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.
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 Id1–3 genes (6).

Recent investigations have identified novel mutations in BMPR1, 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 its 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 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 BMPR2 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 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 anti-apoptotic Bcl-xS transcripts in PAH patients with and without BMPR2 mutations.
**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 BMP2 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...
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
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, p.R899X and p.R899P plasmids, indicating that the pro-survival effects of BMPRII receptor are greatly reduced in
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 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 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.
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. 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
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.R111Q) 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 $\beta$-gal protein will be produced. An interaction between BMPRII and ALK1 generates both $\beta$-gal and luciferase proteins. (B) The efficiency of the interaction of BMPRII 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 $\pm$ 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.

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

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 diseaserelated 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 (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-1 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 scraped off using scalpel blade. The adjacent adventitia was stripped off and the medial explants were cut into 4–9 mm² sections. These segments were then plated into T25 cm² flasks and were allowed to adhere after 2 h (40,41). Cells were grown to confluency and used for experiments.

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). 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 determination of the efficiency of protein–protein interactions between BMPR1 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.

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 BMPR1 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.

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


