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## Selective BMP-9 Inhibition Partially Protects Against Experimental Pulmonary Hypertension

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**Running title:** Loss of BMP-9 and pulmonary hypertension

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## ABSTRACT

**Rationale:** Although many familial cases of pulmonary arterial hypertension (PAH) exhibit an autosomal dominant mode of inheritance with the majority having mutations in essential constituents of the bone morphogenetic protein (BMP) signaling, the specific contribution of the long-term loss of signal transduction triggered by the type 2 BMP receptor (BMPR2) remains poorly characterized.

**Objective:** To investigate the role of BMP9, the main ligand of ALK1/BMPR2 heterocomplexes, in pulmonary hypertension (PH).

**Method and Results:** The absence of BMP9 in *Bmp9*<sup>-/-</sup> mice and its inhibition in C57BL/6 mice using neutralizing anti-BMP9 antibodies substantially prevent against chronic hypoxia induced PH judged by right ventricular systolic pressure (RVSP) measurement, right ventricular hypertrophy, and pulmonary distal arterial muscularization. In agreement with these observations, we found that the BMP9/BMP10 ligand trap ALK1<sub>ECD</sub> administered in monocrotaline (MCT) or Sugeng/hypoxia (SuHx) rats substantially attenuate proliferation of pulmonary vascular cells, inflammatory cell infiltration and regresses established PH in rats. Our data obtained in human pulmonary endothelial cells derived from controls and PAH patients indicate that BMP9 can affect the balance between endothelin-1, apelin and adrenomedullin. We reproduced these *in vitro* observations in mice chronically exposed to hypoxia, with *Bmp9*<sup>-/-</sup> mice exhibiting lower mRNA levels of the vasoconstrictor peptide endothelin (ET)-1 and higher levels of the two potent vasodilator factors apelin and adrenomedullin (ADM) compared with *Bmp9*<sup>+/+</sup> littermates.

**Conclusion:** Taken together, our data indicate that the loss of BMP9, by deletion or inhibition, has beneficial effects against PH onset and progression.

**Keywords:** Pulmonary hypertension • Pulmonary vascular remodeling • Endothelial cell • BMPR-2 • Growth and differentiation factor 2 (GDF2)-BMP9

### Nonstandard Abbreviations and Acronyms:

ADM	Adrenomedullin
ALK1	Activin receptor-like kinase 1
AU	Arbitrary unit
BMPR2	Bone morphogenetic protein receptor type 2
CO	Cardiac output
CTR	Control
EC	Endothelial cell
ET	Ejection time
ET-1	Endothelin-1
HHT	Hereditary hemorrhagic telangiectasia
iPAH	Idiopathic pulmonary arterial hypertension
IL	Interleukin
i.p.	Intraperitoneal
LV	Left ventricle
mPAP	Mean pulmonary arterial pressure
MCT	Monocrotaline
PAs	Pulmonary arteries
PAH	Pulmonary arterial hypertension
PA-SMC	Pulmonary artery-smooth muscle cell
RV	Right ventricle
RVH	Right ventricular hypertrophy
RVSP	Right ventricular systolic pressure
S	Septum
SuHx	Sugen/hypoxia
TPVR	Total pulmonary vascular resistance
WT	Wild type

## INTRODUCTION

Pulmonary endothelial dysfunction associated to pulmonary arterial hypertension (PAH) is a key pathogenetic mechanism that could be detrimental for disease susceptibility and development of pulmonary vascular remodeling<sup>1-3</sup>. Despite recent progress in the treatment of PAH<sup>4</sup>, most patients still die from the disease or fail to respond adequately to medical therapy with a 5-years survival of 59%<sup>5</sup>. Current treatments can relieve some PAH symptoms and slow the progress of the disease in some patients, but they have limited impact on the progressive pulmonary vascular remodeling that eventually culminates in right heart failure. Since 2000, heterozygous germline mutations in the *Bone Morphogenetic Protein Receptor type 2 (BMPR2)* gene have been identified as critical genetic factors predisposing to pulmonary vascular remodeling and PAH development with low penetrance<sup>6-8</sup>. Still, the specific contribution of the long-term loss of signal transduction triggered by this signaling pathway remains poorly characterized.

Among bone morphogenetic proteins (BMPs), BMP9 and BMP10 are two high affinity ligands for ALK1 and BMPR2 present in a heterotetrameric complex on pulmonary endothelial cells (ECs)<sup>9</sup>, and thereby are key actors of vascular development and homeostasis<sup>10</sup>. Perturbation in the BMP9/BMP10 signaling pathway has emerged as essential in endothelial (dys)function and vascular remodeling, in particular in PAH and hereditary hemorrhagic telangiectasia (HHT)<sup>11,12</sup>. In line with this notion, several works have highlighted a potential role for this pathway in the regulation of vascular tone<sup>13</sup> and in the modulation of ET-1<sup>14,15</sup> and apelin, two potent vasoreactive mediators for the pulmonary vasculature<sup>16,17</sup>. Even if *Bmp9*-KO mice are viable and present no obvious defects, these mice exhibit an altered lymphatic maturation that leads to drainage deficiency<sup>18</sup>. Although new biomedical therapeutics targeting BMPs or the BMP signaling pathway hold promise in future treatment strategies for PAH and other life-threatening diseases, our current understanding of the molecular signaling pathways activated by BMPs and their exact pathogenic roles remains limited<sup>11</sup>.

Therefore, we hypothesized that long-term loss of endothelial BMP9 signaling in rodents could alter pulmonary vascular tone and remodeling, thereby reducing their susceptibility to pulmonary hypertension.

## METHODS

The authors declare that all supporting data are available within the article and its online supplementary files. Because of space limitations, a detailed description of the Materials and Methods is presented in the Online Supplement.

## RESULTS

### *Mice deficient in BMP9 (*Bmp9*<sup>-/-</sup>) are less susceptible to chronic hypoxia-induced PH*

To gain information about the role of BMP9 in the susceptibility of mice to develop PH, we used mice congenitally deficient in the expression of the BMP9 gene (*Gdf2*). The loss of active circulating BMP9 levels in *Bmp9*<sup>-/-</sup> mice was confirmed using a BMP responsive (BRE) reporter assay (**Figure 1A**). Consistent with these findings, a 2-fold decrease in p-Smad1/5/8 and p-P38 were found in lungs of *Bmp9*<sup>-/-</sup> versus *Bmp9*<sup>+/+</sup> littermates and no compensatory upregulation of other BMP family members was present in these mice deficient in BMP9 (**Online Figure I**). *Bmp9*<sup>-/-</sup> mice in the C57BL/6 background are viable and present no obvious defects except a defect in lymphatic maturation<sup>17, 18</sup>. Our present findings indicate that *Bmp9*<sup>-/-</sup> mice are less susceptible to pulmonary vascular remodeling than *Bmp9*<sup>+/+</sup> littermates when exposed to chronic hypoxia (CHx). Indeed, the elevations in right ventricular systolic pressure (RVSP) (**Figure 1B**) and right ventricular hypertrophy (RV/LV+S weight ratio) (**Figure 1C**) following chronic hypoxia were lower in the *Bmp9*<sup>-/-</sup> than the *Bmp9*<sup>+/+</sup> mice. No significant differences were observed in heart rate or systemic pressures between *Bmp9*<sup>-/-</sup> and *Bmp9*<sup>+/+</sup> mice (heart rate: 320 ± 34 versus 324 ± 44 bpm, respectively, NS; systemic blood pressure: 110 ± 20 versus 105 ± 18, n = 10, respectively, NS). Similar findings have also been observed with intravenous (*i.v.*) administration of the active monocrotaline metabolite (monocrotaline pyrrole, MCTp) to *Bmp9*<sup>-/-</sup> and *Bmp9*<sup>+/+</sup> mice (**Online Figure II**). Following chronic hypoxia, the less pronounced RVSP elevation in the *Bmp9*<sup>-/-</sup> was also associated with a decreased number and thickness of muscularized distal pulmonary arteries versus *Bmp9*<sup>+/+</sup> mice (**Figure 1D**). Consistent with these observations, we did not observe a significant increase in the number of proliferating cell nuclear antigen positive (PCNA<sup>+</sup>) cells or an accumulation of F4/80<sup>+</sup> cells (monocytes/macrophages) in the perivascular area of muscularized vessels in *Bmp9*<sup>-/-</sup> mice under normoxic versus hypoxic conditions in contrast to *Bmp9*<sup>+/+</sup> mice (**Figure 1E**).

#### *Administration of neutralizing anti-BMP9 antibodies in mice prevents chronic hypoxia-induced PH*

To further demonstrate the role of BMP9 in the susceptibility of mice to develop PH, we study the effect of suppressing BMP9 action in adult C57BL/6 mice (WT) with neutralizing anti-BMP9 antibodies (kindly obtained from Dr. M Yan). Weekly administration of neutralizing anti-BMP9 antibodies (5mg/kg, i.p.) after hypoxia exposure completely inhibited BMP circulating ALK1-dependent activity (**Figure 2A**). Consistent with our hypothesis and our findings obtained in *Bmp9* deficient mice, we found that C57BL/6 mice chronically exposed to hypoxia and treated with neutralizing anti-BMP9 antibodies were less susceptible to the development of chronic hypoxia-induced PH than C57BL/6 mice treated with immunoglobulin G (IgG). The severity of the disease was assessed by RVSP measurement (**Figure 2B**), by right ventricular hypertrophy (**Figure 2C**), and by pulmonary distal arterial muscularization (**Figure 2D**). We also noted that under hypoxic conditions, cell proliferation and the degree of inflammatory cell infiltration were significantly decreased in lungs from C57BL/6 mice chronically exposed to hypoxia and treated with neutralizing anti-BMP9 antibodies when compared with lungs of C57BL/6 mice chronically exposed to hypoxia and treated with IgG (**Figure 2E**).

#### *Efficacy of treatment with the soluble extracellular ALK1 domain (ALK1<sub>ECD</sub>) on the progression of established PH in two complementary and well-established models of severe PH in rats.*

Next, these results prompted us to test the efficacy of the soluble extracellular ALK1 domain (ALK1<sub>ECD</sub>), a ligand trap targeting ALK1's ligands thus both BMP9 and BMP10<sup>19</sup>, on the development of monocrotaline (MCT)-induced PH in Wistar rats. Treatment with ALK1<sub>ECD</sub> (4mg/kg, i.p.) was started 1 week after a subcutaneous MCT injection and was repeated one week later (3mg/kg, i.p.) (**Figure 3A**). We first validated that the weekly ALK1<sub>ECD</sub> administration completely inhibited BMP circulating ALK1-dependent activity (**Figure 3B**). On day 21, in MCT-injected rats treated with human IgG, a marked increase in mean pulmonary arterial pressure (mPAP), total pulmonary vascular resistance (TPVR), RV/(LV+ S) ratio (**Figure 3C**), percentage medial wall thickness, and numbers of muscularized distal pulmonary arteries (**Figure 3D**) were found compared with controls. However, MCT-injected rats receiving ALK1<sub>ECD</sub> exhibited reduced mPAP, TPVR and RV/(LV+ S) ratio as compared to MCT-injected rats receiving IgG (**Figure 3C**).

Consistent with these results, the percentages of medial wall thickness and of muscularized distal pulmonary arteries were substantially decreased in MCT-injected rats receiving ALK1<sub>ECD</sub> when compared to control rats (**Figure 3D**). In addition, we found a substantial decrease in collagen deposition in the RV myocardium of MCT-injected rats receiving ALK1<sub>ECD</sub> when compared to control rats, an observation that is consistent with the more preserved cardiac function observed in these ALK1<sub>ECD</sub>-treated rats (**Online Figure IIIA-B**). Interestingly, no significant differences were found in the number of PCNA<sup>+</sup> and CD68<sup>+</sup> (monocyte/macrophages) cells in pulmonary vessel walls in lungs of MCT-injected rats receiving ALK1<sub>ECD</sub> as compared to lungs of control rats receiving IgG (**Figure 3E**).

To validate our findings obtained in the MCT rat model, weekly treatments of Sugren-hypoxia (SuHx) rats with ALK1<sub>ECD</sub> or IgG were next performed (**Figure 4**). Treatment with ALK1<sub>ECD</sub> (4mg/kg, i.p.) was started 5 weeks post-SU5416 injection and was repeated one and two weeks later (3mg/kg, i.p.) (**Figure 4A**). We first validated that the weekly ALK1<sub>ECD</sub> administration completely inhibited BMP circulating ALK1-dependent activity (**Figure 4B**). Eight weeks post-SU5416 injection, SuHx rats develop severe experimental PH, as reflected by a marked increase in values of mPAP TPVR, and Fulton index (**Figure 4C**). Although only a trend towards lower mPAP values was found, SuHx rats treated with ALK1<sub>ECD</sub> exhibit less severe PH when compared to SuHx rat-treated with IgG, as reflected by significant lower values of TPVR and Fulton index (**Figure 4C**). Consistent with these findings and those obtained in the MCT rat model, we also noted a beneficial effect of ALK1<sub>ECD</sub> treatments on the pulmonary arterial muscularization (**Figure 4D**), numbers of PCNA<sup>+</sup> and CD68<sup>+</sup> cells per vessel (**Figure 4E**), as well as on RV function and collagen deposition in the RV myocardium (**Online Figure IIIC-D**) when compared with SuHx rat-treated with IgG.

#### *BMP9 regulates in vitro and in vivo the balance of key vascular tone regulators*

BMP9 has been described as a potential vasoconstriction factor in the chick chorioallantoic membrane (CAM) assay<sup>13</sup> and as a BMP ligand that can down-regulate the expression of the vasodilator factor apelin<sup>16</sup>,<sup>17</sup> and increase the vasoconstrictor peptide endothelin (ET)-1<sup>14, 15</sup>. An *in silico* reanalysis of the microarray data (accession number E-MTAB-2495) obtained from Long and coworkers<sup>20</sup>, in which human pulmonary ECs were stimulated with 1ng/mL of recombinant BMP9 for 4 hours, revealed two key pathways that are susceptible to regulate vascular contraction, namely the “cGMP-PKG signaling pathway” and the “Vascular

*smooth muscle contraction pathway*". Based on these sets of genes and their known effectors, we set-up a list of genes that allowed us to identify 23 genes that were significantly regulated by BMP9 (**Online Figure IV**), including apelin and adrenomedullin (ADM), two potent vasodilator factors. We thus investigated whether recombinant human (rh)BMP9 could alter the balance between pulmonary endothelium-derived relaxing factors and contracting factors *in vitro* using freshly isolated pulmonary ECs established from lung tissue obtained from control and PAH patients (both idiopathic and *BMPR2* mutation carriers). Interestingly, we found that rhBMP9 induced a dose-dependent production of ET-1, and a dose-dependent decreased production of apelin and ADM in control pulmonary ECs (**Figure 5A**). A similar profile was observed in pulmonary ECs derived from PAH patients except for ADM level that was already significantly reduced at the basal level in comparison to control cells (**Figure 5A**). Based on these *in vitro* findings, we then assessed the levels of the synthesis of potent vasoreactive factors in lungs of *Bmp9* <sup>-/-</sup> and *Bmp9* <sup>+/+</sup> mice under normoxic and hypoxic conditions. Consistent with our findings showing that *Bmp9* <sup>-/-</sup> mice are less susceptible to chronic hypoxia-induced PH, we found a decreased ET-1 mRNA level and an increased apelin and ADM mRNA levels in *Bmp9* <sup>-/-</sup> mice chronically exposed to hypoxia compared with *Bmp9* <sup>+/+</sup> mice chronically exposed to hypoxia (**Figure 5B**). Since these mediators can regulate the function and the thickness of the underlying smooth muscle cells and because myosin light chain (MLC) is a major regulatory molecule for smooth muscle contraction, we next studied its expression in lungs of *Bmp9* <sup>-/-</sup> mice under normoxic and hypoxic conditions. Interestingly, we found that MLC protein expression is decreased in lungs of *Bmp9* <sup>-/-</sup> mice chronically exposed to hypoxia compared with *Bmp9* <sup>+/+</sup> mice chronically exposed to hypoxia (**Figure 5C-D**), a phenomenon that was associated with decreased activity of the Rho/Rho-associated protein kinase (ROCK) signaling pathway (**Online Figure V**). Consistent with these findings obtained in lungs of *Bmp9* <sup>-/-</sup> mice, a substantial decrease in MLC protein expression was found in lungs of MCT- and SuHx rats receiving ALK1<sub>ECD</sub> when compared to lungs of MCT- and SuHx rats receiving IgG (**Figure 5E-F**).

## DISCUSSION

Our study identifies BMP9, also known as growth and differentiation factor 2 (GDF2), as a major factor for controlling functions of pulmonary vascular cells (**Figure 6**). Using three different approaches of suppressing BMP9 action in rodents (*Bmp9* <sup>-/-</sup> mice, administration of neutralizing anti-BMP9 antibodies or of ALK1<sub>ECD</sub> ligand trap for BMP9 and BMP10 in rats) in different complementary and well-established animal models of PH, we demonstrate that the selective loss of BMP9 partially protects against experimental pulmonary hypertension.

These results seem at first glance counter-intuitive, as it would have been expected that less ligands for the BMPR2 receptor should mimic the loss of BMPR2 signaling found in PAH patients. In accordance with this hypothesis, it was recently proposed to enhance this pathway by exogenous BMP9 in PAH <sup>20</sup>. Even if there are some arguments that support this notion <sup>21-24</sup>, our functional data indicate the opposite and further illustrate that BMP signaling is complex and involves many ligands and many receptor combinations that could explain these unexpected results, especially in the context of PAH. Indeed, it has been found that *BMPR2* mutations could alter BMP signaling in a BMP ligand- or receptor-specific manner <sup>25-27</sup>. While writing this article, it was published that BMP9 could be a sensitive biomarker that segregates portopulmonary hypertension from other forms of PAH <sup>28</sup>. Together, these results clearly support that BMP9 signaling pathway is involved in PAH development but also that the molecular mechanisms involved in PAH are complex.

BMP9 is mainly produced by the liver and circulates in blood under a biologically active form and has been proposed to act as a vascular quiescence factor <sup>13</sup>. Several works also support a potential role for this pathway in the regulation of vascular tone. Indeed, it was shown by our group and others that BMP9 induces the expression of ET-1, a strong vasoconstrictor <sup>14, 15</sup>, and decreases the expression of apelin, a potent vasodilator for the pulmonary vasculature <sup>16, 17</sup>. We also previously showed that addition of BMP9 induced vasoconstriction in the chicken chorionallantoic membrane <sup>13</sup>. So, one hypothesis that could explain why the loss of BMP9 protects from PH development is that BMP9 could act as a potent modulator of vasoconstriction in the pulmonary circulation. This hypothesis is consistent with the dilation phenotype observed in *Alk1* +/- or

*Eng* +/- mice <sup>29, 30</sup> or *Alkl* mutants, *vbg*, in zebrafish <sup>31</sup>. This hypothesis is further supported by the fact that we found that *Bmp9* -/- pups presented a partial reopening of the *ductus arteriosus* just after birth that could due to a defect in vasoconstriction <sup>32</sup>. Interestingly, patent *ductus arteriosus* (PDA) is a serious congenital heart disease that can lead to irreversible pulmonary hypertension <sup>33</sup>. We confirm here the down-regulation of apelin and show, for the first time, the down-regulation of adrenomedullin by BMP9, two known critical actors that can impair pulmonary endothelial function <sup>34, 35</sup>.

We also confirm here that BMP9 induces a dose dependent ET-1 expression in human pulmonary endothelial cells and regulate its lung production in rodents. However, although BMP9 and other BMPs have been shown to induce ET-1 <sup>14, 15</sup>, unexpectedly BMPR2 down-regulation is also associated with an increase in ET-1 expression <sup>15, 36</sup>, supporting the notion that BMP9 stimulated ET-1 production by pulmonary endothelial cells is not sufficient alone to lead to PH development and strengthening the evidence for the importance of BMP receptor balance in the control of vascular tone.

The role of BMP9 has recently been studied in a bronchopulmonary dysplasia model induced by chronic exposure to hyperoxia in neonatal rats. Consistent with our present findings, BMP9 treatment has been found to improve aberrant alveolar development and reduced lung inflammation and fibrosis but did not improve aberrant vascularization (arterial medial wall thickness and muscularization) and right ventricular hypertrophy <sup>37</sup>. In line with this notion, BMP9 and BMP10 can synergize with TNF $\alpha$  to induce the up-regulation of endothelial selectins and adhesion molecules and the secretion of various key pro-inflammatory mediators <sup>38, 39</sup>, and are therefore likely to contribute to the proinflammatory signature of the dysfunctional endothelium in PAH <sup>40</sup>.

In the present study, we provide definitive evidence that the selective BMP9 loss or inhibition partially prevent and protects against experimental pulmonary hypertension. In addition, this study offers important physiopathological insights into the role of BMP9 in the synthesis of potent vasoreactive factors by the pulmonary endothelium *in vitro* and *in vivo*, namely ET-1, apelin and adrenomedullin and may have important implications for human PAH.



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## DISCLOSURES

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## Figure Legends:

**Figure 1: Susceptibility of mice deficient in BMP9 (*Bmp9*<sup>-/-</sup>) to the development of chronic hypoxia-induced PH.** (A) Analyses of the luciferase reporter activity under the control of the BRE promoter in the sera of *Bmp9*<sup>+/+</sup> and *Bmp9* knockout mice (*Bmp9*<sup>-/-</sup>) (n = 5–6). The relative firefly luciferase activity was normalized to renilla luciferase activity. (B) Right ventricular systolic pressures (RVSP) and (C) right ventricular hypertrophy expressed by the Fulton index in *Bmp9*<sup>-/-</sup> and *+/+* mice (n = 7–13). (D) Representative images of Hematoxylin and eosin (H&E), alpha-smooth muscle actin ( $\alpha$ -SMA) staining and quantification of the percentage of muscularized distal pulmonary arteries (intra-alveolar vessels < 200  $\mu$ m) and of wall thickness in lungs from *Bmp9*<sup>-/-</sup> and *+/+* mice (n = 3–6). (E) Representative images of PCNA and F4/80 immunostaining, and quantification of the percentage of PCNA and F4/80 positive cells (arrow heads) per vessels in lungs from *Bmp9*<sup>-/-</sup> and *+/+* mice. Scale bars: 50  $\mu$ m. Data are presented as mean  $\pm$  SEM (n = 4–5). Comparisons were made using 1-way ANOVA with Tukey's post hoc tests. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001 versus *Bmp9*<sup>+/+</sup> mice under normoxia; #P < 0.05, ##P < 0.01, versus *Bmp9*<sup>+/+</sup> mice under chronic hypoxia. LV, left ventricle; PCNA, proliferating cell nuclear antigen RV, right ventricle; S, septum.

**Figure 2: Efficacy of chronic treatment with the neutralizing anti-BMP9 antibodies in the mouse model of hypoxia-induced PH.** (A) Analyses of the luciferase reporter activity under the control of the BRE promoter in the sera of C57BL/6 mice treated with either the neutralizing anti-BMP9 or IgG antibodies (n = 6–7). The relative firefly luciferase activity was normalized to renilla luciferase activity. (B) Right ventricular systolic pressures (RVSP) and (C) right ventricular hypertrophy expressed by the Fulton index in C57BL/6 mice treated with either the neutralizing anti-BMP9 or IgG antibodies (n = 5–7). (D) Representative images of Hematoxylin and eosin (H&E), alpha-smooth muscle actin ( $\alpha$ -SMA) staining and quantification of the percentage of muscularized distal pulmonary arteries (intra-alveolar vessels < 200  $\mu$ m) and of wall thickness in lungs from C57BL/6 mice treated with either the neutralizing anti-BMP9 or IgG antibodies (n = 4–5). (E) Representative images of PCNA and F4/80 immunostaining, and quantification of the percentage of PCNA and F4/80 positive cells (arrow heads) per vessels in lungs from C57BL/6 mice treated with either the neutralizing anti-BMP9 or IgG antibodies. Scale bars: 50  $\mu$ m. Data are presented as mean  $\pm$  SEM (3–5). Comparisons were made using 1-way ANOVA with Tukey's post hoc tests. \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001 versus C57BL/6 mice treated with IgG antibodies under normoxia; #P < 0.05, ##P < 0.01, ###P < 0.001, versus C57BL/6 mice treated with IgG antibodies under chronic hypoxia. LV, left ventricle; PCNA, proliferating cell nuclear antigen RV, right ventricle; S, septum.

**Figure 3: Efficacy of chronic treatment with the BMP9/BMP10 ligand trap ALK1<sub>ECD</sub> in the rat model of monocrotaline-induced PH.** (A) Experimental design. (B) Analyses of the luciferase reporter activity under the control of the BRE promoter in the sera of Control + IgG and MCT-injected rats receiving either ALK1<sub>ECD</sub> or IgG (n = 4–6). The relative firefly luciferase activity was normalized to renilla luciferase activity. (C) Mean pulmonary arterial pressures (mPAP), total pulmonary vascular resistance (TPVR), and right ventricular hypertrophy expressed by the Fulton index in Control + IgG and MCT-injected rats receiving either ALK1<sub>ECD</sub> or IgG (n = 8–11). (D) Representative images of Hematoxylin and eosin (H&E), alpha-smooth muscle actin ( $\alpha$ -SMA) staining and quantification of the percentage of muscularized distal pulmonary arteries (intra-alveolar vessels < 200  $\mu$ m) and of wall thickness in lungs from Control + IgG and MCT-injected rats receiving either ALK1<sub>ECD</sub> or IgG (n = 5–7). (E) Representative images of PCNA and CD68 immunostainings, and quantification of the percentage of PCNA and CD68 positive cells (arrow heads) per vessel in lungs from Control + IgG and MCT-injected rats receiving either ALK1<sub>ECD</sub> or IgG. Scale bars: 50  $\mu$ m. Data are presented as mean  $\pm$  SEM (n = 6). Comparisons were made using 1-way ANOVA with Tukey's post hoc tests. \*P < 0.05, \*\*P < 0.01, \*\*\*P

< 0.001, \*\*\*\*P < 0.0001 *versus* Control rats treated with IgG antibodies; #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 *versus* MCT rats treated with IgG antibodies. ALK1, activin receptor-like kinase 1; ECD, extracellular domain; LV, left ventricle; MCT, monocrotaline; PCNA, proliferating cell nuclear antigen RV, right ventricle; S, septum.

**Figure 4: Efficacy of chronic treatment with the BMP9/BMP10 ligand trap ALK1<sub>ECD</sub> in the Sugén-hypoxia (SuHx) rat model of severe PH.** (A) Experimental design. (B) Analyses of the luciferase reporter activity under the control of the BRE promoter in the sera of Control + IgG and SuHx rats receiving either ALK1<sub>ECD</sub> or IgG (n = 4-7). The relative firefly luciferase activity was normalized to renilla luciferase activity. (C) Mean pulmonary arterial pressures (mPAP), total pulmonary vascular resistance (TPVR), and right ventricular hypertrophy expressed by the Fulton index in Control + IgG and SuHx rats receiving either ALK1<sub>ECD</sub> or IgG (n = 4-7). (D) Representative images of Hematoxylin and eosin (H&E), alpha-smooth muscle actin (α-SMA) staining and quantification of the percentage of muscularized distal pulmonary arteries (intra-alveolar vessels < 200 μm) and of wall thickness in lungs from Control + IgG and SuHx rats receiving either ALK1<sub>ECD</sub> or IgG (n = 4-6). (E) Representative images of PCNA and CD68 immunostainings, and quantification of the percentage of PCNA and CD68 positive cells (arrow heads) per vessel in lungs from Control + IgG and SuHx rats receiving either ALK1<sub>ECD</sub> or IgG (n = 3-7). Scale bars: 50 μm. Data are presented as mean ± SEM. Comparisons were made using 1-way ANOVA with Tukey's post hoc tests. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001 *versus* Control rats treated with IgG antibodies; #P < 0.05, ##P < 0.01, ###P < 0.001, ####P < 0.0001 *versus* MCT rats treated with IgG antibodies. ALK1, activin receptor-like kinase 1; ECD, extracellular domain; LV, left ventricle; MCT, monocrotaline; PCNA, proliferating cell nuclear antigen RV, right ventricle; S, septum.

**Figure 5: Effects of BMP9 on the expression of ET-1, apelin, adrenomedullin.** Gene expression analysis of ET-1, apelin and adrenomedullin (A) in freshly isolated pulmonary endothelial cells derived from lung tissue obtained from control (n=5) and PAH patients (n=7) exposed to increasing doses of recombinant human (rh)BMP9 and (B) in lungs of *Bmp9* <sup>-/-</sup> and *Bmp9* <sup>+/+</sup> mice under normoxic and hypoxic conditions (n=7-11). (C) Representative Western blots and quantification of the myosin light chain (MLC)/GAPDH ratio in lungs of *Bmp9* <sup>-/-</sup> and *Bmp9* <sup>+/+</sup> mice under normoxic and hypoxic conditions (n = 5-11). (D, E, F) Confocal immunofluorescence analyses of MLC, SM22 or alpha-smooth muscle actin (α-SMA), and DAPI in lungs from (D) *Bmp9* <sup>-/-</sup> and *Bmp9* <sup>+/+</sup> mice under normoxic and hypoxic conditions (n=5) and from (E) MCT-injected rats receiving either ALK1<sub>ECD</sub> or IgG (n = 5) and from (F) SuHx rats receiving either ALK1<sub>ECD</sub> or IgG (n = 4). Scale bars: 50 μm. Data are presented as mean ± SEM. Comparisons were made using 1-way ANOVA with Tukey's post hoc tests. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001 *versus* cells exposed to 0 ng/mL of rhBMP9 or *Bmp9* <sup>+/+</sup> mice under normoxia; #P < 0.05, ##P < 0.01, ####P < 0.0001 *versus* *Bmp9* <sup>+/+</sup> mice under chronic hypoxia. ALK1, activin receptor-like kinase 1; DAPI, 4',6-diamidino-2-phenylindole, dihydrochloride; ECD, extracellular domain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; L, lumen; MCT, monocrotaline.

**Figure 6: Schematic diagram summarizing our findings.** ADM, adrenomedullin; ALK1, activin receptor-like kinase 1; BMPR2, Bone morphogenetic protein receptor type 2; ECD, extracellular domain; ET-1, endothelin-1; MLC, myosin light-chain.

## Novelty and Significance

### What Is Known?

- Autosomal dominant mutations in the *BMPR2*, *ACVLR1 (ALK1)*, *GDF2 (BMP9)*, and in the *BMP10* genes predispose to heritable pulmonary arterial hypertension (PAH).
- BMP9 and BMP10 are two high affinity ligands for ALK1 and BMPRII present in a heterotetrameric complex on pulmonary endothelial cells.
- Perturbation in the BMP9/BMP10 signaling pathway have emerged as essential in endothelial (dys)function and vascular remodeling.

### What New Information Does This Article Contribute?

- Using three different approaches of suppressing BMP9 action in rodents, we provide definitive evidence that the selective BMP9 loss or inhibition does not predispose, but partially prevent or protect against experimental pulmonary hypertension.
- We show that BMP9 regulates the endothelial synthesis/release of potent vasoreactive factors *in vitro* and *in vivo*, namely endothelin-1, apelin and adrenomedullin.
- Further studies are needed to better understand the differences between experimental models and the human predisposition to PAH identified in subjects carrying *BMP9* germline mutations.

These findings offer new insight into the complexity of endothelial BMP9 signaling in PAH and may have important implications for human PAH. Our data support the notion that blockade of BMP9 signaling can protect the pulmonary endothelium and attenuate the structural and functional remodeling of the lung vasculature. The apparent ambivalent role of the BMP9/BMP10 signaling pathway should stimulate work to better understand this feature and the underlying mechanisms.















