SMAD1 Deficiency in Either Endothelial or Smooth Muscle Cells Can Predispose Mice to Pulmonary Hypertension

Chul Han,* Kwon-Ho Hong,* Yong Hwan Kim, Mi-Jung Kim, Cheol Song, Myung Joon Kim, Seong-Jin Kim, Mohan K. Raizada, S. Paul Oh

Abstract—A deficiency in bone morphogenetic protein receptor type 2 (BMPR2) signaling is a central contributor in the pathogenesis of pulmonary arterial hypertension (PAH). We have recently shown that endothelial-specific Bmpr2 deletion by a novel L1Cre line resulted in pulmonary hypertension. SMAD1 is one of the canonical signal transducers of the BMPR2 pathway, and its reduced activity has been shown to be associated with PAH. To determine whether SMAD1 is an important downstream mediator of BMPR2 signaling in the pathogenesis of PAH, we analyzed pulmonary hypertension phenotypes in Smad1 conditional knockout mice by deleting the Smad1 gene either in endothelial cells or in smooth muscle cells using L1Cre or Tagln-Cre mouse lines, respectively. A significant number of the L1Cre(+);Smad1 (14/35) and Tagln-Cre(+);Smad1 (4/33) mutant mice showed elevated pulmonary pressure, right ventricular hypertrophy, and a thickening of pulmonary arterioles. A pulmonary endothelial cell line in which the Bmpr2 gene deletion can be induced by 4-hydroxy tamoxifen was established. SMAD1 phosphorylation in Bmpr2-deficient cells was markedly reduced by BMP4 but unaffected by BMP7. The sensitivity of SMAD2 phosphorylation by transforming growth factor-β1 was enhanced in the Bmpr2-deficient cells, and the inhibitory effect of transforming growth factor-β1-mediated SMAD2 phosphorylation by BMP4 was impaired in the Bmpr2-deficient cells. Furthermore, transcript levels of several known transforming growth factor-β downstream genes implicated in pulmonary hypertension were elevated in the Bmpr2-deficient cells. Taken together, these data suggest that SMAD1 is a critical mediator of BMPR2 signaling pertinent to PAH, and that an impaired balance between BMP4 and transforming growth factor-β1 may account for the pathogenesis of PAH. (Hypertension. 2013;61:00-00.) • Online Data Supplement

Key Words: BMPR2 ■ conditional knockout mice ■ pulmonary arterial hypertension ■ pulmonary endothelial cells ■ SMAD1

Pulmonary arterial hypertension (PAH) is a rare but fatal vascular lung disease that is characterized by increased pulmonary vascular resistance and sustained elevation of mean pulmonary arterial pressure, leading to right ventricular hypertrophy and right heart failure. PAH is subclassified into idiopathic PAH, heritable PAH, drug- and toxin-induced PAH, persistent pulmonary hypertension of the newborn, and PAH associated with other diseases, such as congenital heart defect, portal hypertension, HIV infection, connective tissue disease, schistosomiasis, and chronic hemolytic anemia.1 Pathological features of PAH include a narrowing and thickening of small pulmonary vessels and plexiform lesions. PAH patients exhibit pulmonary vascular remodeling of all layers of pulmonary arterial vessels: intimal thickening, smooth muscle cell (SMC) hypertrophy or hyperplasia, adventitial fibrosis, and occluded vessels by in situ thrombosis.2

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implying the wide-ranging influence of BMPR2 deficiency on PAH. Furthermore, BMPR2 signaling plays an important role in the survival of endothelial cells (ECs) and in the migration and proliferation of SMCs.\textsuperscript{2,6} Taken together, impaired BMP signaling as a result of BMPR2 deficiency would be a considerable risk factor for the development of PAH.

TGF-\(\beta\) is a large cytokine family that contributes to diverse cellular processes, including cell proliferation, migration, apoptosis, pattern formation, and immunosuppression.\textsuperscript{7} TGF-\(\beta\) family members include TGF\(\beta\), BMPs, growth and differentiation factors, activins/inhibins, and müllerian inhibiting substance.\textsuperscript{2} TGF-\(\beta\) signal transduction is initiated by the binding of ligands to a heteromeric complex of transmembrane serine/threonine type 2 and type 1 receptors, which in turn activates receptor-regulated SMADs: SMAD2/3 for TGF-\(\beta\)/activins and SMAD1/5/8 for BMPs. Receptor-regulated SMADs then form a complex with a common partner, SMAD4 (Co-SMAD), which translocates to the nucleus and regulates the transcription of target genes. On the contrary, there is mounting evidence demonstrating that TGF-\(\beta\) BMP signaling can be transduced through mediators other than SMADs, such as the mitogen-activated protein kinases (MAPKs), including p38MAPK, p42\,44MAPK (ERK1/2), and c-Jun-N-terminal kinase/stress-activated protein kinase.\textsuperscript{8} For instance, exogenous BMP ligands stimulate the phosphorylation of p38MAPK and p42\,44MAPK and affect the proliferation of SMCs.\textsuperscript{9}

Genetic studies with human subjects as well as mouse models clearly indicate that a deficiency in BMPR2 is a crucial genetic factor in PAH development.\textsuperscript{10–13} However, downstream mediators for BMPR2 signaling in PAH pathogenesis remain unknown. About 20% of \textit{Bmpr2} mutations occurred in the cytoplasmic tail domain, such as R899X, that does not impact SMAD phosphorylation, thereby indicating that SMAD proteins may not be the essential mediators of BMPR2 signaling in PAH pathogenesis. West et al\textsuperscript{13} demonstrated that transgenic mice overexpressing the \textit{Bmpr2}(R899X) transgene in the SMCs of adult mice exhibited pulmonary hypertensive (PH) phenotypes, suggesting that SMAD may not be associated with PAH caused by BMPR2 deficiency. On the contrary, Yang et al\textsuperscript{10} showed that SMAD1 phosphorylation was reduced in the pulmonary arterial SMCs of PAH patients with \textit{Bmpr2} mutation.

We investigated a cell-type specific role of SMAD1 in PAH pathogenesis by conditionally deleting the \textit{Smad1} gene either in ECs or in SMCs using a \textit{L1Cre} or \textit{Tagln-Cre} line, respectively. \textit{Smad1} deletion in either cell type resulted in the elevation of pulmonary pressure and the muscularization of pulmonary arteries, suggesting that SMAD1 is indeed a critical downstream molecule in PAH. Using pulmonary ECs (pECs), we further demonstrated that \textit{Bmpr2} deletion not only reduces the level of BMP4-mediated SMAD1/5 phosphorylation but also elevates the level of TGF-\(\beta\)-mediated SMAD2/3 phosphorylation, suggesting that the prevalence of TGF-\(\beta\) signaling in BMPR2/SMAD1 deficiency may contribute to the pathogenesis of PAH.

**Materials and Methods**

Detailed methods about mouse strains, mating scheme, hemodynamic analysis, right ventricular hypertrophy, pulmonary vessel morphometry, methods for establishment of immortalized pulmonary ECs, culture conditions, semiquantitative reverse transcription polymerase chain reaction, Western blotting analysis, and statistical analysis are described in the online-only Data Supplement.

**Results**

**Smad1 Deletion in Pulmonary ECs or SMCs by \textit{L1Cre} or \textit{Tagln-Cre}**

To investigate the role of SMAD1 in the pathogenesis of PAH, we exploited conditional knockout (cKO) approaches for deleting the \textit{Smad1} gene in ECs or SMCs by \textit{L1Cre} or \textit{Tagln-Cre} lines, respectively, because \textit{Smad1} null mice are embryonic lethal.\textsuperscript{14} Both \textit{L1Cre(+)};\textit{Smad1}\textsuperscript{f/f} and \textit{Tagln-Cre(+)};\textit{Smad1}\textsuperscript{f/f} mice were viable and did not exhibit any visible morphological defects as compared with their Cre-negative littermates. The Cre activity was monitored in several organs of 2-month-old mice, including the lung, heart, liver, kidney, and spleen, by detecting the \textit{Smad1}-null allele. As expected based on our previous reports,\textsuperscript{12,15} the Cre-mediated \textit{Smad1} deletion was detected primarily in the lungs of \textit{L1Cre(+)};\textit{Smad1}\textsuperscript{f/f} mice, whereas it was found in most organs of \textit{Tagln-Cre(+)};\textit{Smad1}\textsuperscript{f/f} mice (Figure S1 in the online-only Data Supplement).

**Elevated Pulmonary Pressure and Right Ventricular Hypertrophy Exhibited in Some Mice With \textit{Smad1} Deletion in ECs or SMCs**

To assess pulmonary pressure, we measured the right ventricular systolic pressure (RVSP) of \textit{L1Cre(+)};\textit{Smad1}\textsuperscript{f/f} and \textit{Tagln-Cre(+)};\textit{Smad1}\textsuperscript{f/f} mice as well as of their age-matched \textit{Cre(−)} control male and female mice at various ages (2–24 months). Although the RVSPs of the controls were clustered in the 20–26 mm Hg range, those of the \textit{L1Cre(+)};\textit{Smad1}\textsuperscript{f/f} and \textit{Tagln-Cre(+)};\textit{Smad1}\textsuperscript{f/f} mice were scattered across a wide range from 19 to 42 mm Hg (Figure 1A). \(F\) test for equal variance between \textit{L1Cre} or \textit{Tagln-Cre} and control groups showed significant differences (\(P<0.0001\)) with these \textit{Smad1} mutant groups having wider spread of RVSPs than the control group. Brown-Forsythe test also showed that variances in the 3 groups (\(P<0.005\)), as well as in the combinations of 2 groups are not homogeneous: Control versus \textit{L1Cre} (\(P=0.004\)), \textit{L1Cre} versus \textit{Tagln-Cre} (\(P=0.035\)), and Control versus \textit{Tagln-Cre} (\(P=0.053\)). When we combined RVSPs of control mice used in this study and others from our laboratory (n=81 mice, Table S1 in the online-only Data Supplement), the 99% confidence upper limit is calculated as 29.4. About 40% (14/35) of the \textit{L1Cre(+)};\textit{Smad1}\textsuperscript{f/f} mice and 12% (4/33) of the \textit{Tagln-Cre(+)};\textit{Smad1}\textsuperscript{f/f} mice had RVSPs greater than this boundary point, and we designated them as the PH group (Figure 1A). The Fulton index, the ratio of RV free wall weight over septum plus left ventricular free wall weight, was used to estimate RV hypertrophy. When we grouped the mutant mice into PH and non-PH groups, the Fulton index was greater in the PH mice than in the non-PH mice (Figure 1B), indicating that sustained elevation of pulmonary pressure might have resulted in RV hypertrophy in the PH
groups. There was no significant correlation of RVSPs with sex and age (Figure S2), and also no difference in systemic blood pressure among Cre-negative controls and both Smad1-cKO mice (Figure S3).

Increased Number of Anti-Smooth Muscle α-Actin-Positive Distal Arteries and Medial Wall Thickness in Smad1 Mutant Mice

To examine whether the elevated RVSP and RV hypertrophy in the Smad1-cKO mutants are associated with pulmonary vascular remodeling, anti-smooth muscle α-actin-positive pulmonary arteries ranging from 30 to 70 μm in diameter were counted, and the wall thickness was measured. In comparison with the Cre(−)-Smad1f/f mice (Figure 2A and 2B) and the non-PH group LtCre(+)-Smad1f/f mice (Figure 2C), the PH group LtCre(+)-Smad1f/f mice (Figure 2D) had a higher number of anti-smooth muscle α-actin-positive pulmonary arterioles and thicker arterial walls (Figure 2G and 2H). However, both non-PH and PH Tagln-Cre(+)-Smad1f/f mice (Figure 2E and 2F) showed a higher number of muscularized vessels and thicker walls as compared with the Cre(−)-Smad1f/f controls (Figure 2G and 2H). These unexpected data suggest that the thickening of pulmonary arterioles may not be the direct cause of pulmonary hypertension seen in these mutants. We observed higher percentages of perivascular leukocyte infiltration in both LtCre(+)-Smad1f/f (13/20) and Tagln-Cre(+)-Smad1f/f (5/16) mice in comparison with Cre(−) controls (1/7), but plexiform-like complex vascular lesions were not observed.

Isolation of Pulmonary ECs Carrying R26CreER+/Bmpr2f/f Alleles

It has been hypothesized that an opposing balance between TGF-β and BMP signalings is critical for the homeostasis of pulmonary vasculature and that an imbalance in TGF-β/BMP signalings may contribute to the pathogenesis of PAH. To investigate this hypothesis and to examine the extent to which Bmpr2 deficiency impacts this balance, an in vitro model system was established. We isolated pECs from the lung of a R26CreER+/Bmpr2f/f mouse, in which the conditional f allele can be converted to a null f allele by tamoxifen treatment that activates the Cre recombinase function. Three days of culturing the pECs with medium containing 1 μmol/L 4-hydroxy tamoxifen (4TM) efficiently deleted exons 4 and 5 of the Bmpr2 gene, resulting in conversion of the genotype from 2f/2f to 1f/1f (Figure S4A). When the Bmpr2-deleted cells were subsequently cultured with 4TM-free media for 10 days, no overgrowth of undeleted cell populations was observed (Figure S4B). Henceforth, Bmpr2-intact and Bmpr2-deleted cells will be designated as Bmpr2f/f and Bmpr2f/f pECs (Figure S4C). The Bmpr2 transcript level was undetectable, whereas transcripts for other TGF-β/BMP receptors were unchanged in Bmpr2f/f pECs (Figure S4D).

Deletion of the Bmpr2 Gene and Impaired BMP Signaling

To assess the extent to which Bmpr2 deletion affects BMP signaling, BMP4 or BMP7 was added to Bmpr2f/f and Bmpr2f/f cells at various doses (0, 5, 25, and 50 ng/mL) after 16 hours of serum starvation. First, we examined the protein level of BMPR2 in these cells. Although BMPR2 was readily detected in Bmpr2f/f cells, BMPR2 protein was undetectable in Bmpr2f/f cells (Figure 3A and 3B). The level of Smad1/5/8 phosphorylation was augmented in a dose-dependent manner in Bmpr2f/f cells, whereas it was suppressed at all BMP4 doses in Bmpr2f/f cells (Figures 3A and 3C), indicating that the BMP4 signaling is impaired in Bmpr2f/f pECs. BMP7 signaling could be compensated by ACVR2A in Bmpr2-deficient pulmonary artery SMCs. As shown in Figure 3D, phosphorylation of Smad1/5/8 by BMP7 was elevated in a dose-dependent manner in both Bmpr2f/f and Bmpr2f/f cells, demonstrating that BMP2 is not required for Smad1/5/8 phosphorylation by BMP7 in pECs.

Enhanced TGF-β Signaling in Bmpr2-Deficient pECs

To investigate whether impaired BMP4 signaling affects TGF-β1 signaling, we first examined the basal level of SMAD2
phosphorylation in Bmpr2<sup>2f/2f</sup> and Bmpr2<sup>1f/1f</sup> cells cultured in medium containing 10% fetal bovine serum (Figure 4A). The level of pSMAD2 in Bmpr2<sup>2f/2f</sup> cells was higher than that in Bmpr2<sup>2f/2f</sup> cells. Reduced total SMAD2 levels in Bmpr2<sup>1f/1f</sup> cells is likely a result of ubiquitin ligase-mediated degradation of activated SMAD2 (pSMAD2).<sup>18,19</sup> To test whether Bmpr2-deficient cells are more sensitive to TGF-β, we examined SMAD2 phosphorylation as a response to various doses of TGF-β1 treatments (0, 1, and 2 ng/mL; Figure 4B). Although both Bmpr2<sup>2f/2f</sup> and Bmpr2<sup>1f/1f</sup> cells showed a dose-dependent augmentation of pSMAD2, the level of SMAD2 phosphorylation was significantly higher in Bmpr2<sup>1f/1f</sup> cells at 1 and 2 ng/mL TGF-β1 as compared with Bmpr2<sup>2f/2f</sup> cells, suggesting that impaired BMPR2 signaling may potentiate the TGF-β signaling. To rule out the possibility that the observations seen in Bmpr2<sup>1f/1f</sup> cells were attributable to off-target effects of 4TM treatment, we established immortalized Bmpr2<sup>WT</sup> pECs (Figure S5). In the Bmpr2<sup>WT</sup> pECs, 4TM treatment did not affect Bmpr2 expression (Figure S5), SMAD1/5/8 phosphorylation by BMP4 (Figure S6A), and SMAD2 phosphorylation by TGF-β1 (Figure S6B). Reverse transcription polymerase chain reaction analysis showed that transcript levels of several TGF-β1 downstream genes associated with PAH (Col3a1, Mmp9, Sema7a)<sup>20–23</sup> were elevated in 4TM-treated Bmpr2<sup>2f/2f</sup> (ie, Bmpr2<sup>1f/1f</sup>) cells (Figure S7A), but such alterations were not observed in 4TM-treated Bmpr2<sup>WT</sup> cells (Figure S7B).

**Opposing Balance Between TGF-β and BMP Signalings in pECs**

To further investigate whether BMP and TGF-β signaling form an opposing balance in pECs, we examined whether...
TGF-β-induced SMAD2 phosphorylation is suppressed by BMP treatment in pECs. TGF-β1 (0, 0.1, 1, and 2 ng/mL) and BMP4 (25 ng/mL) or BMP7 (25 ng/mL) were treated for 30 minutes after 16 hours of serum starvation in Bmpr22f/2f and Bmpr21f/1f cells. The level of pSMAD2 in 1 and 2 ng/mL of TGF-β1 treatment in Bmpr22f/2f cells was decreased by either BMP4 or BMP7, suggesting that BMP4/7 and TGF-β form an opposing balance in pECs (Figure 5A and 5B). However, the inhibitory effect of BMP4 treatment on TGF-β-mediated SMAD2 phosphorylation was blunted in Bmpr21f/1f cells (Figure 5A). Interestingly, BMP7 could suppress overactivated TGF-β signaling in Bmpr2-deficient pECs as well as in Bmpr2-intact pECs (Figure 5B).

**Discussion**

In this study, we showed that genetic ablation of Smad1 in ECs or SMCs can predispose mice to PAH, suggesting that the SMAD-dependent pathway in both vascular cell layers is critical for BMPR2 signaling pertinent to the pathogenesis of PAH. Our biochemical data from the Bmpr2-deficient pECs demonstrate the presence of an opposing balance between TGF-β1 and BMP4 signalings in pECs, suggesting that not only diminished SMAD1 signaling but also enhanced TGF-β signaling may contribute to PAH development.

A growing body of evidence indicates that BMPR2 is a critical genetic factor in heritable PAH and also for other types of pulmonary hypertension. However, the downstream mediators of BMPR2 signaling have yet to be clearly defined. There have been incongruent reports about the role of SMAD1 as a downstream mediator of BMPR2 in PAH pathogenesis. Yang et al. reported that SMAD1 phosphorylation was diminished in the pulmonary vasculature of patients with a BMPR2 mutation, suggesting that inactivation of SMAD1 plays a role in PAH pathogenesis. In contrast to this, a significant number of BMPR2 mutations causing heritable PAH are within the cytoplasmic tail domain, which does not affect SMAD phosphorylation. Transgenic (Tg) mice overexpressing a tail domain BMPR2 mutant (R899X) exhibited the PH phenotype, suggesting that SMAD may not be involved in PAH development. However, mutant BMPR2 mRNAs with most of the cytoplasmic tail mutations are destroyed through nonsense mutation decay, a cellular mechanism that destroys defective RNA transcripts having a nonsense mutation to block the production of truncated proteins. BMPR2(R899X) overexpressed in Tg(Bmpr2-R899X) mice was not destroyed.
by nonsense mutation decay and showed normal SMAD1 activation. Therefore, the mechanism of PAH development in a Tg(Bmpr2-R899X) mouse is likely to be different from that in a PAH patient with R899X mutation. However, it is possible that some PAH mice with BMPR2-tail domain mutations might be SMAD-independent by interrupting interactions between BMPR2 and the dynein light chain Tctex-1 as well as LIMK1, a key regulator of actin dynamics. 27, 28

Other studies have suggested the SMAD1-independent mechanism of PAH development in SMCs. Hansmann et al 29 presented a novel antiproliferative BMP2-BMPR2-PPARγ-APOE axis in PAH. Mice with targeted deletion of peroxisome proliferator-activated receptor gamma (Pparg) in SMCs spontaneously developed PAH with elevated RVSP, RVH, and increased muscularization of the distal pulmonary arteries. This was independent of SMAD1/5/8 phosphorylation. Li et al 30 suggested a novel role of NOTCH3 in controlling the proliferation of SMCs and in maintaining SMCs in an undifferentiated state. The severity of the disease in human PAH patients and rodent PH models correlated with the amount of NOTCH3 protein in vascular SMCs of the lung, suggesting that the NOTCH3 signaling pathway in SMCs is crucial for the development of PAH.

About 40% and 12% of L1Cre(+);Smad1<sup>f/f</sup> and Tagln-Cre(+);Smad1<sup>f/f</sup> mice, respectively, displayed PH phenotypes, indicating that Smad1 deletion in ECs has a greater impact on PAH pathogenesis than does Smad1 deletion in SMCs. The reason for this is unclear. One possible explanation is that while BMPR2-SMAD1 signaling in SMCs affects only SMC layer, that in pECs affects both EC and SMC layers. Defects in BMPR2-SMAD1 signaling in SMCs increase the proliferation of SMCs, 10 leading to a

Figure 4. Enhanced SMAD2 phosphorylation in Bmpr2-deleted pulmonary endothelial cells. A, Western blot shows elevated levels of pSMAD2 in Bmpr2<sup>2f/2f</sup> cells cultured in media containing 10% fetal bovine serum without additional cytokine treatment. B, While SMAD2 phosphorylation was increased in both Bmpr2<sup>2f/2f</sup> and Bmpr2<sup>1f/1f</sup> cells dose dependently by transforming growth factor-β1 (TGF-β1) treatment for 30 minutes after 16 hours of serum starvation, the response to TGF-β1 treatment was significantly enhanced in Bmpr2<sup>1f/1f</sup> cells. <sup>r</sup> and <sup>P</sup> values in regression analysis are indicated. Mean and standard error of data obtained from triplicate samples are shown at each data point.

Figure 5. Bone morphogenetic protein (BMP) and transforming growth factor-β (TGF-β) signalings form an opposing balance in pulmonary endothelial cells (pECs). A, Western blot analysis shows that SMAD2 phosphorylation by TGF-β1 (0–2 ng/mL) is inhibited by BMP4 (25 ng/mL) in BMPR2-intact 2f/2f cells, but this inhibitory effect of BMP4 is blunted in BMPR2-deficient 1f/1f cells. B, Inhibitory effect of BMP7 (25 ng/mL) on SMAD2 phosphorylation by TGF-β1 is unaffected in BMPR2-deficient pECs. Error bars are SEM (**<sup>P</sup><0.01 and ***<sup>P</sup><0.001).
thickening of vessel walls, whereas those in ECs may result in endothelial dysfunction, leading to neointima formation, a disrupted balance of vasoactive mediators exacerbating vasoconstriction, increased EC permeability allowing serum growth factors to affect SMC proliferation and the release of mitogens such as serotonin from ECs to induce smooth muscle hyperplasia, and enhanced vessel occlusion and in situ thrombosis.

Only a subset of mice with either endothelial or smooth muscle-\textit{Smad1} deletion displayed PH phenotypes. This incomplete penetrance could be a result of functional redundancy among \textit{Smad1}, 5, and 8. Clear genetic interactions and mutual compensations between \textit{Smad1} and \textit{Smad5} in ECs have been reported. Because of the overlapping functions of these SMAD proteins and the embryonic lethality of homozygous mutations of individual \textit{Smad1}, 5, or 4 genes, the association between SMAD mutations and PAH patients may be difficult to detect. Alternatively, strain-specific genetic modifiers that modulate susceptibility to environmental insults may contribute to PAH development. Inflammation has been suggested as such an environmental factor associated with PAH. Inflammatory cells, including macrophages and lymphocytes were accumulated, and many proinflammatory cytokines were elevated in the PAH vascular lesions. Thus, treatment with anti-inflammatory stimuli such as interleukin-6 and 5-lipoxygenase on \textit{Smad1-cKO} mice may increase penetrance. If so, blocking inflammatory activation could have therapeutic benefits for PAH patients. Supporting this view, platelet-activating factor antagonists, a class of anti-inflammatory drugs, inhibited pulmonary vascular remodeling induced by hypoxic hypoxia in rats. Inhibition of 5-lipoxygenase activating protein suppressed hypoxia-induced pulmonary vasoconstriction in vitro and the development of chronic hypoxic pulmonary hypertension in rats.

Recent studies have implied that BMP and TGF-\beta signalings form an opposing balance in pulmonary vascular homeostasis and that the imbalance in TGF-\beta/BMP signaling may play an important role in PAH development. To investigate whether BMPR2 deficiency alters TGF-\beta signaling in pECs, we established immortalized pEC lines from a \textit{R26\textsuperscript{CreER/\textminus};Bmpr2\textsuperscript{-2/2}} mouse, in which tamoxifen treatment induces \textit{Bmpr2} deletion. This inducible system is advantageous because we can minimize variations attributable to both strain and individual differences and passage numbers because \textit{Bmpr2}-intact and \textit{Bmpr2}-deficient cells originate from the same parental cells. Genetic ablation of the \textit{Bmpr2} gene by 4TM is also more efficient and reproducible as compared with knockdown approaches. \textit{Bmpr2}-deleted pECs, as compared with \textit{Bmpr2}-intact pECs, showed a markedly reduced level of pSMAD1/5/8 with BMP4 treatment as well as enhanced SMAD2 phosphorylation not only at the basal level but also with TGF-\beta1 treatment, demonstrating that \textit{Bmpr2} deficiency potentiates TGF-\beta signaling in pECs. Previous studies suggest that the TGF-\beta/BMP counter balance can occur at multiple levels, including activation of inhibitors of the opposing signal, inhibition of transcriptional targets of the opposing signal, and competition for SMAD4. Molecular mechanism for the antagonism warrants future investigation.

Our data from pECs indicate that restoration of the TGF-\beta/BMP balance could be an important therapeutic option for PAH. Supporting this view, recent reports have shown that the inhibition of TGF-\beta signaling by an ALK5 inhibitor (IN-1233) prevented PH in the monocrotaline-treated rat. Calpain\textsuperscript{40} or the inhibition of TGF-\beta signaling via an angiotensin II type 1 receptor\textsuperscript{45} could be alternative therapeutic options for PAH. Our data and a previous report showed that BMP7 could phosphorylate SMAD1/5/8 in \textit{Bmpr2}-deficient pECs or SMCs. Therefore, restoration of pSMAD1/5/8 by BMP7 can be considered as a possible means to correct BMPR2 deficiency.

**Perspectives**

PAH is a rare but fatal disorder. The currently available therapies help to provide symptomatic relief, but a drug treatment based on the mechanisms of the disease has yet to be developed. A deficiency in BMPR2 signaling has been shown to be involved in sporadic as well as heritable forms of PAH. The first step toward developing a therapy is to discern whether a SMAD-dependent or SMAD-independent pathway is crucial for the pathogenesis of PAH. Here, using \textit{cKO} mouse models, we demonstrated that homozygous deletion of \textit{Smad1} in either ECs or SMCs resulted in PH, suggesting that the SMAD-dependent pathway is critical for PAH pathogenesis. Using a novel pulmonary EC line in which \textit{Bmpr2} deletion can be induced, we showed that BMPR2 is essential for SMAD1 phosphorylation by BMP4 but not by BMP7. We further demonstrated the presence of an opposing balance between BMP4 and TGF-\beta1 in the pECs and the causal relationship between \textit{Bmpr2} deficiency and enhanced TGF-\beta signaling. Our findings suggest that an impaired balance between TGF-\beta (SMAD2/3) and BMP4 (SMAD1/5/8) may underlie the pathogenesis of PAH and that correcting the imbalance, that is, either by enhancing SMAD1/5/8 or by inhibiting SMAD2/3, could be an effective strategy for developing a therapy for PAH.

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**Disclosures**

None.

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**Novelty and Significance**

**What Is New?**
- With novel genetic mouse models and a novel pulmonary endothelial cell line, we demonstrate that SMAD1 is a crucial mediator of bone morphogenetic protein (BMP) receptor type 2 signaling in endothelial and smooth muscle cells pertinent to pulmonary arterial hypertension (PAH), and that transforming growth factor-β and BMP form an opposing balance in pulmonary endothelial cells.

**What Is Relevant?**
- We present SMAD-dependent pathway as a central mediator of BMP receptor type 2, the most well-known genetic factor for PAH. An impaired balance between transforming growth factor-β and BMP may underlie the pathogenesis of PAH, and correcting the imbalance either by enhancing BMP or by inhibiting transforming growth factor-β signalings could be an effective therapeutic strategy for PAH.

**Summary**

SMAD1 is an important downstream mediator of the PAH pathogenesis caused by Bmpr2 deficiency.
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SMAD1-deficiency in either endothelial or smooth muscle cells can predispose mice to pulmonary hypertension

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Running Title: SMAD1-deficiency in the pathogenesis of pulmonary hypertension.
EXPANDED MATERIALS AND METHODS

Mouse strains and mating scheme
Mice were maintained under standard specific-pathogen-free conditions, and all animal procedures performed were reviewed and approved by the University of Florida Institutional Animal Care and Use Committee. Generation of the conditional Smad1 allele (Smad1f) and of the Tg(Alk1-Cre)-L1 (L1Cre) line was previously described.1,2 Tg(Tagln-Cre) and R26R mice were purchased from the Jackson Laboratory. Smad1f/f mice were intercrossed with L1Cre or Tagln-Cre lines. The L1Cre(+);Smad1f/+ Cre males were further intercrossed with Smad1f/f;R26R females to produce L1Cre(-);Smad1f/f and L1Cre(+);Smad1f/f mice. Tagln-Cre(-);Smad1f/f and Tagln-Cre(+);Smad1f/f mice were produced by the same breeding scheme. More than half of the control and experimental mice contained the R26R allele to monitor the Cre activity. PCR primer sets and conditions for detecting the conditional as well as null alleles of Smad1 were previously described.14 Primer sets for the genotype of L1Cre, Tagln-Cre, or R26R are shown in Table S2.

Hemodynamic analysis
To evaluate pulmonary artery pressure, right ventricular systolic pressure (RVSP) was measured by right heart catheterization through the right jugular vein.1 Each mouse was anesthetized by isoflurane (1-2 %) and placed in the supine position. A 1-2 cm incision was made in the neck to expose the right jugular vein. The Mikro-Tip pressure transducer (SPR-835, Millar Instrument) was inserted into right external jugular vein and advanced into the right ventricle. Systemic blood pressure was recorded non-invasively using the tail-cuff method. A pneumatic pulse sensor was placed on the tail distal to an occlusion cuff controlled by a Programmed Electro-Sphygmomanometer (PE-300, Narco Bio-Systems), which is connected to the Powerlab system (ADInstruments). All electrical outputs from the tail cuff, the pulse sensor and transducer were recorded and analyzed by the Powerlab 8/30 data acquisition system and associated Chart software (ADInstruments).

Right Ventricular Hypertrophy (RVH)
After hemodynamic analysis, mice were euthanized and using syringe-generated flow, the pulmonary circulation was perfused with PBS containing heparin (3 units/ml). The hearts were isolated and outflow tracts and atria were removed. The right ventricle was cut out from the heart by the spring scissor and the right ventricle and the remaining left ventricle (LV) plus septum (S) were weighed. Right ventricular hypertrophy was determined by the ratio of RV/LV+S.

Pulmonary vessel morphometry
After the hemodynamic analysis, the left lung was inflated with PBS for 20 minutes followed by formalin at a constant inflation pressure of 23cmH2O, fixed with 4% paraformaldehyde overnight, and paraffin-embedded. Each lung sample was transversely sliced into 5 μm thick sections and subjected to immunostaining with anti-smooth muscle α-actin (αSMA) antibody (Sigma-aldrich, mouse monoclonal, 1:800) using M.O.M kit (Vector laboratories) to visualize the vascular smooth muscle layer. To determine muscularization of pulmonary vessels, peripheral blood vessels ranging from 30-70 μm in diameter were counted in at least four fields.
at 20X magnification with a Zeiss Axioplan-2 optical microscope. The counted vessels were categorized as muscularized and nonmuscularized vessels at the level of alveolar ducts. The percentage of muscularized pulmonary vessels was calculated by dividing the number of muscularized vessels by the total number of counted vessels in the same field. To calculate the percentage of wall thickness (WT), circular and fully muscularized vessels were selected. WT1 (the thickness between the outer boundary and the inner boundary of αSMA positive medial layer) was measured at one point of the vessel wall and WT2 at the point which was diametrically opposite, guided by Openlab 5.03 Beta software (Improvision Inc.). External diameter (ED) was also measured at the same vessel. The percentage of medial wall thickness was calculated as (WT1 + WT2) x100/ED.

Establishment of immortalized pulmonary endothelial cells
The lungs from an eight-week-old R26CreER+/−;Bmpr22f/2f mouse and R26+/+;Bmpr22f/2f (Bmpr2WT) were finely minced using a sterile scalpel. The chopped tissues were subjected to serial digestion using 2 ml of a 1X trypsin solution (0.25% trypsin, 0.5 M EDTA [pH 8.0] in DMEM) at 37°C, with frequent shaking, for three times at 8 minutes each. Trypsin digestion was inactivated by adding 6 ml of normal endothelial cell media (ECM; 10% fetal bovine serum, 1 mg/ml heparin, 0.1 mg/ml endothelial mitogen [Biomedical Technologies, Inc.], 1 mM non-essential aminoacids [Cellgro], 1 mM sodium pyruvate, and 50 units/ml penicillin/streptomycin). After the large debris had settled, cells in the supernatant were carefully collected for culture. When cells in the culture reached about 50% confluency post-isolation, they were transfected with 4.0 µg of SV40 DNA: ATCC (VRMC-3), pUCSV40-B2E3 using Lipofectamine (Invitrogen) following the manufacturer’s protocol. After a couple of passages, endothelial cells were sorted out by Fluorescence-Activated Cell Sorting (FACS) using Dio-Ac-LDL (Biomedical Technologies, Inc.) and lectin (Sigma-Aldrich Co.). For deletion of the Bmpr2 gene, the immortalized pECs were cultured with medium containing 1 µM of 4-hydroxy tamoxifen (4TM; Sigma-Aldrich Co.) for 3 days. Thereafter, the cells were cultured in 4TM-depleted growth medium. Primer sequences used for detecting the null allele of Bmpr2 (Bmpr21f) upon 4TM treatment are listed in Table S2.

Pulmonary endothelial cell (pEC) cultures and cytokines treatment
Bmpr2-intact (Bmpr22f/2f), Bmpr2-deleted (Bmpr21f/1f), and Bmpr2WT pECs were grown in ECM and subcultured every 3 days. Cells were plated on 6-well plates (2x10⁵ cells/well) one day before serum starvation, grown in serum-free ECM for 16 hours, and then incubated with fresh serum-free ECM containing TGF-β1 (0, 1, or 2 ng/ml), BMP4 (0, 5, 25, or 50 ng/ml), or BMP7 (0, 5, 25, or 50 ng/ml) for 30 minutes. Recombinant mouse TGF-β1, BMP4, and BMP7 were purchased from R&D Systems. In all conditions, data were collected in triplicate.

Semi-quantitative RT-PCR
To determine the levels of transcripts of endothelial cell-specific markers and genes involved in TGF-β superfamily signaling, Bmpr2-intact (Bmpr22f/2f), Bmpr2-deleted (Bmpr21f/1f), and Bmpr2WT pECs were harvested at 100% confluency from a 25 cm² culture flask. Total RNAs from the cells were extracted using the NucleoSpin RNA purification kit (Clontech). One µg of
RNA was used for the reverse transcription (RT) reaction. cDNAs were synthesized using SuperScript III First-Strand synthesis kit (Invitrogen). Two μl of cDNA was used as a template for PCR amplification for 25 cycles: denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 1 minute. The transcription level of each gene was normalized to Gapdh or β-actin expression. The primers used for RT-PCR analysis are shown in Table S3.

**Western blot analysis**
Pulmonary ECs were harvested with a Chemicon lysis buffer and sonicated. Cell lysates were spun down at 13000 rpm for 15 minutes. The concentration of protein was determined using the Bio-rad DC Assay kit. 50 μg of total protein was fractionated by 10% of SDS-PAGE and transferred to nitrocellulose membranes (Bio-rad). Membranes were incubated with the primary antibody followed by the horseradish peroxidase-linked secondary antibody. A chemiluminescent detection reagent (ECL Plus, Amersham Pharmacia Biotech Inc.) was used to visualize proteins. The antibodies used for Western blotting analysis are the following: SMAD1 (rabbit polyclonal, 1:1000), SMAD2 (mouse monoclonal, 1:1000), pSMAD2 (rabbit polyclonal, 1:1000), pSMAD1/5/8 (rabbit polyclonal, 1:1000) from Cell Signaling, β-actin (mouse monoclonal, 1:10,000) from Sigma-aldrich, BMPR2 (mouse monoclonal, 1:500) from BD Transduction Laboratories, Secondary antibodies include: mouse (1:5000) and rabbit (1:5000) from Sigma-aldrich.

**Statistical analysis**
Equality of group variances was examined by F-test and Brown-Forsythe test to determine a statistical significance of spread between two groups. To determine upper limit of RVSPs in control mice at 95 or 99% confidence, we included RVSPs of control mice published from our laboratory4,5. Upper limits at 95% or 99% confidence were calculated from (mean + 1.645 x SD) or (mean + 2.326 x SD), respectively (Table S1). Regression analysis was performed for the dose-dependency and sensitivity to treatment. The value of independent variable, x, is transformed to a rank ordered by the amount. For example, 0 for no treatment, 1 for low level (25 ng/ml), 2 for high level (50 ng/ml). T-test was used to determine a statistical significance between two groups.
REFERENCES


## ADDITIONAL TABLES

**Table S1.** Statistical analysis of RVSPs of the control mice

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<th>Value</th>
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**Table S2. Primers for genomic DNA PCR for genotyping**

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<th>Forward primers</th>
<th>Reverse primers</th>
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<tr>
<td>Smad1</td>
<td>Smad1-A: CACCTGTGCCCCCTCCAAGT</td>
<td>Smad1-B: GAGCTCTGCTCCGCCACTCA</td>
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<td>Alk1</td>
<td>Alk1-F: CAGCACCTACATCTTGGGTGGA</td>
<td>Alk1-R: ACTGTTTCTTCTCGGAGCCTTGTC</td>
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<td>Bmpr2</td>
<td>Bmpr2-2A: CACACCAGCCTATATCTCTAGATAC</td>
<td>Bmpr2-6R:CACATATCTGTTATGAAACTTGAG</td>
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<td></td>
<td>Bmpr2-2C: TTATTGTAAGTACACTGTTGCTGTC</td>
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<td>L1Cre</td>
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<td>Tagln-Cre</td>
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<td>ROSA26</td>
<td>R26R-F: GTCGTTTTACACGTCGACT</td>
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### Table S3. Primers for RT-PCR

<table>
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<th>Genes</th>
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**Figure S1.** Smad1 deletion in L1Cre(+);Smad1f/f and Tagln-Cre(+);Smad1f/f mice. Deleted Smad1 allele was examined in various organs of Smad1-conditional KO mice and Cre-negative control mice by genomic PCR analysis. Null Smad1 was detected at 300 bp. Genomic segment between 2 loxp sites including exon2 can be deleted by Cre recombinase resulting in shortened genomic fragment that can be amplified by PCR with a primer set (Smad1-A and Smad1-B). A primer set (Alk1-F and Alk1-R) amplifying the wild-type Alk1 locus (190 bp) was used as a control for the PCR amplification.
Figure S2. No correlations of RVSP with gender and age. A-D, Closed circles indicating RVSP of each mouse in L1Cre(+)×Smad1df/f (A) and Tagln-Cre(+)×Smad1df/f (B) were plotted by gender (A, B), or by age (C, D). There was no significant difference in RVSP distributions by gender and age. Dotted horizontal lines in each panel indicate 28.5 mmHg of RVSP.
Figure S3. Systolic blood pressures (SBP) of Smad1-cKO mice were not significantly different from those of Cre(-) control mice.
Figure S4. Characterization of pulmonary endothelial cells in which Bmpr2 gene deletion can be induced by 4TM treatment. A, 4TM treatment for 3 days activated Cre recombinase to delete the genomic segment containing 6R primer binding sequence and exon 4-5, flanked by two loxP sequences (triangles). The conditional allele can produce a PCR amplicon by the 2A-6R but not by 2A-2C primer pairs whereas the deleted allele can produce a PCR amplicon by the 2A-2C but not by the 2A-6R primer pair. Genomic PCR analysis by the 2A-6R pair shows gradually diminished PCR band by three days of 4TM treatment. B, PCR analysis with mixture of 2A, 6R, and 2C on genomic DNA isolated from pECs treated with 4TM for three days followed by further growth in TM-free media for 3 and 10 days, showing maintenance of Bmpr2-deleted cells. C and D, RT-PCR analysis show that the mRNA levels of endothelial marker genes (C) and TGF-β family type I and II receptors (D) are maintained in Bmpr2-deleted (1f/1f) cells, demonstrating the 4TM treatment and Bmpr2-deletion did not affect EC characteristics and expressions of other TGF-β mediators but Bmpr2. ‘W’ indicates a negative control using distilled water instead of cDNA.
Figure S5. Establishment of Bmpr2-wildtype (Bmpr2WT) pulmonary ECs. Immortalized Bmpr2WT pECs were treated with 4TM for 3 days followed by culturing in 4TM-free media for longer than 10 days as it was done for Bmpr2/2 pECs. RT-PCR analysis shows that unchanged transcript levels of Bmpr2 and other EC specific marker genes, including Pecam1, VE-cad, Flk1, Agp2. β-actin was used for equal loading of cDNA template. Triangle indicates no reverse transcriptase control.
**Figure S6.** No effect of 4TM treatment on SMAD phosphorylations by BMP4 or TGF-β1. **A** and **B**, Bmpr2WT pECs were grown in 4TM-free media for more than 10 days after 3 days of 4TM treatment. Cells were serum-starved for 16 hours in chemically defined growth factor- and serum-free ECM, and treated with BMP4 (0 or 25 ng/ml; A) or TGF-β1 (0, 1, or 2 ng/ml; B) for 30 minutes. Treatment with 4TM did not affect SMAD1/5/8 phosphorylation by BMP4 (A), and SMAD2 phosphorylation by TGF-β1 (B).
Figure S7. Elevated transcript levels of TGF-β1 downstream genes implicated in PAH in the Bmpr2-deficient cells. Total RNAs extracted from 4TM-untreated and -treated R26CreER/+;Bmpr22f2f (A) and Bmpr2WT (B) pECs were subjected to reverse transcriptase reaction followed by PCR amplification with Bmpr2, Col3a1, Mmp9, and Sema7a. β-actin was used for equal loading of cDNA template, and triangle indicates no reverse transcriptase control.