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General Article

GENERAL ARTICLE

BMPRII deficiency impairs apoptosis via the BMPRII-ALK1-BclX-mediated pathway in pulmonary arterial hypertension

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Abstract

Pulmonary arterial hypertension (PAH) is a devastating cardiovascular disorder characterized by the remodelling of pre-capillary pulmonary arteries. The vascular remodelling observed in PAH patients results from excessive proliferation and apoptosis resistance of pulmonary arterial smooth muscle cells (PASMCs) and pulmonary arterial endothelial cells (PAECs). We have previously demonstrated that mutations in the type II receptor for bone morphogenetic protein (BMPRII) underlie the majority of the familial and inherited forms of the disease. We have further demonstrated that BMPRII deficiency promotes excessive proliferation and attenuates apoptosis in PASMCs, but the underlying mechanisms remain unclear. The major objective of this study is to investigate how BMPRII deficiency impairs apoptosis in PAH. Using multidisciplinary approaches, we demonstrate that deficiency in the expression of BMPRII impairs apoptosis by modulating the alternative splicing of the apoptotic regulator, B-cell lymphoma X (Bcl-x) transcripts: a finding observed in circulating leukocytes and lungs of PAH subjects, hypoxia-induced PAH rat lungs as well as in PASMCs and PAECs. BMPRII deficiency elicits cell specific effects: promoting the expression of Bcl-xL transcripts in PASMCs while inhibiting it in ECs, thus exerting differential apoptotic effects in these cells. The pro-survival effect of BMPRII receptor is mediated through the activin receptor-like kinase 1 (ALK1) but not the ALK3 receptor. Finally, we show that BMPRII interacts with the ALK1 receptor and pathogenic mutations in the *BMPR2* gene abolish this interaction. Taken together, dysfunctional BMPRII responsiveness impairs apoptosis via the BMPRII-ALK1-Bcl-xL pathway in PAH. We suggest Bcl-xL as a potential biomarker and druggable target.

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Introduction

Pulmonary arterial hypertension (PAH) is a devastating and incurable cardiovascular disorder characterized by the remodelling of pre-capillary pulmonary arteries. The condition is characterized by abnormal proliferation and apoptosis resistance of pulmonary arterial smooth muscle cells (PASMCs), pulmonary arterial endothelial cells (PAECs) and fibroblasts, leading to elevated pulmonary artery pressure, right-heart failure and premature death (1,2).

Heterozygous loss of function mutations in the bone morphogenetic protein type II receptor (*BMPR2*) gene (3) underlie the majority (>80%) of familial and heritable PAH (HPAH) and can be identified in a substantial proportion (~40%) of isolated PAH cases (3,4). We have determined that disease-associated nonsense mutations trigger degradation of the *BMPR2* transcript via the nonsense-mediated decay (NMD) process. This contributes to a stoichiometric imbalance in the receptor complex and dysfunctional signalling (5). Furthermore, we have found a number of heterozygous mutations in *SMAD1*, *SMAD4* and *SMAD9* genes in European and Japanese cohorts, which were excluded for *BMPR2* mutation. Each mutation impacts upon transcript integrity, reduces bone morphogenetic protein (BMP) signalling and diminishes expression of the *Id*1–3 genes (6). Recent investigations have identified novel mutations in *BMP9*, *EIF2AK4*, *ATP13A3*, *AQP1* and *SOX17* genes, but they represent an infrequent cause of the disease (7,8). Furthermore, reduced type II receptor for bone morphogenetic protein (BMPRII) expression has been observed in non-genetic forms of PAH in humans (9) and animal models (10). These results imply that the impaired BMPRII pathway may cause susceptibility to abnormal vascular homeostasis leading to PAH.

A balance between pro- and anti-apoptotic factors determines cell fate. The B-cell lymphoma 2 (Bcl2) family proteins are well known for their roles in regulation of apoptosis. Bcl-2, Bcl-xL and Bcl-w proteins constitute the anti-apoptotic members of this family, while Bax, Bid, Bad and Bcl-xS proteins belong to the pro-apoptotic group. The expression of anti-apoptotic regulators such as the Bcl2 gene is increased in both sporadic and familial PAH lung tissues (11). A member of this family of genes is B-cell lymphoma X (Bcl-x), which generates both pro- (Bcl-xS) and anti-apoptotic (Bcl-xL) isoforms via alternative splicing (12). In normal PASMCs, BMP signalling downregulates the expression of Bcl2 (13). We and others have shown that SMAD-independent pathways such as p38MAPK and NF-κB are activated in experimental models of PAH and that these pathways have been shown to enhance the expression of Bcl-xL isoform (14), thereby exerting an anti-apoptotic phenotype. We have previously demonstrated that apoptosis is greatly reduced in mouse PASMCs harbouring the pathogenic *BMPR2* (p.R899X) mutation and the BMPRII deficiency potentiates the transforming growth factor β (TGFβ) signalling pathway. TGFβ induces apoptosis in human B cells by transcriptional activation of Bcl-xL (15). Taken together, these observations indicate a relationship between BMP/TGFβ signalling pathways and the regulation of Bcl2 family of proteins. However, how dysfunctional BMPRII signalling regulates the alternative splicing of Bcl-x transcripts in PAH remains to be investigated.

We and others have demonstrated that in PASMCs isolated from PAH subjects (PAH-PASMCs), the anti-proliferative and pro-apoptotic effects of BMPs (2,4,7,9) are greatly reduced (6,16–18). In vascular endothelial cells, BMP ligands elicit a pro-proliferative response (19). In addition, BMPs protect vascular and blood-derived endothelial cells from apoptosis (4) and this protection seems to be mediated by the BMPRII receptor, as inactivating mutations in the receptor trigger endothelial cell apoptosis (20). We have previously demonstrated that BMPRII deficiency elicits pro-proliferative and anti-apoptotic phenotype in PASMCs via the TGFβ-associated kinase 1, but the underlying mechanisms by which apoptosis resistance is controlled in PAH remain unclear.

In this report, we have investigated the underlying mechanisms leading to apoptosis resistance in PAH. We have shown that the expression of anti-apoptotic Bcl-xL transcripts is greatly increased in the lungs and circulating leukocytes of PAH patients with and without *BMPR2* mutations. The expression of Bcl-xL transcripts is also increased in hypoxic PAH rat lungs and in PAH-PASMCs harbouring the *BMPR2* (p.R899X) mutation. Selective inhibition of Bcl-xL protein by a small molecule agent induces apoptosis in PAH-PASMCs. In endothelial cells, short interfering RNA (siRNA) knock-down of either the *BMPR2* or the activin receptor-like kinase 1 (*ALK1*) gene promotes endothelial cell apoptosis. Overexpression of the BMPRII receptor activates the expression of Bcl-xL and inhibits caspase 3/7 activities. The pro-survival effect of BMPRII receptor is mediated through the ALK1 but not the ALK3 receptor. Finally, we have shown that BMPRII interacts with the ALK1 receptor and mutations in the *BMPR2* gene abolish this interaction and reduce BMP-responsive reporter activation. Taken together, these observations suggest that the BMPRII-ALK1-Bcl-xL pathway regulates cell-specific apoptosis in PAH. While determination of the ratio of Bcl-xL and Bcl-xS transcripts offers the opportunity for a novel biomarker, targeting the BMPRII-Bcl-xL axis may provide a novel therapeutic intervention in PAH.

Results

The expression of anti-apoptotic Bcl-xL transcripts is increased in circulating leukocytes and lungs of PAH patients

Alternative splicing of the Bcl-x gene, a key regulator of apoptosis, generates the pro-apoptotic (Bcl-xS) and anti-apoptotic (Bcl-xL) isoforms (Fig. 1A) (12). To determine whether alternative splicing of Bcl-x transcripts is altered in PAH patients, semi-quantitative polymerase chain reaction (PCR) analyses were performed on circulating leukocytes of six PAH patients and seven control subjects. Out of the six PAH patients studied, five patients harboured mutations in the *BMPR2* gene. The *BMPR2* mutations were nonsense (p.L287X), frameshift (c.A796fsX6) and deletion (c.A497-503del, c.768 + 3delA), all of which are likely to introduce a premature termination codon into the reading frame. A substantial reduction in the pro-apoptotic transcript, Bcl-xS, and an increase in the anti-apoptotic Bcl-xL isoform were observed in PAH cases compared to control subjects (Fig. 1B–C). We next determined the relationship between defective BMPRII signalling and Bcl-x splicing in lung tissues of a PAH patient who had undergone combined heart and lung transplantation. The PAH patient studied harboured a nonsense (p.T835X) *BMPR2* mutation. Consistent with the leukocyte data, we observed significantly increased expression of the anti-apoptotic isoform Bcl-xL over Bcl-xS in PAH lungs compared to the control (Fig. 1D–E). These results indicated that there might be a correlation between preferential expression of the anti-apoptotic Bcl-xL over the pro-apoptotic Bcl-xS transcripts in PAH patients with and without *BMPR2* mutations.

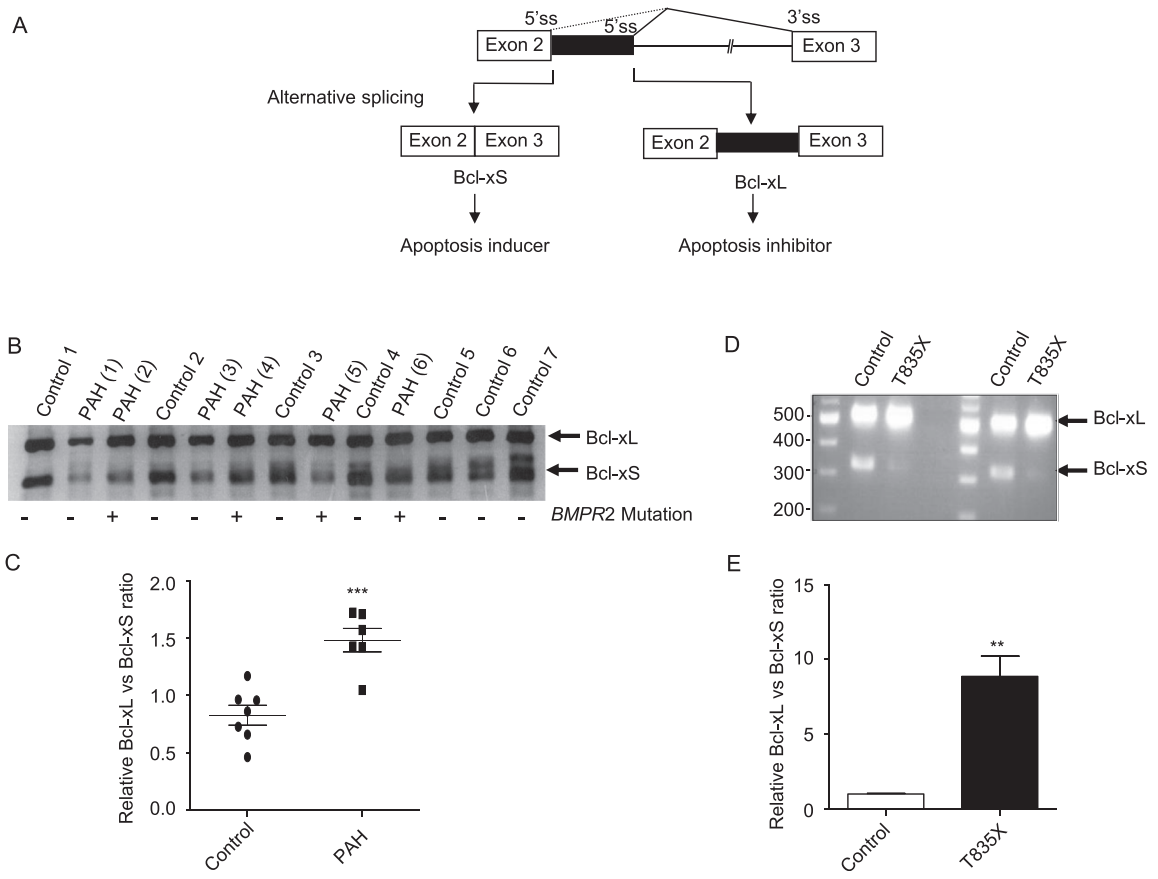

Figure 1. Increased expression of Bcl-xL transcripts in circulating leukocytes and lungs of PAH patients. (**A**) Alternative splicing of the Bcl-x gene. Selection of the upstream 5′ splice site (5'ss) generates the anti-apoptotic Bcl-xL long isoform, while the downstream 5′ splice site produces the pro-apoptotic short isoform, Bcl-xS. (**B**) Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of Bcl-xS and Bcl-xL transcripts derived from circulating leukocytes of PAH patients with and without *BMPR2* mutations. Controls 1–6 are RNAs derived from healthy subjects while Control 7 is commercially available RNA (Clontech). (**C**) Quantification of the RT-PCR image. (**D**) RT-PCR analysis of Bcl-x transcripts in human lung tissue from a PAH subject harbouring a *BMPR2* mutation (p.835X) who underwent combined heart and lung transplantation. The control lung RNAs are from a commercially available source (Clontech). (**E**) Quantification of RT-PCR by image analysis. The mean densities of bands derived from three independent experiments are shown. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control as indicated.

BMPRII deficiency promotes the anti-apoptotic Bcl-xL expression in hypoxia-induced PAH rat lungs

As we observed an increased Bcl-xL expression in PAH cases that do not harbour a *BMPR2* mutation, we were keen to determine whether the expression of Bcl-xL was upregulated in a non-genetic PAH experimental model. Therefore, the hypoxia-induced rat model of PAH (17) was used to investigate the relationship between reduced levels of the BMPRII receptor and the alternative splicing of Bcl-x. Two weeks of chronic hypoxia reduced the level of *BMPR2* expression (Fig. 2A) and increased the ratio of Bcl-xL to Bcl-xS and Bcl-xL to GAPDH in rat lung tissues (Fig. 2B–D), confirming a link between BMPRII deficiency and preferential expression of the anti-apoptotic Bcl-xL transcripts.

The expression of anti-apoptotic Bcl-xL transcripts is increased in both primary PAH-PASMCs and hTERT-PASMCs harbouring a pathogenic *BMPR2* mutation

Having observed an increased level of Bcl-xL expression in PAH patient samples and in PAH rat lungs, we were keen to investigate alternative splicing of the Bcl-x gene in cells including PASMCs and ECs, which are involved in the remodelling of small pulmonary arteries. We first investigated the expression of Bcl-x transcripts in human PASMCs. These cells were isolated from explanted lung samples from a PAH patient harbouring a pathogenic mutation (p.R899X) in the *BMPR2* gene. PAH-PASMCs and commercially available wild-type PASMCs were grown in tissue culture plates until confluency and their total RNAs were isolated. Semi-quantitative PCR was carried out to determine the expression of Bcl-x transcripts. Low-level expression of Bcl-x transcripts was observed in wild-type PASMCs while the expression of Bcl-xL transcripts was significantly increased in PAH-PASMCs (Fig. 3A–B). We further investigated the expression of Bcl-xL transcripts in PAH-PASMCs immortalized by the expression of human telomerase (hTERT) gene (Nasim *et al.*, unpublished data). The expression of Bcl-xL transcripts was also increased in these cells (Fig. 3A–B).

Dysregulated BMPRII-mediated signalling impairs caspase activity in PASMCs

BMPRII-mediated signalling elicits pro-apoptotic effect in PASMCs (17). To determine the relationship between pro-apoptotic effects of BMPRII and the preferential expression of Bcl-xL transcripts (Fig. 3C), we took advantage of PASMCs

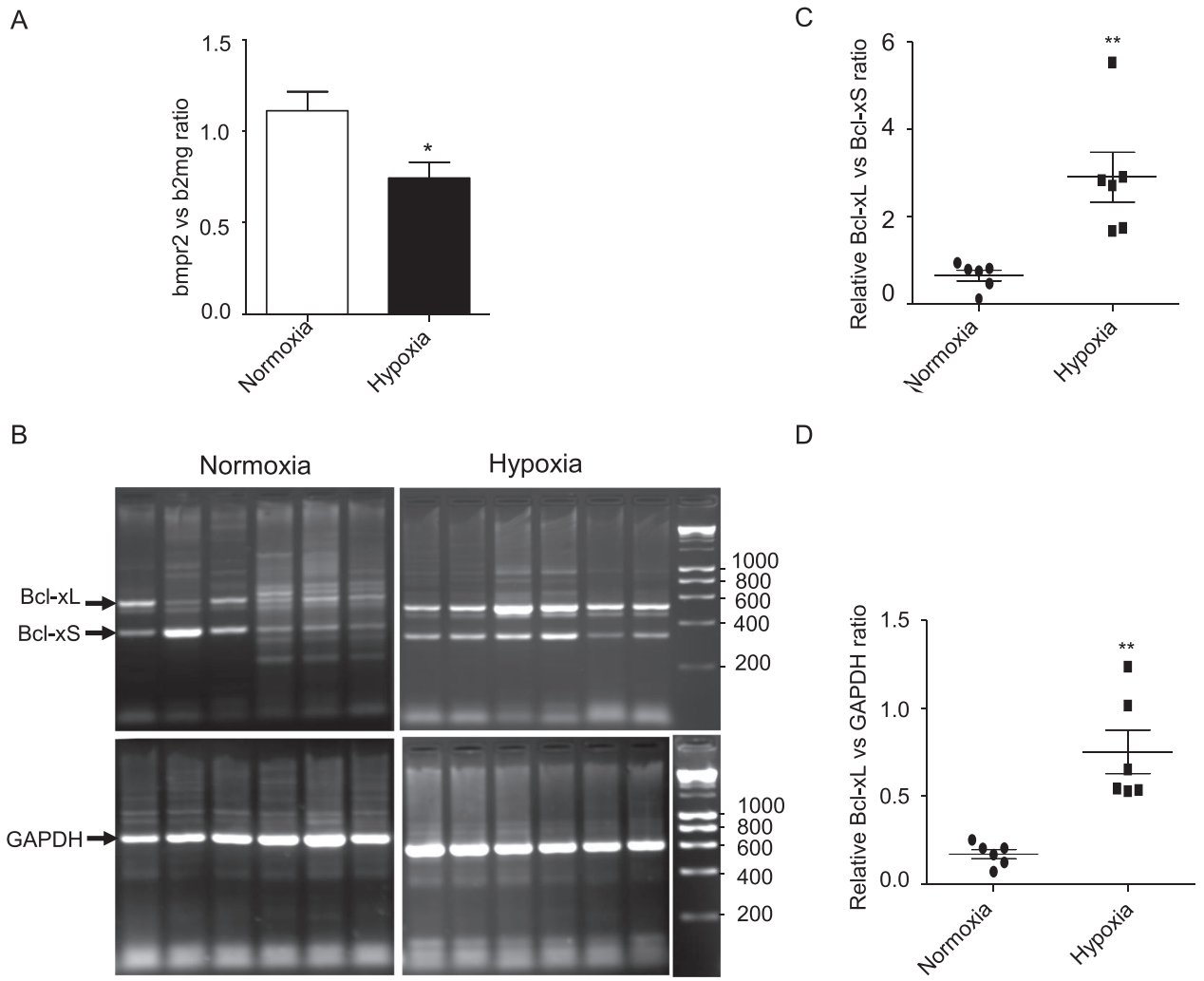

Figure 2. Determination of the alternative splicing of Bcl-x pre-mRNA in hypoxic PAH rat lungs. (**A**) Quantification of the *BMPR2* transcript relative to β-2-microglobulin (b2mg) in hypoxic rat lungs by quantitative PCR. (**B**) As in Figure 1D, with lung samples derived from normoxic and hypoxic rats ($n = 6$). (**C**) Quantification of the Bcl-xL versus Bcl-xS ratio of RT-PCR. (**D**) Quantification of the relative Bcl-xL versus GAPDH ratio of RT-PCR. $^{**}P < 0.01$ compared to normoxia.

derived from knock-in (K-I) mice harbouring the PAH-associated *BMPR2* nonsense mutation (p.R899X). These mice were asymptomatic at the age of 3 months but developed age-related PAH at the age of 6 months (4). PASMCs were isolated from asymptomatic wild-type (*bmpr2* $^{+/+}$) and mutant (*bmpr2* $^{R899X+/-}$) mice and the rate of apoptosis was determined by measuring cysteinyl aspartate proteases (caspase) activity using commercially available kits (Promega) following manufacturer's instructions. Consistent with our previous observations, the basal activity level of caspases 3 and 7 was attenuated in mutant cells compared to wild-type (Supplementary Material, Fig. S1) (17). We earlier demonstrated that staurosporine (ST), a known inducer of apoptosis, showed differential effects, with ST-induced caspase activity seemingly greater in mutant cells than wild type (17). We further characterized the pro-apoptotic effect of BMP signalling on ST-induced apoptosis by stimulating the cells with BMP9 ligand (Fig. 3D). In wild-type cells, the caspase activity was further increased following ligand stimulation, which is consistent with the previous finding of BMP-dependent caspase activation (21). The pro-apoptotic effect of BMP stimulation was absent in BMPRII-deficient cells. In these cells, BMP9 inhibited ST-induced cell death in a dose-dependent manner (Fig. 3D).

Selective inhibition of Bcl-xL by a chemical agent induces apoptosis in PAH-PASMCs

We hypothesized that BMPRII dysfunction potentiates the apoptotic-resistant phenotype through the upregulation of Bcl-xL and that selective inhibition of Bcl-xL induces apoptosis (Fig. 3E). We used PAH-PASMCs harbouring the *BMPR2* (p.R899X) mutation as these cells showed reduced level of apoptosis compared to wild type (17). PASMCs were treated with 2,3-DCPE hydrochloride for 48 h and the caspase activity was determined. The compound significantly increased apoptosis in PAH-PASMCs in a concentration-dependent manner (Fig. 3F). As expected, it also induced apoptosis in wild-type PASMCs.

BMPRII-mediated signalling promotes the expression of Bcl-xL transcripts in PAECs and HEK293T cells

Having characterized the expression of Bcl-xL transcripts in PASMCs, we then determined Bcl-x splicing in PAECs following knock-down of either BMPRII or ALK1 receptors. siRNAs targeting either the *BMPR2* or *ALK1* gene significantly inhibited the expression of Bcl-xL transcripts (Fig. 4A–B). qPCR

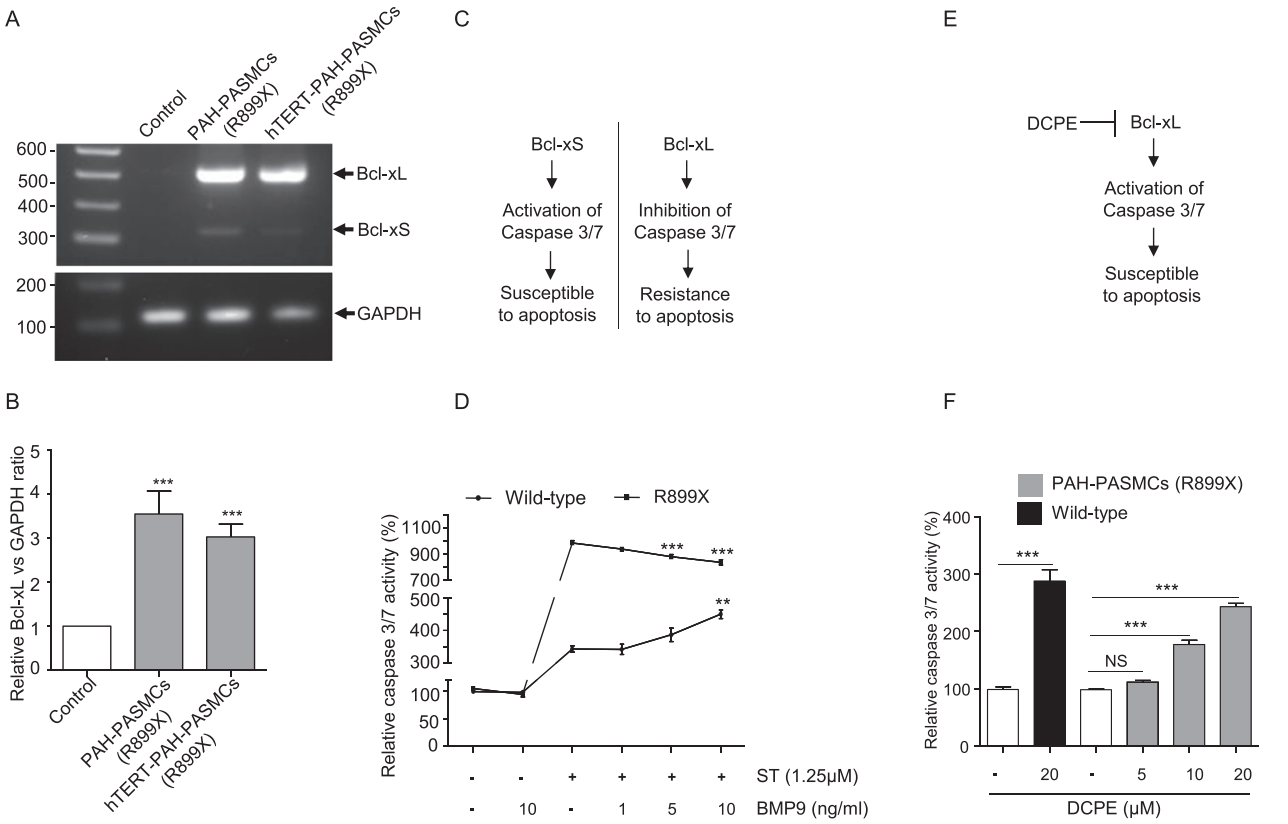

Figure 3. *BMPR2* mutation impairs apoptosis through upregulating the expression of Bcl-xL transcripts in PASMCs. (**A**) Determination of the alternative splicing of Bcl-x pre-mRNA in human PASMCs. RT-PCR analysis of Bcl-x transcripts in human PASMCs from a PAH subject harbouring the *BMPR2* mutation (p.R899X). The hTERT-PAH-PASMCs are the p.R899X-PAH PASMCs that were immortalized with the expression of catalytic subunit of hTERT gene (Nasim *et al.*, unpublished data). (**B**) Quantification of RT-PCR ($n = 4$). (**C**) Diagram depicting the roles of Bcl-xL and Bcl-xS isoforms in apoptosis. (**D**) Differential effects of BMP9 ligand on apoptosis as determined by caspase 3/7 activities in PASMCs derived from wild-type and mutant mice harbouring the pathogenic *BMPR2* mutation (p.R899X) ($n = 6$). Apoptosis was induced by the established chemical ST (1.25 μM). $^{**}P < 0.01$, $^{***}P < 0.001$ compared to cells treated with ST only. (**E**) Diagram depicting the role 2,3-DCPE hydrochloride (TOCRIS), which selectively inhibits the activity of Bcl-xL isoforms on apoptosis. (**F**) Selected inhibition of Bcl-xL by 2,3-DCPE compound induces apoptosis in PAH-PASMCs in a dose-dependent manner ($n = 4$). Concentrations of the BMP9 ligand and DCPE compound are indicated below graphs. $^{***}P < 0.001$ compared to untreated cells. Data are presented as mean ± SEM from three to six independent experiments.

analyses of *BMPR2* and *ALK1* transcripts showed reduced level of these transcripts in cells transfected with siRNAs compared to that of controls (Supplementary Material, Fig. S2A–B). Of note, little or no expression of Bcl-xS transcripts was observed in PAECs. Next, we determined the effect of BMPRII overexpression on Bcl-x expression. For this experiment, we selected the human embryonic kidney (HEK293T) cells as the transfection efficiency is higher in these cells compared to PASMCs and PAECs. Cells were co-transfected with a plasmid harbouring the wild-type BMPRII receptor. We found that the expression of Bcl-xL transcripts was increased in cells overexpressed with the BMPRII receptor compared to the untreated control (Fig. 4C–D). While we found that HEK293T cells treated with BMP9 ligand (10 ng/ml) increased the level of Bcl-xL transcripts, the ligand elicited no discernible effect on the preferential expression of Bcl-x in PAECs (Supplementary Material, Fig. S3A–B).

Knock-down of either BMPRII or ALK1 promotes endothelial cell apoptosis

As BMPRII-mediated signalling has been shown to protect human PAECs from apoptosis (20), we used siRNAs against either BMPRII or ALK1 and determined their effect on endothelial cell apoptosis by measuring caspase 3/7 activities. Knock-down of either BMPRII or ALK1 increased caspase 3/7 activities, indicating pro-survival roles of BMP signalling in PAECs (Fig. 4E).

BMPR2 mutations found in PAH patients reduce pro-survival effects

We were interested to investigate the effect of a wide range of mutations identified in PAH patients on pro-survival activity. To investigate this, we employed HEK293T cells as in these cells BMPRII-mediated signalling increased the expression of pro-survival Bcl-xL isoform. The pro-survival effect of BMPRII-mediated signalling was investigated by transfecting HEK293T cells with plasmids harbouring either BMPRII wild type or a wide range of mutant receptors (Fig. 4F). Cell viability was measured using Cell-Titre Glo Cell Viability Assay Kit (Promega) following manufacturer's instructions. Cell viability was increased in cells transfected with the wild-type receptor suggesting a pro-survival role of BMPRII-mediated signalling in HEK293T cells (Fig. 4G). In contrast, the cell viability was significantly reduced in cells transfected with the mutant p.C118W, p.D485G, p.N51K, p.S532X, pR899X and p.R899P plasmids, indicating that the pro-survival effects of BMPRII receptor are greatly reduced in

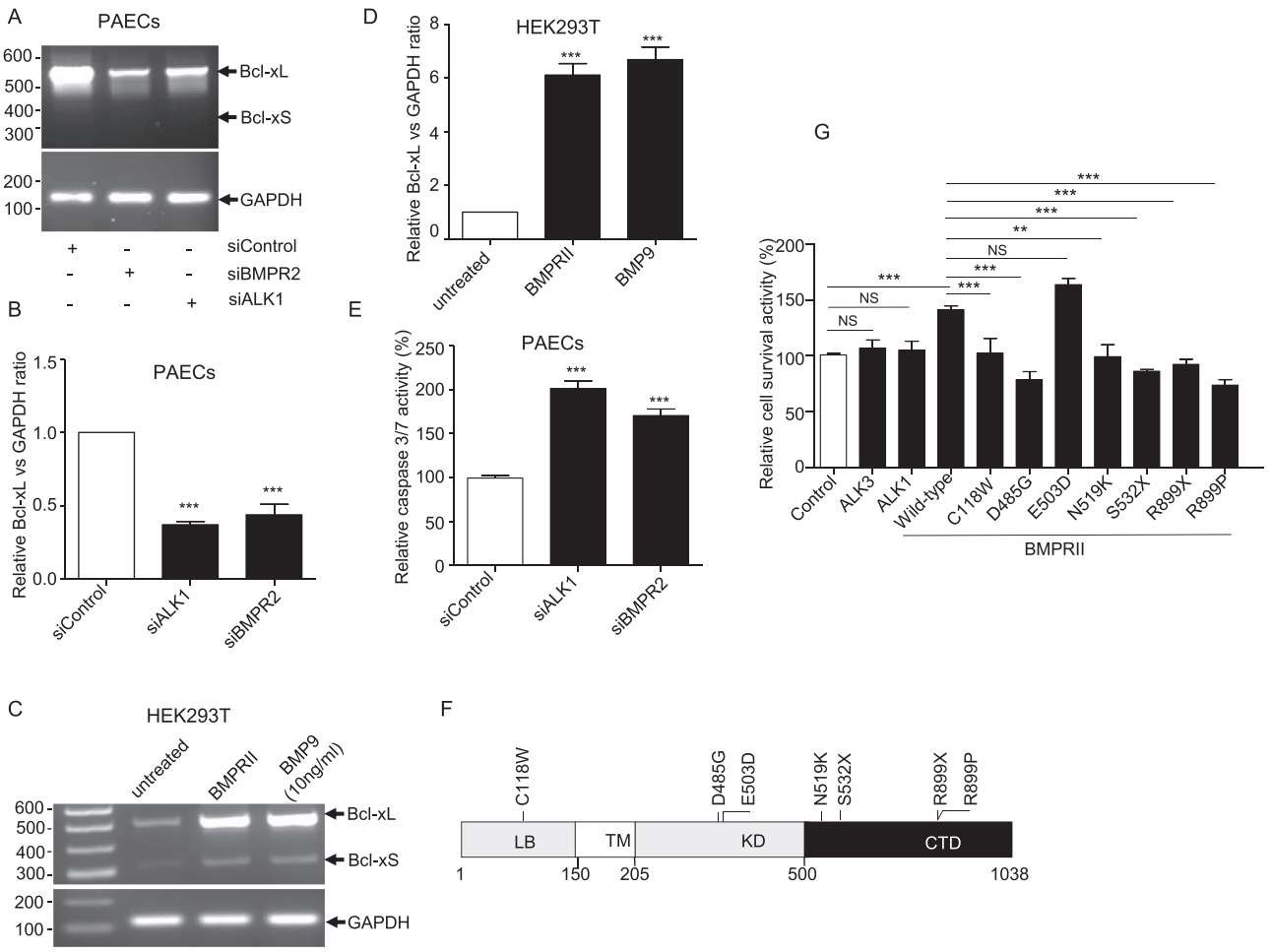

Figure 4. Pro-survival effects of BMPRII are mediated through Bcl-xL and pathogenic *BMPR2* mutations impair the pro-survival activity. (**A**) Either BMPRII or ALK1 downregulates the expression of Bcl-xL transcripts in PAECs. RT-PCR analysis of Bcl-x transcripts in human PAECs transfected with 30 nM of siRNAs targeting either the *BMPR2* or *ALK1* gene. Cells were harvested 48 h after siRNA transfection. (**B**) Quantification of RT-PCR ($n = 4$). (**C**) RT-PCR analysis of Bcl-x transcripts in HEK293T cells either transfected with a construct containing the *BMPR2* gene or stimulated with BMP9 (10 ng/ml) ligand. (**D**) Quantification of RT-PCR ($n = 4$). (**E**) Either BMPRII or ALK1 deficiency by means of siRNA knock-down promotes apoptosis in PAECs as determined by caspase 3/7 activities ($n = 9$). Cells were treated with siRNAs for 48 h. (**F**) Diagram of the BMPRII domain structure depicting the ligand binding (LB), transmembrane (TM), kinase (KD) and C-terminal extension (CTD). The numbers below indicate relative amino acids, while the mutations tested are indicated above. (**G**) Mutations in the *BMPR2* gene identified in PAH cases impair cell survival as determined by the Cell-titre Glow Cell Viability Assay (Promega) ($n = 8$). HEK293T cells were transfected either with the wild-type or mutant BMPRII receptors, ALK1 or ALK3 receptor and the relative cell viability was determined. The value derived from untreated control cells was set as 100. $^{***}P < 0.001$ compared to mock transfected cells, NS-non-significant. Data are presented as mean ± SEM from three to six independent experiments.

the presence of pathogenic mutations (Fig. 4G). However, cells transfected with the p.E503D mutant showed no discernible effects. Of note, we previously demonstrated that this mutation was capable of mediating BMP signalling at the level comparable to the wild-type receptor (5). Collectively, these data suggest that *BMPR2* dysfunction elicits cell-specific effects. The receptor dysfunction induces anti-apoptotic effects in PASMCs while it exerts anti-survival effects in PAECs and HEK293 cells.

BMP signalling inhibits caspase 3/7 activities via the ALK1 receptor

Having identified pro-survival effects of BMPRII-mediated signalling in both endothelial and HEK293T cells, we were keen to investigate the underlying mechanisms by which BMPRII regulates anti-apoptotic effects (Fig. 5A). First, we investigated the effect of BMP signalling on apoptosis. Briefly, HEK293T cells were stimulated with BMP4 ligand (10 ng/ml) overnight in the presence of ST and the activities of caspase 3/7 activities were determined. We found that BMP4 stimulation reduced ST-induced caspase activities (Fig. 5B). Next, the BMP signalling promoting activity of BMP4 ligand and BMPRII receptor was tested using the BMP-responsive 3GC2-Lux reporter assay (22) as previously described by us (5). HEK293T cells were stimulated with the ligand (10 ng/ml) at various time points (30 min, 1 h, 3 h, 5 h, 7 h, 9 h and 24 h) with and without BMPRII overexpression and their luciferase and β-galactosidase activities were determined. Reporter activation was observed after 7 h of ligand stimulation and the highest activity was observed at the 24-h time point (Supplementary Material, Fig. S4A–C). Together, these results indicate that the attenuation of caspase activities observed in these cells might be due to the activation of BMP signalling.

Next, we tested whether the anti-caspase effect of BMP signalling is mediated through the ALK3, ALK1 and BMPRII receptors. Overexpression of either BMPRII or type I receptors ALK3 and ALK1 individually has no discernible effect on basal caspase activation (Fig. 5C). Interestingly, co-expression

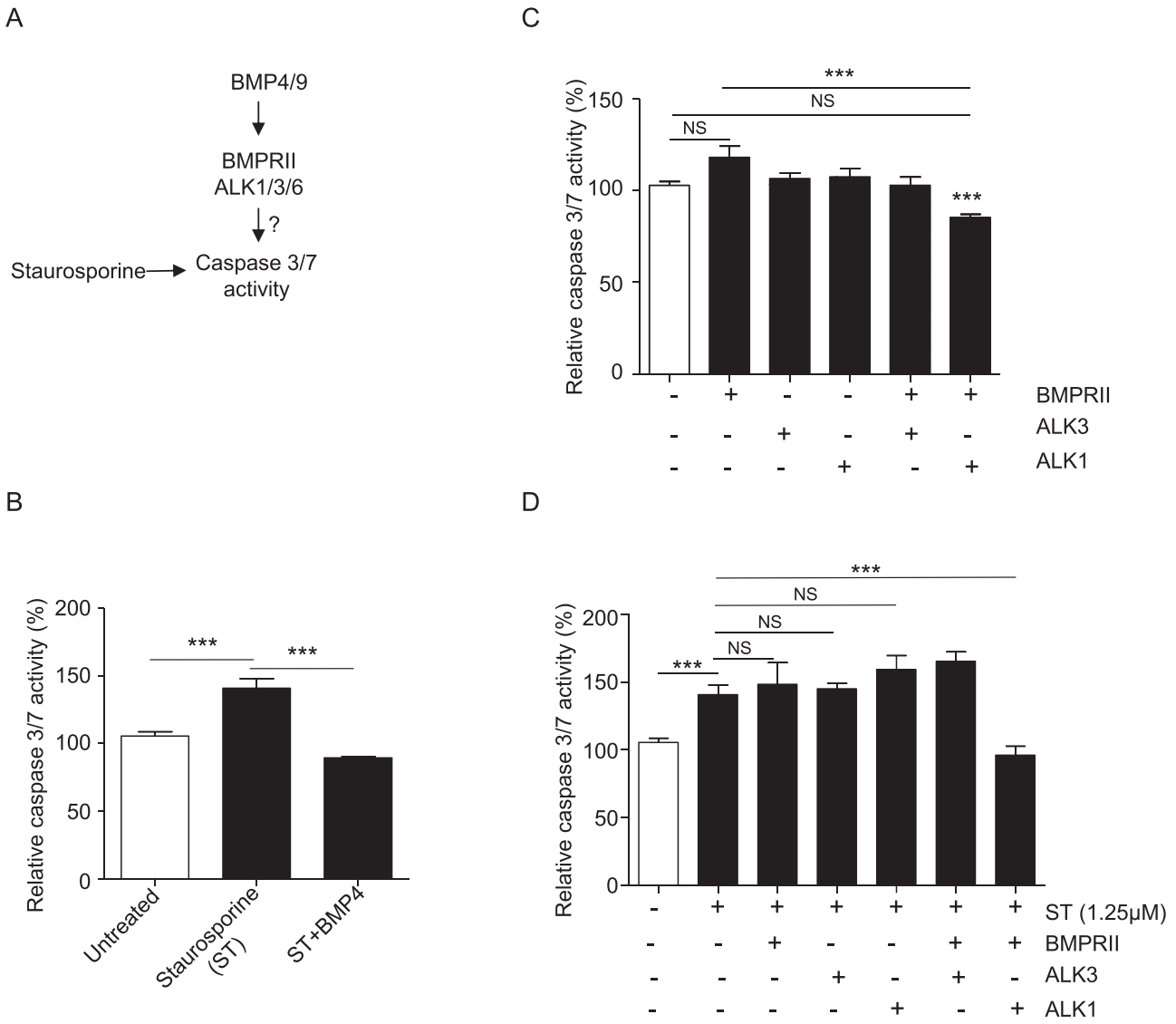

Figure 5. BMP signalling inhibits apoptosis via the BMPRII-ALK1-mediated pathway. (**A**) Diagram depicting the effect of BMP signalling cascade on caspase 3/7 activities. (**B**) BMP signalling inhibits apoptosis in HEK293T cells ($n = 4$) as determined by the Caspase-Glo 3/7 Assay (Promega). Cells treated with apoptosis-inducing agent ST (1.25 μM) and stimulated with BMP4 ligand (10 ng/ml) for 16 h. BMPRII receptor inhibits both basal (**C**) and (**D**) ST-induced apoptosis through the ALK1 receptor ($n = 4$). HEK293T cells transfected with either BMPRII, ALK1 and ALK3 receptors alone or BMPRII in combination with ALK1 and ALK3 receptors. Apoptosis was induced by treating the cells with ST (1.25 μM) for 16 h (D). ***$P < 0.001$ compared to untreated (B), BMPR-II overexpressed (C) and ST-induced (D) cells. Data are presented as mean ± SEM from three to six independent experiments.

of ALK1 and BMPRII significantly inhibits both basal and ST-induced caspase activity HEK293T cells (Fig. 5C–D and Supplementary Material, Fig. S5).

Mutations in either ALK1 or BMPRII receptor impair the BMPRII-ALK1 pathway

Since ALK1 has been shown to form a complex with BMPRII (23), we investigated whether both receptors work synergistically to regulate BMP signalling pathway. To do this, we used the BMP-responsive 3GC2-Lux reporter assay system to monitor BMP signalling (22). Overexpression of BMPRII alone and co-expression of BMPRII with ALK3 synergistically elevated the basal level of luciferase activity, which was consistent with our previous observations (5) (Fig. 6A). Co-expression of BMPRII and ALK1 receptors generated reporter activity to a level comparable to that achieved by co-expression of BMPRII and ALK3 receptors, indicating that both BMPRII and ALK1 regulate BMP signalling in a synergistic manner (Fig. 6A).

Having demonstrated that the BMPRII and ALK1 receptors work synergistically to activate the BMP signalling pathway, we then investigated the effects of ligands including BMP4 and BMP9 on BMPRII receptor (Fig. 6B). We found that HEK293T cells co-expressing BMPRII and stimulated with either BMP4 or BMP9 showed increased reporter activation compared to cells expressing BMPRII alone (Fig. 6B). Furthermore, we found BMP9 but not BMP4 ligand significantly increased ALK1-BMPRII-mediated reporter activation compared to cells transfected with BMPRII and stimulated with either BMP4 or BMP9 ligand (Supplementary Material, Fig. S6A–B). We previously showed that mutations in the *BMPR2* gene significantly reduced BMPRII-ALK3-mediated reporter activation. Among the mutations

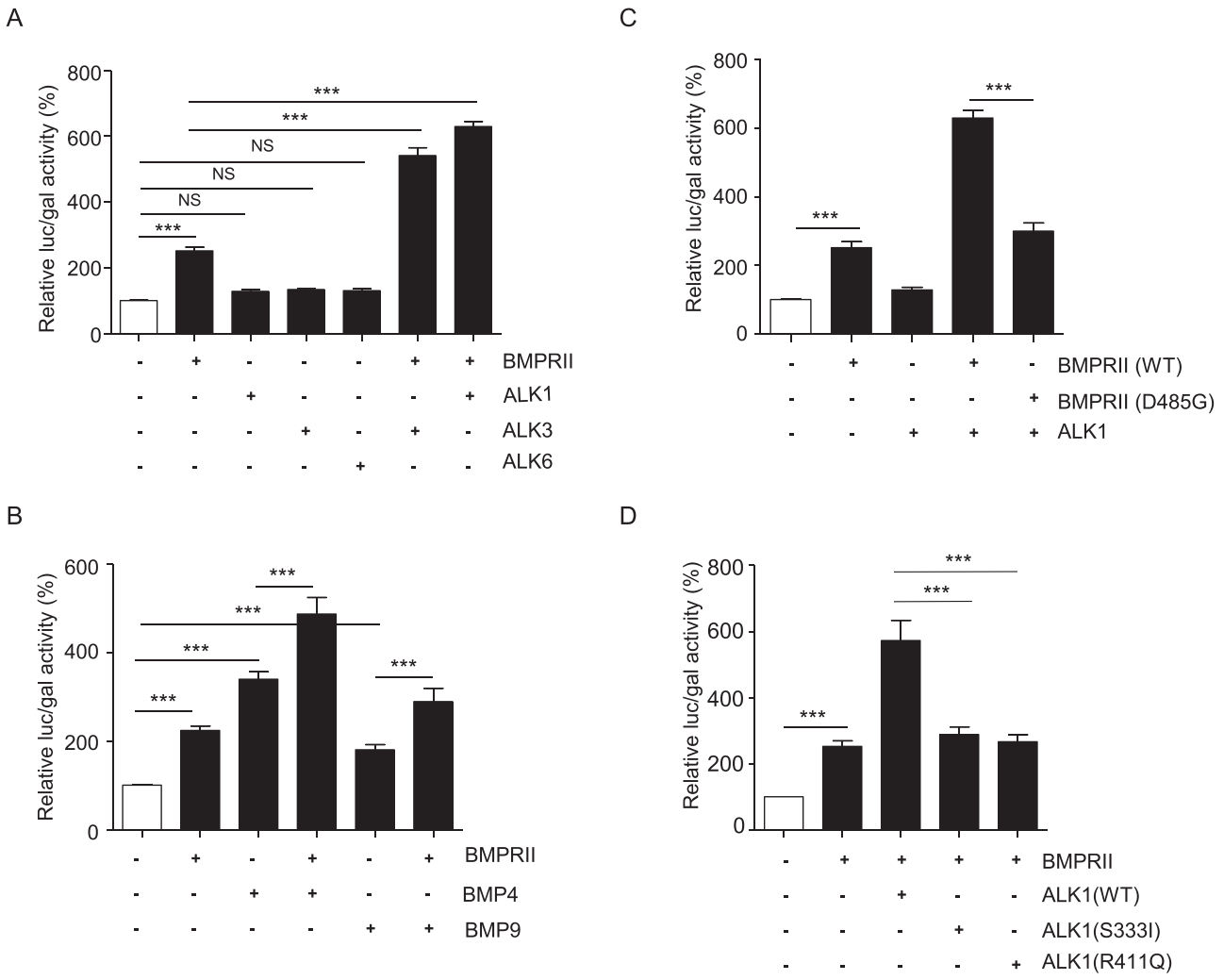

Figure 6. Mutations in either BMPRII or ALK1 receptors impair the BMPRII-ALK1-mediated signalling. (**A**) The efficiency of BMPRII-ALK1 signalling is comparable to that of BMPRII-ALK3 as determined by the BMP-responsive 3GC2-Lux reporter assay ($n = 8$). HEK293T cells transfected with the reporter plasmids together with BMPRII, ALK1, ALK3 and ALK6 receptors. (**B**) Promotion of BMPRII-mediated signalling by BMP4 and BMP9 ligands. HEK293T cells transfected with the reporter plasmids together with BMPRII and stimulated with either BMP4 (10 ng/ml) or BMP9 (10 ng/ml) ligands for overnight ($n = 8$). (**C**) Mutation in the kinase domain of BMPRII receptor (p.D485G) significantly reduced BMPRII-ALK1-mediated reporter activation. HEK293T cells transfected in combination with either wild-type or mutant BMPRII receptor with and without ALK1 ($n = 8$). (**D**) As (C), cells transfected in combination with wild-type and mutant ALK1 receptor with and without BMPRII ($n = 6$). The luc-gal activity of the untreated control cells was set as 100. Data are presented as mean ± SEM from three to six independent experiments. ***$P < 0.001$ compared to either untreated or as indicated.

investigated, the p.D485G mutant was unable to interact with either ALK3 or ALK6 receptor and failed to activate the BMP-responsive reporter in the absence and presence of ALK3 and ALK6 receptor overexpression and BMP4 ligand stimulation (5). In this study, we found that in the presence of this mutation, BMPRII-ALK1-mediated reporter activation was also significantly reduced both in the absence and presence of BMP9 stimulation (Fig. 6C and Supplementary Material, Fig. S7A). Similarly, while testing the effect of ALK1 mutations (p.S333I and p.R111Q) identified in hereditary haemorrhagic telangiectasia patients (24–26), we found that BMPRII-ALK1-mediated reporter activation was significantly reduced (Fig. 6D). Furthermore, BMP9-stimulated reporter activation was significantly reduced in cells overexpressing ALK1 mutations (p. D179A, p.S333I and p.R111) compared to the wild-type receptor (Supplementary Material, Fig. S7B). Collectively, these data suggest that mutations in either the BMPRII or ALK1 receptor impinge upon the BMPRII-ALK1 pathway.

Mutations found in the *BMPR2* gene identified in PAH cases impair BMPRII-ALK1 interactions

We previously showed that the BMPRII receptor interacts with both ALK3 and ALK6 receptors and pathogenic *BMPR2* mutations impair these interactions (17). In this study, we first investigated whether BMPRII interacted with the ALK1 receptor and then determined the effect of *BMPR2* mutations on BMPRII-ALK1 interactions. For this investigation, we employed our previously established cell-based protein–protein interactions assay, which was successfully used for determining the efficiency of interactions of BMPRII with ALK3 and ALK6 receptors (5,27). The assay system was based on the mammalian version of yeast two hybrid screen (27). Briefly, the activation domain (AD) was fused with the intracellular part of the BMPRII receptor, while the ALK1 lacking the extracellular domain was fused with DNA-binding domain (DBD). The dual reporter was co-transfected with both the ALK1-DBD and BMPRII-AD. In the event of no

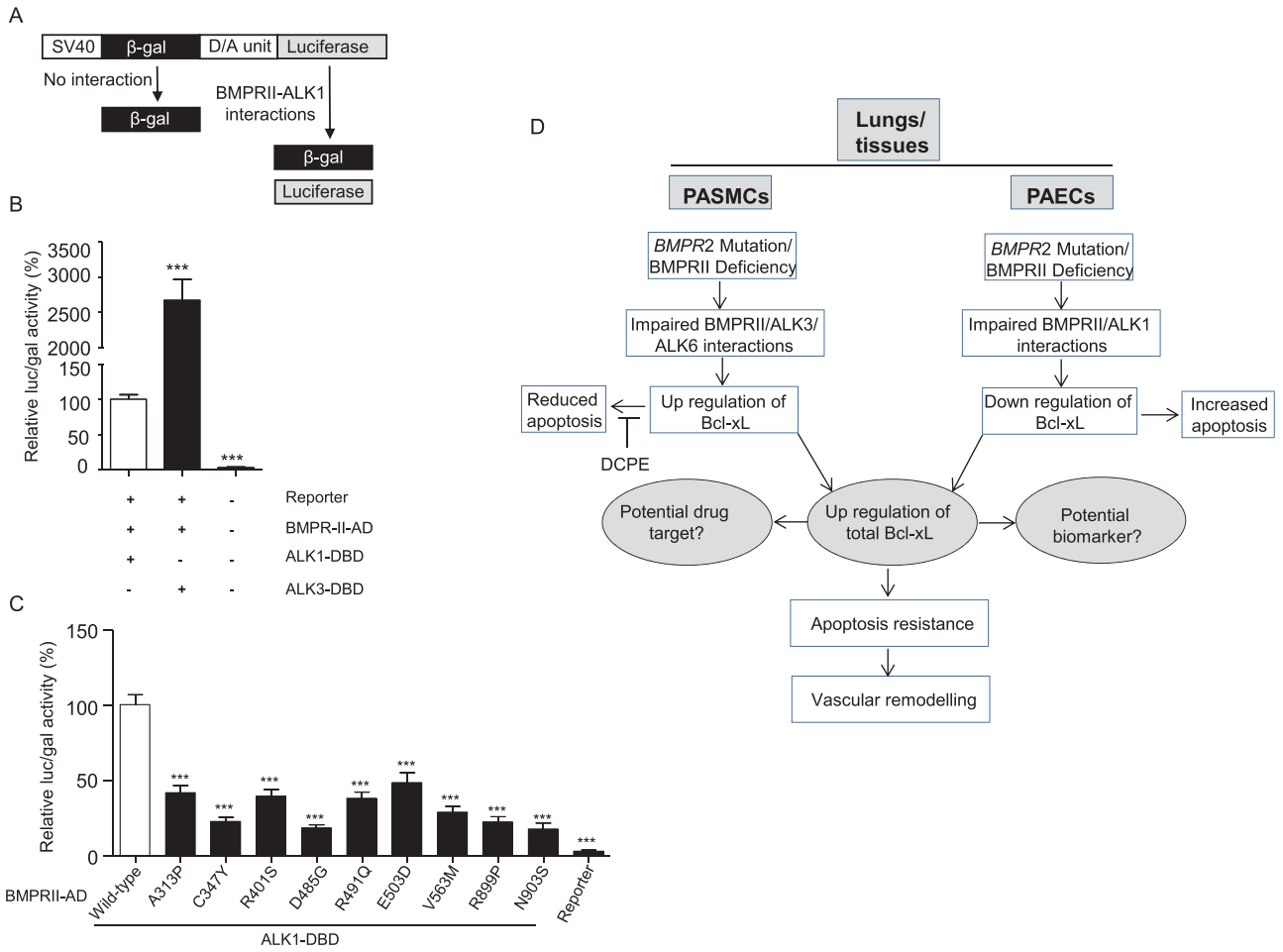

Figure 7. Interaction of ALK1 and BMPRII determined by mammalian two-hybrid protein–protein interaction assay (27). (**A**) Outline of the BMPRII-ALK1 interactions assay, which is based on our previously established mammalian version of yeast two-hybrid screen (27). The assay was developed such that in the event of no interaction, only β-gal protein will be produced. An interaction between BMPRII and ALK1 generates both β-gal and luciferase proteins. (**B**) The efficiency of the interaction of BMPR-II with either ALK3 or ALK1. HEK293T cells transfected with plasmids encoding BMPRII fused to the activation domain (BMPRII-AD) and ALK1 fused to the DNA-binding domain (ALK1-DBD) compared to reporter alone (TN114). The efficiency of the interaction between BMPRII and ALK1 was set as 100 ($n = 8$). (**C**) Mutations in the *BMPR2* gene identified in both familial and sporadic PAH cases significantly reduce BMPRII-ALK1 interactions ($n = 10$). As (B), cells were co-transfected in combination with either wild-type or mutant BMPR-II receptor together with ALK1-DBD and pTN114 reporter. The relative luc-gal ratio in cells overexpressing mutant BMPRII and ALK1 compared to the wild-type BMPRII receptor. Data are presented as mean ± SEM from three to six independent experiments. SV40, SV40 promoter; D/A unit, deactivation/activation unit for downstream transcription (27). ***$P < 0.001$ compared to reporter transfected with BMPR-II-AD and ALK1-DBD plasmids. AD indicates activation domain; DBD, DNA-binding domain. (**D**) Model depicting the regulation of apoptosis resistance in PAH. In PASMCs, either *BMPR2* mutations or BMPRII receptor deficiency potentiate the expression of Bcl-xL transcripts leading to apoptosis resistance. In contrast, in PAECs, the receptor deficiency reduces the Bcl-xL expression leading to anti-survival effects. The pro-survival phenotype of PAECs is mediated via the BMPRII and ALK1 receptors. Both ALK1 and ALK3 receptors work synergistically with the BMPRII and in the event of pathogenic *BMPR2* mutations, the efficiency of interactions of BMPRII with type I receptors is greatly reduced (5,17), which increases the susceptibility of PASMCs and PAECs to undergo impaired apoptotic pathway but not sufficient to trigger PAH. The presence of an additional stimulus may further exacerbate the apoptosis-resistance process leading to vascular remodelling. Thus, the Bcl-x gene may represent a potential biomarker and druggable target for PAH. Straight arrows indicate activation; T-shaped line indicates inhibition.

interaction, the cells generate only the upstream β-galactosidase protein (Fig. 7A). In the event of BMPRII-ALK1 interactions, both luciferase and β-galactosidase activities are generated.

Co-expression of BMPRII and ALK1 constructs generated both luciferase and β-galactosidase read outs indicating an interaction between these two proteins (Fig. 7B). However, we found that the efficiency of interactions of BMPRII with ALK1 was 26-fold weaker compared to the interactions between BMPRII and ALK3 receptors. We next investigated the effects of a wide range of *BMPR2* mutations found in patients suffering from either PAH or congenital heart defects on BMPRII-ALK1 interactions. All mutations tested significantly impaired the efficiency of interactions between ALK1 and BMPRII proteins (Fig. 7C).

Discussion

The vascular remodelling observed in PAH lungs is caused by abnormal apoptosis of PASMCs and PAECs, but the underlying mechanisms by which apoptosis is controlled in PAH have remained elusive. Here, we report a novel mechanism that demonstrates that the dysregulated apoptosis in PAH is controlled via the BMPRII-Bcl-xL axis.

This study shows in a number of ways that *BMPR2* haploinsufficiency modulates the preferential expression of the anti-apoptotic Bcl-xL over the pro-apoptotic Bcl-xS transcripts both in PAH patients and in an animal model. First, we found preferential expression of the anti-apoptotic Bcl-xL transcripts

in circulating leukocytes of PAH patients with and without *BMPR2* mutations. Second, the increased expression of Bcl-xL transcripts was also observed in the lungs of a PAH patient who had undergone combined heart and lung transplantation. The *BMPR2* mutations included in this study are nonsense, insertion and deletion mutations, and hence transcripts harbouring these alleles are likely to be degraded through the NMD pathway, creating a state of haploinsufficiency (5). Finally, in the chronic hypoxic rat lungs that showed a diminished level of *BMPR2* transcripts, the ratios of Bcl-xL to Bcl-xS and Bcl-xL to GAPDH were increased. Consistent with our observations, elevated levels of Bcl-xL protein were also found in pulmonary artery of chronic hypoxia–treated rats with vascular remodelling (28).

Many signalling pathways such as p38MAPK, ERK1/2, JAK/-STAT, TNF, NF-κB and PI3K are activated and/or dysregulated in PAH patients. Previous observations suggest that each of these pathways is capable of contributing to the preferential expression of Bcl-xL transcripts. For example, ERK1/2 activates the p90RSK, which potentiates the transcription factor CREB and induces the expression of Bcl-xL and Bcl-2 transcripts (29,30). Additionally, ECs derived from idiopathic PAH patients show elevated levels of phosphorylated ERK1/2 and Bcl-xL expression leading to apoptosis resistance (31). TNF-α has been shown to activate both pro-apoptotic and anti-apoptotic pathways. While it induces apoptosis by activating caspase-8 and -10, its anti-apoptotic activity is mediated via NF-κB, which induces the expression of anti-apoptotic Bcl-2 family of proteins (32–34). The PI3K/AKT pathway inhibits the expression of Bcl-xL transcripts by inhibiting the activation of FoxO transcription factor (35,36). These observations suggest that regulation of Bcl-xL transcripts may also occur through a process that is independent of *BMPR2* mutation.

Having established that the expression of Bcl-xL transcripts increased *in vivo*, we then proved that BMPRII controls the expression of Bcl-xL transcripts. Firstly, we showed that in PAH-PASMCs harbouring the pathogenic *BMPR2* (p.R899X) mutation, the expression of anti-apoptotic Bcl-xL transcripts was greatly increased, indicating that BMPRII dysfunction might potentiate resistance to apoptosis. Secondly, siRNA knock-down of either the *BMPR2* or *ALK1* genes inhibited the expression of Bcl-xL in PAECs. Finally, overexpression of either BMPRII receptor or BMP9 stimulation increased the expression of Bcl-xL in HEK293T cells. These observations suggest cell-specific effects of BMPRII dysfunction on the preferential expression of Bcl-xL transcripts. BMPRII deficiency promotes the expression of Bcl-xL transcripts in PASMCs, while inhibits it in ECs. Thus, the increased expression of Bcl-xL transcripts may contribute to differential apoptotic effects in these cells.

Consistent with previous observations, we found that dysfunctional BMPRII signalling exerted anti-apoptotic phenotype in PASMCs while deficiency of this receptor led to apoptosis in PAECs (4,17,23). We observed that overexpression of BMPRII receptor promoted cell survival and this effect was greatly reduced in the presence of pathogenic *BMPR2* mutations. We provided extensive evidence that the pro-survival effect of BMPRII signalling was mediated through the ALK1 receptor and that these two receptors worked synergistically. Firstly, caspase activity was greatly reduced following co-expression of BMPRII with ALK1 but not with ALK3 receptor; activation of the BMP9/ALK1/BMPRII axis in endothelial cells showed anti-apoptotic phenotypes (4,37). Secondly, cells co-expressed with BMPRII and ALK1 promoted BMP-responsive reporter activity and mutation either in BMPRII or in ALK1 impaired the reporter activation. Finally, we demonstrated that BMPRII interacted with the ALK1 receptor and in the event of pathogenic *BMPR2* mutations, the efficiency of this interaction was greatly reduced. These observations support that stoichiometric imbalance in the BMPRII-ALK1 receptor complex may contribute to dysfunctional signalling leading to impaired apoptosis (5).

The involvement of anti-apoptotic Bcl-xL protein in developing apoptosis resistance in many cancers is well documented, but the underlying mechanisms by which preferential expression of the Bcl-xL transcripts is regulated in PAH are not known. Based on our observations, we propose that either *BMPR2* mutation or BMPRII receptor deficiency potentiates the expression of Bcl-xL transcripts leading to apoptosis resistance in PASMCs (Fig. 7D). In PAECs, BMPRII deficiency reduces the Bcl-xL expression leading to anti-survival effects. The pro-survival phenotype of PAECs is mediated via the BMPRII and ALK1 receptors. The ALK1 receptor works synergistically with the BMPRII and in the event of pathogenic *BMPR2* mutations, the BMPRII-ALK1-mediated signalling is greatly reduced, which increases the susceptibility of endothelial cells to undergo apoptosis but not sufficient to trigger PAH. A critical reduction in BMPRII-mediated signalling or the presence of an additional stimulus may trigger apoptosis resistance of PASMCs leading to vascular remodelling.

Currently, neither any cure nor biomarker for PAH is known. Antisense RNAs, small molecule agents such as fluoxetine and sodium nitroprusside and established drugs including ABT-263 (Navitoclax), reversed vascular remodelling in experimental models of PAH and adult T cell leukaemia (38,39). These agents reduced the level of Bcl-xL expression indicating the potential of targeting this protein for therapeutic intervention. Although increased Bcl-xL expression was observed in preclinical and PAH cases, the major limitation to developing Bcl-xL as a potential biomarker is that only a small number of patient samples were investigated in this study. Further epidemiological studies including various classes of PAH patients matched with healthy subjects together with analytical and clinical validations are required to determine whether Bcl-xL can be used as a novel biomarker in PAH.

In summary, we have demonstrated that BMPRII deficiency impairs apoptosis by modulating the alternative splicing of Bcl-x transcripts, a finding observed in circulating leukocytes, lungs of PAH patients, hypoxic PAH rat lungs as well as in disease-relevant human PASMCs and PAECs. While BMPRII deficiency elicits anti-apoptotic effects in PASMCs, the receptor dysfunction induces pro-apoptotic responses in PAECs. The pro-survival effects of BMPRII are mediated through the ALK1 but not the ALK3 receptor in PAECs/HEK293T cells. BMPRII interacts with the ALK1 receptor and in the event of pathogenic *BMPR2* mutations, this interaction is greatly reduced resulting in impaired signalling events. We propose that assessing Bcl-x transcripts in patient samples offers the opportunity for developing a novel biomarker and that targeting the BMPRII-Bcl-xL axis may provide a novel therapeutic intervention in PAH.

Materials and Methods

Patient and control populations

Ethical approval for these studies was obtained from Papworth Hospital ethical review committee (Ethics Ref 08-H0304–56 + 5) and Trent Multi-Centre Research Ethics Committee (MREC/02/4/003) for the human tissues used and patients gave the written informed consents. Control samples are healthy individuals, all of which have no mutations in the *BMPR2* gene.

Human lung tissues are from a PAH subject harbouring a *BMPR2* mutation (p.835X) who underwent combined heart and lung transplantation. The control human lung RNAs are from a commercially available source (Clontech Laboratories, Inc.).

Isolation of PASMCs

Isolation of PASMCs was described elsewhere (40,41). PAH-PASMCs were isolated from the proximal pulmonary arterial vessel segment (5–8 mm diameter) obtained from the lung sample of an HPAH patient with a pathogenic *BMPR2* mutation (p.R899X) undergoing lung transplantation. PASMCs of wild-type (*bmpr2* $^{+/+}$) and K-I mice harbouring the PAH-associated *bmpr2* nonsense mutation (p.R899X) were derived from explants.

The luminal surface of the pulmonary artery was cut to open and endothelium was gently scrapped off using scalpel blade. The adjacent adventitia was stripped off and the medial explants were cut into 4–9 mm^2 sections. These segments were then plated into T25 cm^2 flasks and were allowed to adhere. Dulbecco's Modified Eagle's medium (DMEM) containing 20% Foetal bovine serum (FBS) and antibiotic (Penicillin/Streptomycin)–antimycotic (Amphotericin B) was added to the flasks after 2 h (40,41). Cells were grown to confluency and used between passages 4 and 10. The phenotype of the isolated cells was confirmed by immunostaining using antibody to smooth muscle-specific α-actin (Sigma, UK).

RNA isolation, cDNA synthesis and reverse transcriptase PCR

RNAs were isolated from mammalian cells and circulating leukocytes using either TRI-Reagent (Sigma) or RNeasy Purification Kit (Qiagen, UK). cDNAs were synthesized using random primers and MMLV Reverse Transcriptase (Promega, UK) following manufacturer's protocol. Total lung tissue RNA was extracted using the TRIzol method (Invitrogen, Thermo Fisher Scientific, UK). A total of 2-µg RNA was then reverse transcribed using Thermoscript reverse transcriptase PCR (RT-PCR) kits (Invitrogen, Thermo Fisher Scientific, UK). The PCR was carried out using Hi-Fidelity Extensor Master Mix (ABgene). Quantitative PCR for determining transcripts of *bmpr2* and β-2 *microglobulin* (β-2 mg) were performed using TaqMan Gene Expression Assay (Applied Biosystems, UK) on either 7900HT Fast Real-Time PCR system (Applied Biosystems, UK) or Step One Plus (Applied Biosystems, UK) according to the manufacturer's protocol.

Cell culture, transient transfection and enzymatic assay

Cell culture and transfections were carried out as described elsewhere (5,42,43). GeneJammer transfection reagent (Stratagene, Agilent Technologies, USA) was used for transfection of the plasmids. The amount of plasmid DNAs transfected into the HEK293 and HEK293T cell lines varied from assay to assay (from 30 ng to 1 µg). Twenty-four hours after transfection, cells were treated with compounds in DMEM containing 0.1% FBS for an additional 24 h. Cell lysate was prepared using 1X reporter lysis buffer (Promega, UK) as described earlier (17,18) and Luciferase and β-galactosidase activities were determined with the Dual-light Reporter Assay (Applied Biosystems, UK) using either an ORION-II or an ORION-L Plate Luminometer (Berthold Technologies, UK) according to the manufacturer's protocols.

Quantification of apoptosis and cell survival activity

To determine the rate of apoptosis in HEK293, PASMCs and ECs, cells were seeded at 0.2X10^5 per well in a white 96-well plate as described elsewhere (17,18). Twenty-four hours later, the medium was replaced by either fresh DMEM/0.1% FBS alone or containing BMP9 (1–10 ng/ml) (R&D Systems), BMP4 (10 ng/ml) (R&D Systems, UK) and 2,3-DCPE hydrochloride (5–20 µM) (TOCRIS, Bioscience, UK) and assays were carried out 24 h after the treatment. For induction of apoptosis, cells were treated with ST (TOCRIS, Bioscience, UK) for overnight at a concentration of 1.25 µM. Apoptosis assays were carried out using Caspase-Glo® 3/7 Assay (Promega, UK) following manufacturer's instruction. Luminescence was recorded using either an ORION-II or an ORION-L Plate Luminometer (Berthold Technologies, UK) at 30- and 60-min intervals. The cell survival activity was determined using CellTiter-Glo Cell Viability Assay (Promega, UK).

siRNA-mediated knock-down of *ALK1* and *BMPR2* genes

siGENOME SMART pool siRNAs (Dharmacon) were used to knock-down *ALK1* and *BMPR2* genes. The siGENOME SMARTpool is a mixture of four siRNAs, all designed to target different regions of the single gene of interest. PAECs were transfected with siRNAs either in 96-well plate (1×10^4) or 6-well plate (1×10^6) using Dharmafect 1 (Dharmacon, USA) following manufacturer's protocol and incubated for 72 h as previously described (43).

Generation of constructs for protein–protein interactions assay

The coding sequence of human ALK1 (accession number BC017715) lacking the first amino acid was cloned into BamHI and XbaI sites of pTN111 vector (27). The resulting plasmid contains an N-terminal DBD adjacent to the human ALK1, the fidelity of which was verified by restriction analyses and sequencing. Efficiency of protein–protein interactions between ALK1 and BMPRII in mammalian cells was determined as previously reported (https://www.nature.com/protocolexchange/protocols/261). In brief, the intracellular domain of BMPR2 was fused with VP-16 AD (5) while ALK1 was fused with a DBD. Plasmids encoding both fusion proteins were transfected into HEK293T cells together with the pTN114 dual-reporter plasmid (5). In the event of an interaction, both reporter proteins will be generated with the ratio of reporter activities producing a measure of the efficiency of protein–protein interactions.

Details of the other expression constructs used in this study are available upon request.

Statistics

Statistical analysis was performed following paired Student's *t*-test. Comparison of multiple means was carried out using analysis of variance followed by Tukey's *post hoc* test.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. Competing interests, a patent application has been filed.

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References

1. Morrell, N.W., Adnot, S., Archer, S.L., Dupuis, J., Jones, P.L., MacLean, M.R., McMurtry, I.F., Stenmark, K.R., Thistlethwaite, P.A., Weissmann, N. *et al.* (2009) Cellular and molecular basis of pulmonary arterial hypertension. *J. Am. Coll. Cardiol.*, **54**, S20–S31.
2. Gaine, S.P. and Rubin, L.J. (1998) Primary pulmonary hypertension. *Lancet*, **352**, 719–725.
3. Lane, K.B., Machado, R.D., Pauciulo, M.W., Thomson, J.R., Phillips, J.A. 3rd, Loyd, J.E., Nichols, W.C. and Trembath, R.C. (2000) Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. The International PPH Consortium. *Nat. Genet.*, **26**, 81–84.
4. Long, L., Ormiston, M.L., Yang, X., Southwood, M., Graf, S., Machado, R.D., Mueller, M., Kinzel, B., Yung, L.M., Wilkinson, J.M. *et al.* (2015) Selective enhancement of endothelial BMPR-II with BMP9 reverses pulmonary arterial hypertension. *Nat. Med.*, **21**, 777–785.
5. Nasim, M.T., Ghouri, A., Patel, B., James, V., Rudarakanchana, N., Morrell, N.W. and Trembath, R.C. (2008) Stoichiometric imbalance in the receptor complex contributes to dysfunctional BMPR-II mediated signalling in pulmonary arterial hypertension. *Hum. Mol. Genet.*, **17**, 1683–1694.
6. Nasim, M.T., Ogo, T., Ahmed, M., Randall, R., Chowdhury, H.M., Snape, K.M., Bradshaw, T.Y., Southgate, L., Lee, G.J., Jackson, I. *et al.* (2011) Molecular genetic characterization of SMAD signaling molecules in pulmonary arterial hypertension. *Hum. Mutat.*, **32**, 1385–1389.
7. Graf, S., Haimel, M., Bleda, M., Hadinnapola, C., Southgate, L., Li, W., Hodgson, J., Liu, B., Salmon, R.M., Southwood, M. *et al.* (2018) Identification of rare sequence variation underlying heritable pulmonary arterial hypertension. *Nat. Commun.*, **9**, 1416.
8. Hadinnapola, C., Bleda, M., Haimel, M., Screaton, N., Swift, A., Dorfmuller, P., Preston, S.D., Southwood, M., Hernandez-Sanchez, J., Martin, J. *et al.* (2017) Phenotypic characterization of EIF2AK4 mutation carriers in a large cohort of patients diagnosed clinically with pulmonary arterial hypertension. *Circulation*, **136**, 2022–2033.
9. Atkinson, C., Stewart, S., Upton, P.D., Machado, R., Thomson, J.R., Trembath, R.C. and Morrell, N.W. (2002) Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. *Circulation*, **105**, 1672–1678.
10. Long, L., Crosby, A., Yang, X., Southwood, M., Upton, P.D., Kim, D.K. and Morrell, N.W. (2009) Altered bone morphogenetic protein and transforming growth factor-beta signaling in rat models of pulmonary hypertension: potential for activin receptor-like kinase-5 inhibition in prevention and progression of disease. *Circulation*, **119**, 566–576.
11. Geraci, M.W., Moore, M., Gesell, T., Yeager, M.E., Alger, L., Golpon, H., Gao, B., Loyd, J.E., Tuder, R.M. and Voelkel, N.F. (2001) Gene expression patterns in the lungs of patients with primary pulmonary hypertension: a gene microarray analysis. *Circ. Res.*, **88**, 555–562.
12. Sugimori, K., Matsui, K., Motomura, H., Tokoro, T., Wang, J., Higa, S., Kimura, T. and Kitajima, I. (2005) BMP-2 prevents apoptosis of the N1511 chondrocytic cell line through PI3K/Akt-mediated NF-kappaB activation. *J. Bone Miner. Metab.*, **23**, 411–419.
13. Zhang, S., Fantozzi, I., Tigno, D.D., Yi, E.S., Platoshyn, O., Thistlethwaite, P.A., Kriett, J.M., Yung, G., Rubin, L.J. and Yuan, J.X. (2003) Bone morphogenetic proteins induce apoptosis in human pulmonary vascular smooth muscle cells. *Am. J. Physiol. Lung Cell Mol. Physiol.*, **285**, L740–L754.
14. Konig, H.G., Kogel, D., Rami, A. and Prehn, J.H. (2005) TGF-β1 activates two distinct type I receptors in neurons: implications for neuronal NF-{kappa}B signaling. *J. Cell Biol.*, **168**, 1077–1086.
15. Spender, L.C., O'Brien, D.I., Simpson, D., Dutt, D., Gregory, C.D., Allday, M.J., Clark, L.J. and Inman, G.J. (2009) TGF-beta induces apoptosis in human B cells by transcriptional regulation of BIK and BCL-XL. *Cell Death Differ.*, **16**, 593–602.
16. Morrell, N.W., Yang, X., Upton, P.D., Jourdan, K.B., Morgan, N., Sheares, K.K. and Trembath, R.C. (2001) Altered growth responses of pulmonary artery smooth muscle cells from patients with primary pulmonary hypertension to transforming growth factor-beta(1) and bone morphogenetic proteins. *Circulation*, **104**, 790–795.
17. Nasim, M.T., Ogo, T., Chowdhury, H.M., Zhao, L., Chen, C.N., Rhodes, C. and Trembath, R.C. (2012) BMPR-II deficiency elicits pro-proliferative and anti-apoptotic responses through the activation of TGFβ-TAK1-MAPK pathways in PAH. *Hum. Mol. Genet.*, **21**, 2548–2558.
18. Ogo, T., Chowdhury, H.M., Yang, J., Long, L., Li, X., Torres Cleuren, Y.N., Morrell, N.W., Schermuly, R.T., Trembath, R.C. and Nasim, M.T. (2013) Inhibition of overactive transforming growth factor-β signaling by prostacyclin analogs in pulmonary arterial hypertension. *Am. J. Respir. Cell Mol. Biol.*, **48**, 733–741.
19. Valdimarsdottir, G., Goumans, M.J., Rosendahl, A., Brugman, M., Itoh, S., Lebrin, F., Sideras, P. and ten Dijke, P. (2002) Stimulation of Id1 expression by bone morphogenetic protein is sufficient and necessary for bone morphogenetic protein-induced activation of endothelial cells. *Circulation*, **106**, 2263–2270.
20. Teichert-Kuliszewska, K., Kutryk, M.J., Kuliszewski, M.A., Karoubi, G., Courtman, D.W., Zucco, L., Granton, J. and Stewart, D.J. (2006) Bone morphogenetic protein receptor-2 signaling promotes pulmonary arterial endothelial cell survival: implications for loss-of-function mutations in the

pathogenesis of pulmonary hypertension. *Circ. Res.*, **98**, 209–217.
21. Lagna, G., Nguyen, P.H., Ni, W. and Hata, A. (2006) BMP-dependent activation of caspase-9 and caspase-8 mediates apoptosis in pulmonary artery smooth muscle cells. *Am. J. Physiol. Lung Cell Mol. Physiol.*, **291**, L1059–L1067.
22. Ishida, W., Hamamoto, T., Kusanagi, K., Yagi, K., Kawabata, M., Takehara, K., Sampath, T.K., Kato, M. and Miyazono, K. (2000) Smad6 is a Smad1/5-induced smad inhibitor. Characterization of bone morphogenetic protein-responsive element in the mouse Smad6 promoter. *J. Biol. Chem.*, **275**, 6075–6079.
23. Scharpfenecker, M., van Dinther, M., Liu, Z., van Bezooijen, R.L., Zhao, Q., Pukac, L., Lowik, C.W. and ten Dijke, P. (2007) BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. *J. Cell Sci.*, **120**, 964–972.
24. Trembath, R.C., Thomson, J.R., Machado, R.D., Morgan, N.V., Atkinson, C., Winship, I., Simonneau, G., Galie, N., Loyd, J.E., Humbert, M. *et al.* (2001) Clinical and molecular genetic features of pulmonary hypertension in patients with hereditary hemorrhagic telangiectasia. *N. Engl. J. Med.*, **345**, 325–334.
25. Trembath, R.C. (2001) Mutations in the TGF-β type 1 receptor, ALK1, in combined primary pulmonary hypertension and hereditary haemorrhagic telangiectasia, implies pathway specificity. *J. Heart Lung Transplant*, **20**, 175.
26. Harrison, R.E., Flanagan, J.A., Sankelo, M., Abdalla, S.A., Rowell, J., Machado, R.D., Elliott, C.G., Robbins, I.M., Olschewski, H., McLaughlin, V. *et al.* (2003) Molecular and functional analysis identifies ALK-1 as the predominant cause of pulmonary hypertension related to hereditary haemorrhagic telangiectasia. *J. Med. Genet.*, **40**, 865–871.
27. Nasim, M.T. and Trembath, R.C. (2005) A dual-light reporter system to determine the efficiency of protein–protein interactions in mammalian cells. *Nucleic Acids Res.*, **33**, e66.
28. Suzuki, Y.J., Nagase, H., Wong, C.M., Kumar, S.V., Jain, V., Park, A.-M. and Day, R.M. (2007) Regulation of Bcl-xL expression in lung vascular smooth muscle. *Am. J. Resp. Cell Mol. Biol.*, **36**, 678–687.
29. Xiang, H., Wang, J. and Boxer, L.M. (2006) Role of the cyclic AMP response element in the bcl-2 promoter in the regulation of endogenous Bcl-2 expression and apoptosis in murine B cells. *Mol. Cell Biol.*, **26**, 8599–8606.
30. Shabestari, R.M., Safa, M., Alikarami, F., Banan, M. and Kazemi, A. (2017) CREB knockdown inhibits growth and induces apoptosis in human pre-B acute lymphoblastic leukemia cells through inhibition of prosurvival signals. *Biomed. Pharmacother.*, **87**, 274–279.
31. Tu, L., Dewachter, L., Gore, B., Fadel, E., Dartevelle, P., Simonneau, G., Humbert, M., Eddahibi, S. and Guignabert, C. (2011) Autocrine fibroblast growth factor-2 signaling contributes to altered endothelial phenotype in pulmonary hypertension. *Am. J. Resp. Cel Mol. Biol.*, **45**, 311–322.
32. Kasof, G.M., Lu, J.J., Liu, D., Speer, B., Mongan, K.N., Gomes, B.C. and Lorenzi, M.V. (2001) Tumor necrosis factor-alpha induces the expression of DR6, a member of the TNF receptor family, through activation of NF-kappaB. *Oncogene*, **20**, 7965–7975.
33. Feng, B., Cheng, S., Hsia, C.Y., King, L.B., Monroe, J.G. and Liou, H.C. (2004) NF-kappaB inducible genes BCL-X and cyclin E promote immature B-cell proliferation and survival. *Cell Immunol.*, **232**, 9–20.
34. Dai, Y., Desano, J., Tang, W., Meng, X., Meng, Y., Burstein, E., Lawrence, T.S. and Xu, L. (2010) Natural proteasome inhibitor celastrol suppresses androgen-independent prostate cancer progression by modulating apoptotic proteins and NF-kappaB. *PLoS One*, **5**, e14153.
35. Qian, J., Zou, Y., Rahman, J.S., Lu, B. and Massion, P.P. (2009) Synergy between phosphatidylinositol 3-kinase/Akt pathway and Bcl-xL in the control of apoptosis in adenocarcinoma cells of the lung. *Mol. Cancer Ther.*, **8**, 101–109.
36. Zhang, X., Zhuang, T., Liang, Z., Li, L., Xue, M., Liu, J. and Liang, H. (2017) Breast cancer suppression by aplysin is associated with inhibition of PI3K/AKT/FOXO3a pathway. *Oncotarget*, **8**, 63923–63934.
37. David, L., Mallet, C., Mazerbourg, S., Feige, J.J. and Bailly, S. (2007) Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. *Blood*, **109**, 1953–1961.
38. Zhang, M., Mathews Griner, L.A., Ju, W., Duveau, D.Y., Guha, R., Petrus, M.N., Wen, B., Maeda, M., Shinn, P., Ferrer, M. *et al.* (2015) Selective targeting of JAK/STAT signaling is potentiated by Bcl-xL blockade in IL-2–dependent adult T-cell leukemia. *Proc. Natl. Acad. Sci.*, **112**, 12480.
39. Montero, J. and Letai, A. (2018) Why do BCL-2 inhibitors work and where should we use them in the clinic? *Cell Death Differ.*, **25**, 56–64.
40. Long, L., MacLean, M.R., Jeffery, T.K., Morecroft, I., Yang, X., Rudarakanchana, N., Southwood, M., James, V., Trembath, R.C. and Morrell, N.W. (2006) Serotonin increases susceptibility to pulmonary hypertension in BMPR2-deficient mice. *Circ. Res.*, **98**, 818–827.
41. Morrell, N.W., Upton, P.D., Kotecha, S., Huntley, A., Yacoub, M.H., Polak, J.M. and Wharton, J. (1999) Angiotensin II activates MAPK and stimulates growth of human pulmonary artery smooth muscle via AT1 receptors. *Am. J. Physiol.*, **277**, L440–L448.
42. Nasim, M.T. and Eperon, I.C. (2006) A double-reporter splicing assay for determining splicing efficiency in mammalian cells. *Nat. Protoc.*, **1**, 1022–1028.
43. Chowdhury, H.M., Siddiqui, M.A., Kanneganti, S., Sharmin, N., Chowdhury, M.W. and Nasim, M.T. (2018) Aminoglycoside-mediated promotion of translation readthrough occurs through a non-stochastic mechanism that competes with translation termination. *Hum. Mol. Genet.*, **27**, 373–384.