function and positive regulator of BMPR-II expression, we recently examined the therapeutic delivery of BMP9 to target endothelial dysfunction in PAH.⁴¹ *In vitro*, BMP9 prevents the enhanced apoptosis observed in *BMPR2* mutation-bearing endothelial cells and promotes monolayer integrity through the formation of tight junctions. *In vivo*, BMP9 prevents and reverses established disease in a range of rodent models, including spontaneous disease in a mouse model bearing a knock-in of the PAH-associated *R899X-Bmpr2* mutation. From these studies, we propose that BMP9 constitutes a potential therapy that overcomes the underlying endothelial dysfunction that causes PAH and can overcome the deficient BMPR-II signaling that is a consequence of the major genetic defect in most HPAH patients (Fig. 3). Our anticipation is that, by restoring the balance in BMP signaling, this approach will redress the imbalance of other dysregulated pathways that are targeted by current therapies.

Another theoretical approach to activate the endothelial ALK1/BMPR-II pathway would be to develop a peptide mimetic that possesses BMP9/10-like function. An example of such an approach was recently demonstrated for a peptide derived from BMP7.⁴² Although it is considered unlikely by some that a small peptide mimetic would be able to substitute for a BMP dimer in the assembly of two type I and two type II receptors, all of which are required for signalling,^{43–45} the BMP7 peptide mimetic was shown to have similar effects to full-length BMP7 ligand in reversing established fibrosis in five mouse models of acute and chronic renal injury.⁴² Although the findings of this study have been challenged by researchers from several groups, synthetic peptides that specifically activate the endothelial ALK1/BMPR-II pathway still represent promising potential drug candidates for treating PAH.

Since BMP9 exerts its beneficial effect on the endothelium in an ALK1-dependent manner, recombinant BMP10 protein, which is the only other BMP ligand that signals through ALK1, could also have a similar therapeutic potential for treating PAH. There are several advantages of BMP10-based therapy. Firstly, unlike BMP9, it does not show osteogenic activity either in vitro signalling assays, or in an in vivo bone-forming screen,⁴⁶ and may therefore be potentially safer than BMP9 for treating cardiovascular disease. Secondly, BMP10 binds to ALK1 and BMPR-II with higher affinities than BMP9,⁴⁰ making it likely to be more potent and more specific for ALK1 and BMPR-II.

In spite of these advantages, there are still several hurdles to overcome before BMP10 can be developed into a therapy for PAH. For example, the activity of circulating BMP10 in humans is still under investigation. BMP10 is bound tightly by its prodomain, which inhibits BMP10 signalling activity when applied in molar excess.⁴⁷ It has therefore been proposed that BMP10, similar to TGF β , exists in a latent

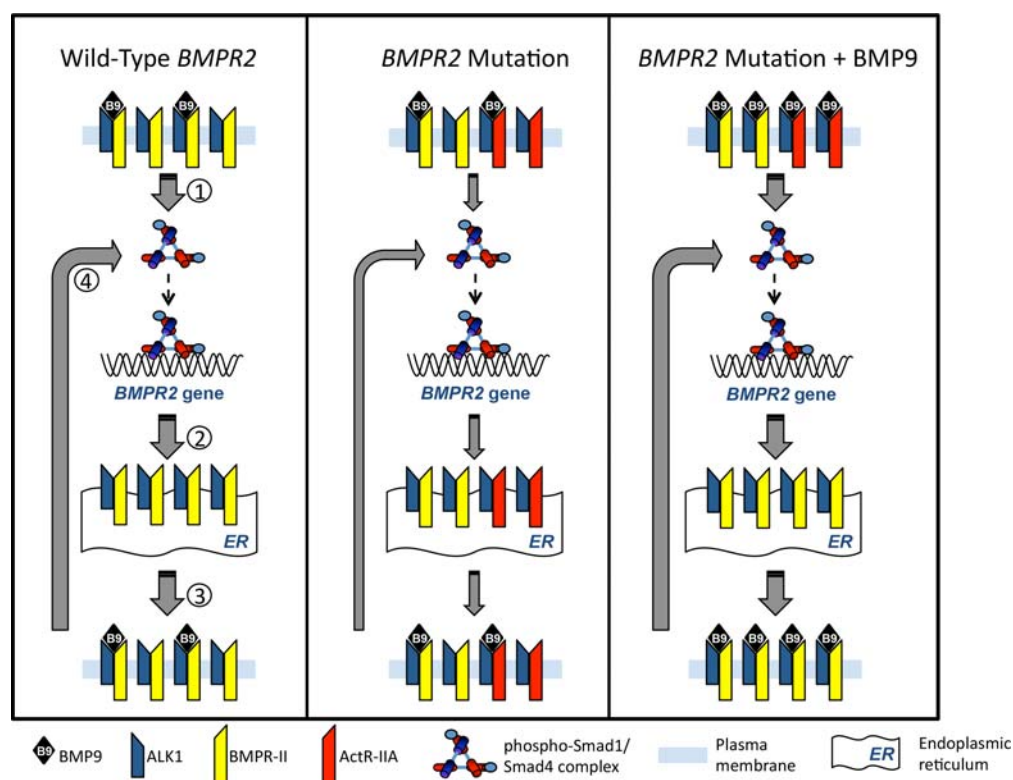


Figure 2. Proposed mechanism for the restoration of cell surface BMPR-II expression and signalling using exogenous BMP9 therapy. *Left panel:* Under normal conditions an individual possesses two wild-type alleles for BMPR2. Under these circumstances BMP9 signalling involves signalling via the ALK1:BMPR-II ligand receptor complex and activation of the Smad1/4 transcriptional complex.¹ This promotes BMPR-II mRNA transcription and synthesis² and trafficking of newly-synthesised BMPR-II to the cell surface where it complexes with ALK1, which has been recycled via the endosomal pathway.³ In the presence of BMP9, this feed forward pathway continues in an autoregulatory loop.⁴ *Middle panel:* in patients with a heterozygous mutation in BMPR2 leading to haploinsufficiency, cell surface BMPR-II is reduced and in its place, ActR-IIA can form a complex with available ALK1 but this does not promote autoregulatory BMPR-II production in response to endogenous concentrations of BMP9, which remain unchanged. The reduced signalling through BMPR-II leads to reduced BMPR-II levels of the receptor at the cell surface. *Right Panel:* Administration of exogenous BMP9 to PAH patients with a heterozygous mutation in BMPR2, increases the circulating concentration of BMP9 which increases signalling via the Smad1/4 complex to induce BMPR-II protein expression. This shifts the equilibrium of the BMPR-II:ActR-IIA ratio in favour of BMPR-II associating with the available ALK1 and thus restores the autoregulatory production of BMPR-II in response to BMP9, thus restoring normal endothelial BMP9 signalling.

form in the circulation and requires an additional activation step to achieve bioactivity, such as cleavage of the prodomain by BMP1.⁴⁷ This hypothesis is further supported by the fact that, although circulating BMP10 could be consistently detected and measured by ELISA^{48,49} and by pull-down coupled with proteomics,⁵⁰ early studies could not detect BMP10 activity in the circulation.^{48,51} However, one recent report was able to demonstrate BMP10 activity in mouse serum.⁴⁹

A further question to be addressed is whether the presence of the prodomain on therapeutically administered BMP10 is important for the stability and half-life of the ligand. A better understanding of the physiology of BMP10 is required *in vivo*, since BMP10 is a much less well-studied BMP ligand and most of the data are from embryos of mouse and zebrafish. The data on the role of BMP10 in adult physiology and in PAH are limited, and such information will inform the development and testing of BMP10-based drugs.

PHYSIOLOGICAL ROLES OF BMP9 AND BMP10

During embryonic development and into adult life, BMP9 and BMP10 are both expressed in a highly tissue-specific pattern. BMP9 is expressed in the mouse fetal liver from E9.75–10⁴⁹ and expression remains high in the adult liver.^{52,53} The highest levels of expression are observed in mRNA from intrahepatic biliary epithelial cells and hepatocytes.⁵³ In contrast, BMP10 is highly expressed in the

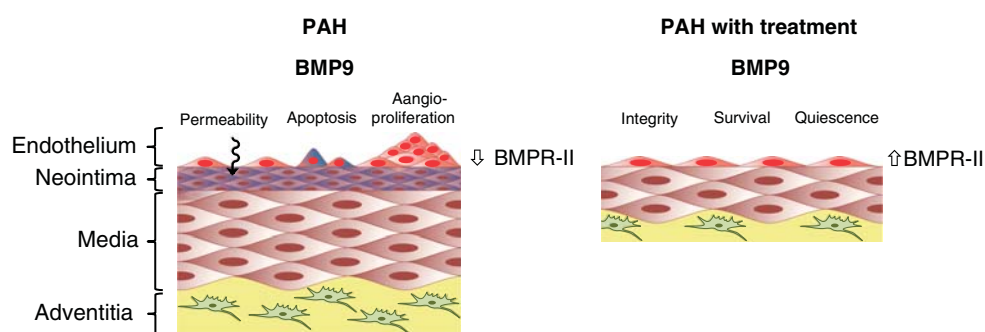


Figure 3. Impact of BMP9 therapy on pulmonary endothelial cell function. In the lungs of PAH patients, loss of BMPR-II leads to endothelial dysfunction, including increased vascular permeability, apoptosis and aberrant angioproliferation. Therapeutic delivery of recombinant BMP9 promotes endothelial quiescence, survival and vascular integrity, while simultaneously enhancing BMPR-II expression.

developing embryonic mouse heart, with much lower levels of expression detected in lung and liver.⁵⁴ The expression of BMP10 is high in the trabecular myocardium between days E9.5 – E13.5, a period that coincides with cardiac growth and chamber maturation.⁵⁴ The expression of BMP10 becomes restricted to the atria by day E18.5 and in adults, only the right atrium expresses BMP10.^{49,55}

BMP9 is secreted and circulates at levels of 2–12 ng/ml in human plasma⁵⁶ and 1.18–1.84 ng/ml in human serum,⁵¹ based on cell-based luciferase bioassays. Plasma BMP9 levels in mice increase just before birth and peak postnatally at levels of 6 ng/ml on Day 15 before declining to a steady state of approximately 1.5–2 ng/ml.⁵³ Similar to other BMPs, BMP9 is synthesised as a 429 amino acid precursor (pre-pro-BMP9) comprising a 22 amino acid signal peptide, a 297 amino acid prodomain and a 110 amino acid mature protein.^{53,57} The precursor is then cleaved by serine endoproteases to produce a mature protein dimer (25 kDa) which can remain non-covalently associated with two pro-domains (33 kDa each) forming a 100 kDa complex.⁵³ Unlike the pro-regions of TGFβs and GDF8, which whilst bound, inhibit their ligands *in vitro* and *in vivo*,^{58–61} the mature BMP9 dimer retains its biological activity when in complex with the pro-domain.^{62,63} The bound pro-domain is proposed to enhance the stability of BMP9 *in vivo*.⁶² Approximately 60% of the 100 kDa circulating pro-BMP9 complex is cleaved and active, whereas the remaining 40% is unprocessed and may be activated through a local furin cleavage.⁵³

The role of BMP10 as a secreted ligand is more controversial, since some studies suggest that BMP9 is solely responsible for the ALK1-activating BMP activity in plasma.^{48,56} However, a recent study has reported that BMP10 is present in mouse (0.5–2 ng/ml) and human (1–3 ng/ml) serum, suggesting that BMP10 may be present in the circulation at physiologically important levels.⁴⁹ Whether BMP10 in the circulation is processed and circulates in a similar manner to BMP9 is not yet clear. However, evidence supporting a functional role for circulating BMP10 has been revealed in zebrafish embryos, showing that BMP10 is necessary for maintaining vascular stability and endothelial Smad signalling in the vessels proximal to the heart.⁶⁴ Loss of BMP10 phenocopies the cranial vascular abnormalities of the Alk1 zebrafish mutants,⁶⁵ although as discussed later, this differs from the phenotype of the Bmp10 knockout mouse.

The overlapping expression of BMP10 and Alk1 at about E8.5^{49, 66} compared to the expression of BMP9 in the liver at E9.75–10 has been argued as evidence for the developmental regulation of BMP10 signalling through Alk1.⁴⁹ As BMP9 and BMP10 both activate ALK1, this may represent temporally important roles of the ligands. For example, Alk1^{-/-} mice die at E10.5 due to defects in angiogenesis,⁶⁷ which may represent a failure of both BMP9 and BMP10 signalling, as discussed below. In comparison, Alk1 + /- mice develop normally, but exhibit nosebleeds and vascular anomalies. This reflects the pathology of ALK1 mutations causing HHT in man, although the disease penetrance is lower in heterozygous mouse models.

Intriguingly, BMP9 knockout mice develop to adulthood without any overt phenotype,⁴⁸ although further analysis has revealed lymphatic vessel defects⁶⁸ and delayed closure of the ductus arteriosus.⁶⁹ The lack of a severe vascular phenotype in BMP9^{-/-} mice appears to be due to functional redundancy with BMP10, confirmed by defective retinal vascularisation in BMP9^{-/-} mice

Table 2. Summary of similarities and differences between BMP9 and BMP10 in physiology

	BMP9	BMP10
-/- mice phenotype	Normal, lymphatic vessel defect	Lethal, impaired cardiac development
Adult expression	Liver, into circulation	Right atrium
Circulating form and levels	2–10 ng/ml (by activity) ~300 pg/ml (ELISA) ¹⁰⁸	Presence shown by ELISA and proteomics; Only 1 in 3 reports can detect activity
Function	Vascular quiescence factor	Flow-dependent arterial quiescence
Bone-forming activity	Highest among 14 BMPs	Undetected
Endothelial cell signalling	Controlling a similar set of target genes with similar potency ⁴⁸	
Affinity for ALK1/BMPRII		Higher

when BMP10 is also neutralised.^{48,49} Of note, ALK1-Fc administration, which inhibits both BMP9 and BMP10, phenocopies this sprouting defect.⁴⁹ As circulating BMP10 levels are elevated slightly in BMP9-/- mice, one might speculate that BMP10 may compensate for the absence of BMP9 in the circulation.⁴⁸

Consistent with the restricted cardiac expression, BMP10-/- embryos die around E9.5–E10.5 due to hypoplastic cardiac development, probably as a result of reduced myocyte proliferation.⁵⁵ Although original reports reported an absence of vascular defects,⁵⁵ recent data indicate that the dorsal aorta and cardinal veins are fused in BMP10-/- mice.⁴⁹ Furthermore, cloning of the BMP9 coding region into the BMP10 knockout mouse does not rescue the cardiac defect whereas early vascular development appears normal, suggesting that BMP10 mediates cardiac development via a mechanism that is independent of the signalling capacity of BMP9.⁴⁹ This could be due to differences in the selectivity for type II receptors.⁴⁹ Intriguingly, ectopic expression of BMP10 driven by the alpha-myosin heavy chain promoter in the mouse myocardium leads to cardiac hypotrophy by 6 weeks of age.⁷⁰ The level of BMP10 expression achieved in the hearts was high, so it is not clear whether this represents a physiologically relevant effect of BMP10.⁷⁰ Table 2 summarizes the similarity and the difference between BMP9 and BMP10 in physiology.

POTENTIAL SIDE EFFECTS OF BMP9/10 THERAPY

One potential concern associated with the delivery of exogenous BMP ligands is the activation of other BMP receptors in non-endothelial cells, much of which is a dose-related phenomenon. It is therefore important to consider the roles of BMP9 in tissue ossification/calcification, hepatic function and tumour regulation.

Osteogenesis, chondrogenesis and adipogenesis: The potent orthotopic bone-forming activity of BMP9 may be a potential complication of BMP9 therapy in PAH. BMP9 induces the differentiation of mesenchymal stem cells (MSCs - adult stem cells found in bone marrow) into osteocytes, chondrocytes and adipocytes⁴⁶ and is one of the most potent osteogenesis-inducing BMP ligands in MSCs.^{46,71–74} The osteogenic induction of bone marrow MSCs is mediated via both ALK1 and ALK2⁷⁵ and is reported to involve BMP9 dependent induction of angiogenic HIF1a signaling.⁷⁶

The osteogenic response to BMP9 in skeletal muscle has been achieved through several modes of local ligand expression. Delivery of BMP9 into skeletal muscle via direct sonoporation,⁷⁷ injection of transfected MSCs⁷⁸ or C2C12 cells^{46,79} or injection of an adenovirus engineered to express BMP9⁴⁶ all elicited ectopic bone formation in muscle tissue. These processes are attributed to the differentiation of local multipotent MSCs or osteoblastic progenitor cells.^{80–82} Ad-BMP9 stimulates lamellar bone in mice by 3 months,⁸³ although studies have reported evidence of calcification after 9 days of exposure.⁸⁴ It is notable that the majority of these studies involve a local inflammatory stimulus and it appears that the heterotopic ossification response to BMP9 requires injury to skeletal muscle.⁸⁵ Another important consideration would be the concentration of BMP9 achieved during therapeutic administration. All of the above studies involved high concentrations of BMP9 or uncontrolled local overexpression. Our recent study employed intraperitoneal dosing of BMP9 injections, and we observed no evidence of heterotopic calcification after 4 weeks of daily intraperitoneal BMP9 injection, or indeed 3 weeks of intramuscular injection.⁴¹ The concentration of BMP9 is likely to be important to achieve therapeutic activation of BMPRII/ALK1, but avoidance of ALK2 activation at higher concentrations.

A recent study demonstrated that BMP9 promotes calcification of vascular smooth muscle cells in the context of high phosphate levels.⁸⁶ In this study, the authors propose ALK1 as the receptor mediating this calcification, although this conclusion was derived from the use of an ALK1-Fc ligand trap to inhibit BMP9 rather than molecular dissection of the receptor signalling in VSMCs. BMP9 stimulated calcification at concentrations of 5 ng/ml or greater, representing concentrations at which BMP9 can stimulate both ALK1 and ALK2. In contrast, no calcification was observed at 0.5 ng/ml BMP9, a concentration that would exclusively activate ALK1. Indeed, BMP9 and BMP10 might be expected to exert endothelial-mediated vascular protective effects in atherosclerosis, since endothelial-specific knockout of BMPRII led to enhanced inflammation and atherosclerosis in ApoE-knockout mice.⁸⁷ As BMP10 appears to lack osteogenic activity in muscle tissue, at least when transduced with an adenovirus,⁴⁶ BMP10 may represent an attractive therapeutic alternative to BMP9, providing that BMP10 proves an effective therapy in animal models of PAH.

Liver function: The ability of BMP9 to stimulate hepatocyte proliferation is long established, being one of the earliest observations of BMP9 function.⁸⁸ Early studies demonstrated that BMP9 binds to specific receptors in HepG2 cells,⁸⁹ consistent with the expression of the low affinity type I receptor, ALK2 and the type II receptors, BMPRII, ActRIIA and ActRIIB.⁹⁰ Furthermore, both the HLE (human, non-differentiated hepatoma) cell line and hepatic stellate cells express ALK1, indicating these cells may exhibit high sensitivity to BMP9, though this has yet to be established.^{91,92} Of note, BMP9 stimulates canonical Smad signalling and proliferation in HepG2, Hep3B and HuH7 cells.⁹³ The lowest BMP9 concentration examined in this report was 1 ng/ml and the responses were completely inhibited by low concentrations of the ALK2/3/6 inhibitor, LDN193189, implying these responses are not through ALK1.⁹³ The data from normal hepatocytes suggest that BMP9 may participate in liver repair but in conditions of liver carcinogenesis, BMP9 may promote tumour growth as discussed below.

In vivo studies have highlighted a positive role for hepatic BMP9 in energy metabolism. BMP9 inhibits hepatic glucose production and activates the expression of key enzymes of lipid metabolism.⁶³ Furthermore, recombinant BMP9 improved glucose homeostasis *in vivo* in diabetic and non-diabetic rodents.⁶³ However, these effects were observed at doses of BMP9 that were at least 1000 times higher than those employed in our PAH models.⁴¹ Conversely, BMP9 is reduced in the livers of insulin-resistant rats and administration of an anti-BMP9 antibody induced glucose intolerance and insulin resistance in fasted rats.⁹⁴ Consistent with this observation, administration of insulin in combination with high glucose induced BMP9 expression in the livers of 12h-fasted rats.⁹⁴ This promotion of hepatic insulin sensing is associated with reduced expression of a rate-limiting enzyme of gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK) in hepatocytes.⁶³

In addition to effects on the liver, BMP9 may regulate glucose utilization by skeletal muscle as it activates Akt2 kinase in differentiated myotubes.⁶³ Akt2 is essential for the activation of insulin-induced glucose uptake by muscle and Akt2 signalling is impaired in insulin resistance.⁹⁵⁻⁹⁷ In differentiated L6 myotubes, Akt2 is activated by Smad5 and this has been proposed as the mechanism for BMP9 action,⁹⁸ though a direct effect of BMP9 signalling through Smad5 was not confirmed. Overall, the positive effect of BMP9 on hepatic glucose sensing may be of benefit in patients given the perspective that metabolic dysfunction is a component of the pathophysiology of PAH.^{99,100}

Tumour regulation: Variable effects of BMP9 on tumours and tumour cell growth have been reported. For example BMP9 promotes proliferation of tumour-derived cell lines from ovary¹⁰¹ and liver⁹³ but induces apoptosis in prostate cancer cells¹⁰² and restricts osteosarcoma cell proliferation and migration.^{103,104} Furthermore, neutralisation of BMP9 with Endoglin-Fc restricts colonic tumours in mice.¹⁰⁵ The key receptor promoting the proliferative response is ALK2 in ovarian and liver cancer cell lines, shown through siRNA transfection and inhibition by low concentrations of the ALK2/3/6 inhibitor, LDN193189.^{93,101} The tumour promoting and inhibitory responses are unlikely to be due to ALK1 activation, as prostate cancer cells do not express ALK1 yet BMP9 promotes apoptosis.¹⁰² In some instances, the proliferation of tumour cell lines is promoted by autocrine BMP9 production.¹⁰¹ Thus the impact of BMP9 on tumour cells appears to predominantly via ALK2 and at high local concentrations of BMP9.

In addition to effects on tumour cells, it is proposed that BMP9, and possibly BMP10, promote tumour angiogenesis.^{106,107} The proangiogenic response to BMP9 appears to be context dependent, as BMP9 is reported to promote proliferation and angiogenic processes in embryonic mouse endothelial cells and transformed endothelial cell lines,^{106,107} but inhibits angiogenesis in primary adult endothelial cell lines.^{35,36,41} The possible role of BMP9 in tumour angiogenesis has led to the

development of Dalantercept, a soluble ALK1 ligand trap, as an anti-tumour angiogenesis therapy. When applied clinically, the potential capacity of BMP9/10 to sustain tumour angiogenesis would need to be balanced against the possible transformational effect of BMP9 in treating patients with life-limiting PAH.

In summary, despite the potential challenges of enhancing signalling downstream of loss-of-function mutations in BMPR-II there are now several approaches that could be taken forwards into the clinic as novel therapeutic approaches in PAH. Of these, the direct enhancement of endothelial BMPR-II/ALK1 signalling with BMP9/10 offers an immediate solution that could be rapidly tested with the appropriate cautions in patients with this life-limiting disease.

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