

BONE MORPHOGENETIC PROTEIN SIGNALING IN HERITABLE VERSUS

IDIOPATHIC PULMONARY HYPERTENSION

BMP signaling in Pulmonary Hypertension

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ABSTRACT

Mutations in gene encoding for bone morphogenetic protein type 2 receptor (*BMPR-2*) have been reported in pulmonary arterial hypertension (PAH), but their functional relevance remains incompletely understood.

BMP receptors expression was evaluated in human lungs and in cultured pulmonary artery smooth muscle cells (PASMCs) isolated from 19 idiopathic PAH patients and 9 heritable PAH patients with demonstrated *BMPR-2* mutations. BMP4-treated PASMCs were assessed for Smad and p38MAPK signaling associated to mitosis and apoptosis.

Lung tissue and PASMCs from heritable PAH patients presented with decreased *BMPR-2* expression and variable increases in *BMPR-1A* and *BMPR-1B* expressions, while a less important decreased *BMPR-2* expression was observed in PASMCs from idiopathic PAH patients. Heritable PAH PASMCs showed no increased phosphorylation of Smad1/5/8 in the presence of BMP4, which actually activated the p38MAPK pathway. Individual responses varied from one mutation to another. PASMCs from PAH patients presented with an *in vitro* proliferative pattern, which could be inhibited by BMP4 in idiopathic PAH, not in heritable PAH. PASMCs from idiopathic PAH and more so from heritable presented an inhibition of BMP4-induced apoptosis.

Most heterogenous *BMPR-2* mutations are associated with defective Smad signaling compensated for by an activation of p38MAPK signaling, accounting for PASMC proliferation and deficient apoptosis.

Abstract Word Count: 200

Key words: Bone morphogenetic protein type 2 receptor; intracellular signal transduction; mutation; pulmonary hypertension; smooth muscle cells; vascular remodeling.

INTRODUCTION

Pulmonary arterial hypertension (PAH) is an uncommon disease with a poor prognosis and mysterious pathobiology, characterized by a progressive increase in pulmonary vascular resistance and eventual right ventricular failure (1). Mutations of bone morphogenetic protein receptor-2 (*BMPR-2*), a member of the TGF- β receptor family have been reported in a high proportion of patients with the heritable form of the disease, and in 10 to 30 % of patients with sporadic idiopathic PAH (2). To date, more than 200 distinct *BMPR-2* mutations have been described, widely dispersed across the gene, with the majority predicting premature truncation of the transcript (3). BMPR signaling involves heterodimerization of two transmembrane serine/threonine-kinase receptor chains, the constitutively active BMPR-2 and the corresponding type 1 receptor, BMPR-1A/ALK3 or BMPR-1B/ALK6 (4,5). With interaction of a ligand, for example BMP4, the activated kinase domain of BMPR-2 phosphorylates the corresponding BMPR-1, which in turn initiates intracellular signaling through the phosphorylation of a set of BMP restricted Smad proteins (Smad1/5/8). Subsequently, these phosphorylated Smads associate with Smad4, translocate to the nucleus and then modulate the transcription of target genes. Alternative Smad-independent signaling pathways involving mitogen activated protein kinase (MAPK), including ERK1/2, JNK and p38MAPK have been reported to be activated by BMP ligands (6). The resulting imbalance is believed to be the cause of a proliferation of pulmonary artery smooth muscle cells (PASMCs) as a major component of pulmonary arteriolar remodeling in PAH (1).

It is of interest that the histopathology and clinical picture of PAH with or without *BMPR-2* mutations appear similar, except for an earlier age of onset, more severe hemodynamic compromise at diagnosis, and less common reversibility at vasodilator testing (7,8). Therefore, the functional consequences of *BMPR-2* mutations remain incompletely

understood, but it may be hypothesized that their phenotypic impact may vary with type of mutation or interaction with alternative signaling pathways.

We have previously reported that PASMCs from idiopathic PAH patients present with an *in vitro* proliferative phenotype (9,10). In the present study, we investigate the effects of BMP4 on Smad and p38MAPK signaling associated with mitosis and apoptosis in cultured PASMCs isolated from idiopathic PAH patients without detected mutations and from heritable PAH patients with mutations. The results are in keeping with the notion of a crucial role for BMP/Smad signaling in the prevention of abnormal growth and apoptosis of PASMCs that is lost in most but not all types of mutations.

METHODS

Tissue samples

Lung tissue and pulmonary arteries were sampled at lung transplantation and sequenced to screen *BMPR-2* mutations. After confirmatory cross-check with medical records, patients with PAH were segregated in two groups, according the presence or the absence of mutations. These 2 groups were respectively called heritable PAH (n=9) and idiopathic PAH (n=19) patients. Pulmonary specimens were also sampled in control subjects (n=10) at lobectomy or pneumonectomy for a suspected localized lung tumor. These control subjects did not bear any *BMPR-2* mutations or polymorphisms.

All PAH patients were in New York Heart Association functional class III or IV and were treated with iv epoprostenol. In the control subjects, transthoracic echocardiography was performed preoperatively to rule out pulmonary hypertension, and pulmonary arteries were sampled at a distance from tumor areas. The study was approved by the local Institutional Review Board.

Screening for mutations in the gene encoding the BMPR-2 receptor

Mutations in the *BMPR-2* gene in lung specimens from patients with PAH (n=28) were screened as previously described (11,12). Briefly, the entire protein-coding region (sequence corresponding to exons 1-13 of the *BMPR-2* gene) was amplified from genomic DNA samples by polymerase chain reaction with specific primers. PCR products were then separated by electrophoresis in a 1% agarose gel and purified using the QIAquick PCR purification kit (QIAGEN, Courtaboeuf, France). Amplified and purified fragments were sequenced with a dye-terminator cycle-sequencing system (ABI PRISM 377, Perkin-Elmer Applied Biosystems).

Culture of human pulmonary artery smooth muscle cells (PASMCs) and pulmonary microvascular endothelial cells (PECs)

Human PASMCs were cultured from explants of pulmonary arteries (1.5 to 10 mm in diameter) derived from previously described patient groups transplanted for heritable and idiopathic PAH, but also from controls. PASMCs were cultured in 10% FCS/DMEM and used between passages 3 and 6, as previously described (13). The phenotype of cultured PASMCs was assessed for expression of muscle-specific contractile and cytoskeletal proteins, including smooth muscle α -actin (α -SMA), desmin, and vinculin (13).

Human PECs were obtained first by Dispase I (Roche Diagnostics, Penzberg, Germany) digestion and after by immunomagnetic purification with anti-platelet endothelial cell adhesion molecule-1 (CD31) monoclonal antibody-labeled Dynabeads (DynaL Biotech, Compiègne, France) of a fragment of lung tissue isolated from heritable and idiopathic PAH patients and controls, as previously described (9,14). To characterize the endothelial phenotype, PECs were labeled with acetylated low-density lipoprotein coupled to a fluorescent carbocyanine dye (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate [Dil-Ac-LDL]; Tebu, Le Perray en Yvelines, France) and stained with antibodies against the endothelial cell-specific lectin *Ulex europaeus* agglutinin-1 (UEA-1; Sigma-Aldrich, Ayrshire, UK) (15). Experiments were also performed with monoclonal antibodies against desmin and vimentin (Dako, Glostrup, Denmark). Cells with positive staining for Dil-Ac-LDL and UEA-1 and negative staining for desmin and vimentin were taken as endothelial cells and constituted more than 95% of our PEC cultures. PECs were used between passages 3 and 6 (9,14).

RNA extraction and cDNA preparation

Total RNA was prepared from snap-frozen human lung tissue samples (weight 100 mg) by homogenization according to the method of Chomczynski and Sacchi (16), using TRIzol reagent (Invitrogen, Cergy-Pontoise, France). Total RNA was extracted from growth-arrested primary cultures of human PASMCs and PECs using Qiagen RNeasy Mini kit

(QIAGEN S.A., Courtabeouf, France), according to the manufacturer's instructions. RNA concentration was determined by standard spectrophotometric techniques and the RNA integrity was assessed by visual inspection of ethidium bromide-stained denaturing agarose gels. First-strand cDNA synthesis was carried out using SuperScript II Reverse Transcriptase System (Life Technologies, Inc.), as previously described (9,14).

Real-time quantitative polymerase chain reaction (RTQ-PCR)

RTQ-PCR primers were designed using the computer program Primer3 (Primer Express Software, Applied Biosystems) for human BMPR-1A, BMPR-1B, BMPR-2, Bax, Bcl2 mRNA and 18s rRNA, as housekeeping gene. To avoid inappropriate amplification of residual genomic DNA, intron-spanning primers were selected. RTQ-PCR was performed in triplicate on an ABI PRISM 7000 (Applied Biosystems, Foster City, CA), in mixtures of 12.5 μ l Sybr Green PCR Master Mix (Applied Biosystems, Warrington, UK), 300 nM (each) primer and 5 μ l of diluted template DNA in a total volume of 25 μ l. Signal detection and analysis of results were performed with ABI PRISM 7000 sequence detection software (Applied Biosystems). Relative quantification was achieved with the comparative $2^{-\Delta\Delta C_t}$ method by normalization with 18s ribosomal RNA. For assays of Bax and Bcl2 mRNAs, PASCs were seeded and synchronized. The cells were then exposed to BMP4 (100 ng/mL) for 4 hours and then used for mRNA extraction and RTQ-PCR.

Protein extraction and BMPR-1A, BMPR-1B and BMPR-2 Western Blotting

Proteins were extracted from snap-frozen tissue samples (weight 100 mg) by homogenization in an appropriate amount of homogenizing buffer [Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) in PBS and 0.1% Triton X-100]. The homogenates were centrifuged at 4°C and the supernatants were collected. After determination of the protein concentration using the method of Bradford (17), 40 μ g of protein from each lung sample were resuspended in 3x Laemmli's buffer, boiled for 5 minutes, and

separated on 10% acrylamide gel by electrophoresis. Proteins were electrophoretically transferred to a nitrocellulose membrane (Sigma-Aldrich, Ayrshire, UK) for 1h at room temperature. After blocking with 5% BSA in 1x Tween (T)-TBS (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; and 0.1% Tween 20) for 2 h at room temperature, the membrane was washed three times with T-TBS at room temperature for 5 min. The membrane was incubated with goat anti-human BMPR-1A, BMPR-1B or BMPR-2 antibody (1:500) (R&D systems, Mineapolis, MN) at 4°C overnight with rocking. Then the membrane was washed three times for 5 min and incubated with the secondary antibody (rabbit antigoat IgG conjugated with horseradish peroxidase; Dako, Glostrup, Denmark; 1:2,000) for 1 h at room temperature. Immunoreactive bands were detected using the enhanced chemiluminescence Western blotting analysis system (Amersham Pharmacia Biosciences, Buckinghamshire, UK) and quantified by laser densitometry. Relative quantification was performed by normalization with β -actin (Sigma-Aldrich CO, St Louis, USA).

Immunoblotting for BMP Signaling Pathways

PASMCs were plated in fresh 10% FCS/DMEM medium for 24 hours, and then quiesced for 48 hours in serum-free medium. BMP4 (100 ng/mL) or vehicle was then added to the cells for 20 minutes. Protein was harvested by washing cells in cold PBS and by scraping in 300 μ L of 1x sample loading buffer [Tris-HCl (pH 7.4), NaCl, NaF, sodium pyrophosphate (all at 25 mM), sodium vanadate (1mM), EDTA, EGTA (both at 2,5mM), phenylmethylsulfonyl fluoride (1mM), aprotinine, leupeptine (both at 5 μ g/mL), SDS, deoxycholate and NP-40 (all at 0,50%)] on ice. The samples were then stored at -20°C. After determination of the protein concentration, using the method of Bradford (17), samples (20 μ g) were resuspended in 3x Laemmli buffer, boiled at 95°C for 5 minutes and electrophoresed on acrylamide gels (10%). Immunoblotting assays were performed as described above with monoclonal mouse anti-human phospho-p38^{MAPK(Thr180/Tyr182)} (1:1000,

Cell Signalling Technology Inc, Danvers, MA), polyclonal rabbit anti-human phospho-Smad1^(Ser463/465)/Smad5^(Ser463/465)/Smad8^(Ser426/428) (1:1000, Cell Signalling Technology Inc, Danvers, MA) and polyclonal goat anti-human total Smad 1/5/8 (1:1000, Santa-Cruz Biotechnology, Santa Cruz, CA). Relative quantification was performed by normalization with total Smad1/5/8 for phospho-Smad1/5/8 and β -actin (Sigma-Aldrich, France) for p38MAPK. A treatment of 100 ng/mL during 20 minutes was chosen based on preliminary studies of BMP4 concentrations in relation to the capacity to activate downstream signaling pathways and to inhibit growth-promoting activity of mitogenic agents on PSMCs (18,19).

PASMC Proliferation Assays

The growth of human cultured PSMCs was determined by [³H]thymidine incorporation, representing DNA synthesis. Briefly, PSMCs were seeded in 24-well plates in 10% FCS/DMEM at a density of 5×10^4 cells/well and allowed to adhere for 24 hours. The medium was then removed and the cells subjected to growth arrest by incubation with serum-free DMEM. After 48 hours, the medium was replaced with fresh DMEM completed with 10% FCS or PDGF (10 ng/mL) in the presence or absence of BMP4 (100ng/mL). PSMC proliferation was also assessed in response to 10% FCS and PDGF (10 ng/mL) alone. For each condition, [³H]thymidine (0.6 μ Ci/ml) was added to each well. After incubation for 24 hours, the cells were washed twice with PBS, treated with ice-cold 10% trichloroacetic acid and neutralized with 0,1N NaOH (0,5 ml/well). [³H]thymidine incorporation into DNA was counted and reported as counts per minute per well.

Apoptosis Assays

Apoptosis evaluation was performed by flow cytometry analysis of the DNA content by propidium iodide incorporation and RTQ-PCR analysis of Bax/Bcl2 ratio. For flow cytometry analysis of DNA content, PSMCs were seeded and treated for 24 h with fresh serum-free DMEM in presence or absence of BMP4 (100ng/mL). Culture medium was

removed and saved. Cells were trypsinized and returned to the medium they had grown in and then centrifuged. Cells were then washed twice in ice-cold phosphate-buffered saline (PBS) and stored at 4°C in 75% ethanol. Fixed cells were centrifuged, washed with PBS and incubated with 200 µl Rnase I (1 mg/mL, Invitrogen, Cergy-Pontoise, France) and 200 µl of propidium iodide (1mg/mL; Sigma, France). Cells were incubated at room temperature for one hour in the dark. Samples were analyzed by flow cytometry. The red fluorescence of single events was recorded using a laser beam at 488 nm excitation λ with 610 nm as emission λ , to measure the DNA index. For Bax/Bcl2 ratio determination, PASMCS were seeded, synchronized and treated for 4 hours as described above. mRNA extraction, cDNA synthesis and RTQ-PCR were performed to determinate the expression of pro-apoptotic Bax and anti-apoptotic Bcl2 genes compared to 18s as housekeeping gene, as described above.

Statistical Analyses

All data are reported as mean \pm SEM. Effects of *BMPR-2* mutations and BMP4 treatment were analysed by repeated-measures ANOVA. When the F-ratio of the ANOVA reached a critical value of $p < 0.05$, nonparametric Mann-Whitney tests were used to compare specific situations (20). A linear squared regression analysis was used to calculate correlations between pulmonary vascular resistance and contents (mRNA and protein) of the investigated BMP signaling molecules (20).

RESULTS

Clinical and Hemodynamic Characteristics of PAH Patients

There were no differences between PAH patients with and without *BMPR-2* mutations in age (41 ± 2 vs 39 ± 3 years), female to male sex ratio (10/9 versus 5/4), mean pulmonary artery pressure (62 ± 2 vs 63 ± 3 mmHg), pulmonary vascular resistance (20 ± 1 vs 20 ± 2 U.m²) and cardiac index (2.19 ± 0.09 vs 2.41 ± 0.25 L.min⁻¹.m²). None of patients presented with reversibility at vasodilator testing.

Identification and description of BMPR-2 mutations (Figure 1)

Germline mutations in the 13 exons encoding *BMPR-2* were identified in the 9 heritable PAH patients. Three heterozygous nonsense mutations were identified in exons 1 (W16X), 2 (W70X) and 3 (S107X), which encode part of the extracellular domain of *BMPR-2*. One mutation consisted in total deletion of exon 1 (Δ exon1). Two mutations were found in exon 5, which encodes the transmembrane domain of *BMPR-2*: one heterozygous nonsense mutation (E195X) and one by loss of 22bp (22bp del). Two heterozygous missense and one nonsense mutations were identified respectively in exons 7 (S301P) and 11 (R491W and Q495X), which encode parts of the kinase domain of *BMPR-2*. No *ALK-1* mutations was found in the PAH patients with and without mutations.

Pulmonary and cellular expression of BMPR's (Figures 2-4)

The expression of *BMPR-1A* was increased in lung tissue from both heritable and idiopathic patients (mARN and protein), but only in PSMCs from idiopathic PAH patients, and was not different from controls in PECs from heritable and idiopathic PAH patients (mRNA). The expression of *BMPR-1B* was increased in PSMCs from heritable PAH patients only (mRNA). The expression of *BMPR-2* was decreased in lung tissue from

heritable PAH only (mRNA and protein), and in PASMCs from both heritable and idiopathic PAH, and was not different from controls in PECs (mRNA). However, as indicated by relatively large SEM's, the increased expressions of BMPR-1A and BMPR-1B, and decreased expressions of BMPR-2 in PASMCs varied greatly from one mutation to another, with no consistent pattern. No correlation was found between pulmonary vascular resistance and lung expressions of BMPR-1A, BMPR-1B and BMPR-2 proteins.

Differential effects of BMP4 on Smad and p38MAPK signaling (Figures 5 and 6)

BMP4 (100ng/mL, 20 minutes) induced the activation (phosphorylation) of Smad1/5/8 in PASMCs isolated from idiopathic PAH patients and controls, indicating that the transmission of BMP signaling was intact in these cells. In contrast, no BMP4-induced phosphorylation of Smad1/5/8 was observed in PASMCs from heritable PAH, with the exception of the 22 bp del mutation. BMP4 activated p38MAPK signaling in PASMCs from heritable PAH patients (with the exception of the PASMC with 22bp del mutation) but not from idiopathic PAH patients or controls.

Effects of BMP4 on PASMC proliferation induced by serum (10%) and PDGF (10 ng/mL) treatment (Figure 7)

PASMCs isolated from heritable and idiopathic PAH exhibited an increased proliferation, as assessed by [³H]-thymidine incorporation, in the presence of serum but not PDGF. The addition of BMP4 induced an inhibition of [³H]-thymidine incorporation in both serum- and PDGF-treated PASMCs from idiopathic PAH and controls, but not of PASMCs from heritable PAH patients. The absence of BMP4-induced inhibition of proliferation was observed on PASMCs from all the heritable PAH patients, excepted in the patient with the 22bp del mutation.

Effects of BMP4 on PASMCM apoptosis (Figure 8)

Flow cytometry analysis showed that BMP4 (10 and 100 ng/mL, 24 hours) increased apoptotic rates in PASMCMs from idiopathic PAH patients and controls but with less extent in PASMCMs from idiopathic PAH and not from heritable PAH patients (Figure 8A). The Bax/Bcl-2 pro-apoptotic ratio was increased by BMP4 at 100 ng/ml in PASMCMs from controls and idiopathic PAH, not from heritable PAH patients (Figure 8B).

DISCUSSION

The present results show that (1) lung tissue and PASMCs, not PECs, from heritable PAH patients present with decreased expressions of *BMPR-2* and variable increases in the expressions of *BMPR1-A* and *BMPR-1B*, while only a (relatively less important) decreased expression in *BMPR-2* is observed in PASMCs from idiopathic PAH patients, (2) PASMCs from heritable PAH patients show no BMP4-induced Smad 1/5/8 phosphorylation but BMP4-induced activation of the p38MAPK pathway, (3) PASMCs from PAH patients present with an *in vitro* proliferative and anti-apoptotic pattern, that can be inhibited by BMP4 in idiopathic PAH but not in heritable PAH and (4) individual responses vary from one mutation to another, with in particular PASMCs from PAH patients with the 22bp del mutation showing no difference as compared to PASMCs from idiopathic PAH patients without identified mutations. The present work confirmed previous studies about *BMPR-2* signaling in PAH and presented for the first time a large *in vitro* comparison of PASMCs with and without naturally occurring *BMPR-2* mutations.

Even though *BMPR-2* mutations are identified in the majority of heritable PAH patients, and carry a significant risk of developing the disease in asymptomatic carriers, two large clinical studies have failed to disclose differences in clinical presentation, hemodynamics and histopathology between heritable or idiopathic PAH patients, except for an earlier onset of the disease, more compromised hemodynamics, and maybe a less frequent reversibility at vasodilator testing in heritable PAH (7,8). These observations suggest heterogenous functional consequences of the various hitherto reported over 200 *BMPR-2* mutations, and also interactions with coexisting signaling abnormalities. In the present study, there was no difference in clinical presentation of heritable and idiopathic PAH, but this is probably related to minor phenotypic differences, individual variability and small sample size.

Our results confirm that the expression of *BMPR-2* is decreased in idiopathic PAH, and much more so in heritable PAH (21). The relative magnitudes of lung tissue and isolated PASMC and PEC expressions were suggestive of a predominant PASMC location of *BMPR-2*. This is in contrast with predominant endothelial cell location previously reported in normal and in lungs from PAH patients (21). However, recent studies on human PASMCs and PECs showed relatively high levels of *BMPR-2* on both cell types, but very low expressions of *BMPR-1A* and *-1B* in PECs consistent with a lack of *BMP4* responsiveness (22). In the present study, the expressions of *BMPR-2* and of *BMPR-1A* and *BMPR-1B* were much lower in PECs as compared to PASMCs with no apparent impact of *BMPR-2* mutations, so that further analysis focused on PASMCs. Moreover, in heritable PAH patients, the level of *BMPR-2* expression was lower than predicted by the state of haploinsufficiency and the process of nonsense mediated decay secondary to the presence of nonsense mutations. These observations are in keeping with the notion that some additional environmental and/or genetic factors may be responsible to further reduce *BMPR-2* expression.

Decreased expressions of *BMPR-2* have also been reported to occur in experimental animal models of pulmonary hypertension such as induced by chronic systemic-to-pulmonary shunting (23), hypoxic exposure (24) and monocrotaline (25). In the latter study, decreased *BMPR-2* expression was described in both lung tissue and PASMCs (25). Surprisingly, exclusive overexpression of *BMPR-2* by gene therapy did not ameliorate monocrotaline-induced PAH in rats (26), indicating that reconstitution of the receptor was unable to restore *BMP* signaling and, thus, did not prevent disease onset or progression. The present results suggest that decreased expression associated to mutations in *BMPR-2* seem to be crucial to explain the pro-proliferative and anti-apoptotic effects in PASMCs.

In the present study, the expressions of BMPR-1A and BMPR-1B tended to increase, though quite variably, with significance for the BMPR-1A in lung tissue of both heritable and idiopathic PAH patients, but only for BMPR-1A on PASMCs from idiopathic PAH patients and BMPR-1B in PASMCs from heritable PAH patients. The expression of BMPR-1A was decreased in a study on patients with various causes of severe pulmonary hypertension, including PAH, but also mitral stenosis and thromboembolic pulmonary hypertension, and this was explained in relation to an overexpression of angiotensin-1 (27). The expression of BMPR-2 was unaltered in that same study (27). We previously reported RTQ-PCR and Western Blotting of unchanged expression of angiotensin-1 in whole lung tissue and in PASMCs from PAH patients (14). In monocrotaline-induced pulmonary hypertension, the expressions of BMPR-2 and BMPR-1B were decreased, while the expression of BMPR-1A remained unchanged (25). An increased expression of BMPR-1B has been reported in one idiopathic PAH patient (28). The reasons for these discrepant results are unclear. Since BMPR-1A/BMPR-2 is the main BMP4 responsive receptor complex allowing for Smad 1/5/8 stimulation (22), the overexpression of BMPR-1A in the PASMCs of the idiopathic PAH patients could be somehow related to the normal BMP4-responsiveness of these cells. As for the overexpression of BMPR-1B in heritable PAH, this could be speculated to be related to alternative p38MAPK signaling activation.

Of the 9 mutations identified in the present study, the W16X, R491X, Q495X have been previously reported (11,29,30). The 6 other mutations (Δ exon1, W70X, S107X, E195X, 22bp del, S301P) across all four domains of the receptor are all novel including one total deletion of exon, three nonsense, one partial deletion and one missense mutation. Previous studies showed that missense mutations in the ligand binding domain by cysteine substitutions impair BMP signaling by mutant receptor mislocalisation in the cytosol (31). Moreover non-cysteine substitutions localise to the cell surface but also exhibit defects in BMP signaling

activity (31). In contrast mutations in the cytoplasmic C-terminal domain only moderately inhibit Smad-signaling (31,32). Therefore, in the present study, all reported mutations would be susceptible to be deleterious by changing the protein sequence at important functional sites of the receptor and the associated protein functions.

Mutations of *BMPR-2* (including R491X) heterogeneously interfere with BMP downstream signaling, but all of them activate proliferative pathways (24). We previously reported that PASMCs isolated from idiopathic PAH patients present with enhanced growth responses to serum, but not to PDGF (10). In the present study, PASMCs with or without *BMPR-2* mutations did not behave differently in this respect, but BMP4-induced growth inhibition and increased apoptosis was markedly more important in PASMCs with *BMPR-2* mutations. It is of interest that all the *BMPR-2* mutations identified in the present study, excepted the 22bp deletion in the transmembrane domain, responded homogeneously to these effects of BMP4, supporting the notion that the majority of *BMPR-2* mutations are functionally linked. Along the same line, decreased *BMPR-2* expression in monocrotaline-induced pulmonary hypertension has been reported to be associated with decreased phosphorylation of Smad1 and a decrease in BMP-induced apoptosis of PASMCs (25).

In the present study, the application of BMP4 was associated in PASMCs with *BMPR-2* mutations with a decreased activation (phosphorylation) of Smad 1/5/8 together with an increased activation (phosphorylation) of the p38MAPK pathway. We selected a dose of BMP4 of 100ng/mL on the basis of available literature (18, 19, 32) and preliminary testing showing maximum efficacy of 100ng/mL as compared to 10ng/mL and 1ng/mL in discriminating PASMCs with and without *BMPR-2* mutations. However, a recent study showed maximum efficacy at a lower dose of 10 ng/mL of BMP4 (33). The reasons for these discrepancies are not clear, and therefore the absence of complete dose-response curves may

be a limitation to our findings. In monocrotaline-induced pulmonary hypertension, decreased BMPR-2 and phospho-Smad1 occurred without change in p38MAPK signaling in PASMCs (25). In hypoxia-induced pulmonary hypertension, downregulation of BMPR-2 did not affect Smad 1/5/8 phosphorylation, and was associated with a decreased p38MAPK signaling (24). Even though part of these differences may be related to model specificities and PASMCs versus whole lung measurements, the present data confirm previous report (31,32) that most mutations of *BMPR-2* are associated with more profound changes in downstream signaling and are associated with increased p38MAPK signaling as a cause of increased PASMC proliferation.

The present results support the notion of altered BMPR-2/Smad signaling as a cause of increased proliferation of PASMCs playing an important role in the remodeling of pulmonary resistance vessels in PAH.

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CONFLICT OF INTEREST DISCLOSURES

The authors have no conflict of interests to disclose.

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FIGURE LEGENDS

Figure 1. Genetic characteristics of patients. Schematic representation of *BMPR-2* functional domains, demonstrating the range of *BMPR-2* mutations studied in this study and indicating the nature of amino acid substitution or nonsense mutations (X).

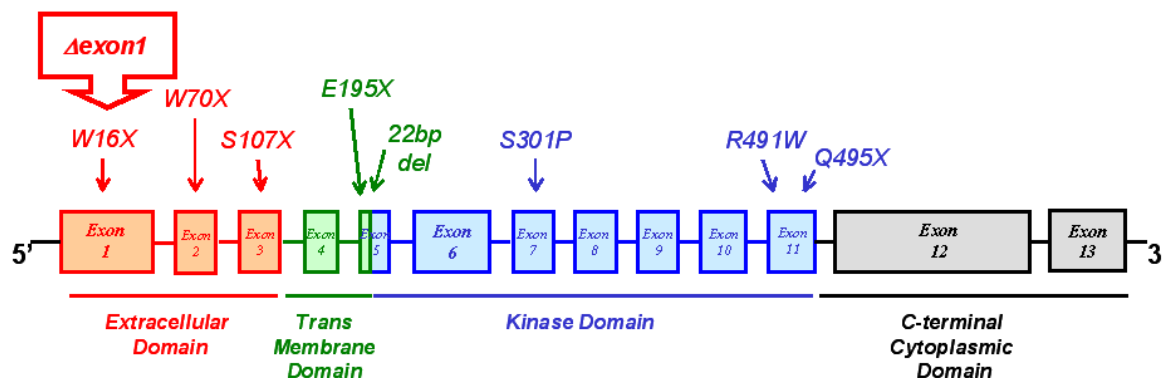


Figure 1

Figure 2. Relative *BMPR-1A* (A, D, G), *BMPR-1B* (B, E, H) and *BMPR-2* (C, F, I) mRNA expression. Whole lung tissue samples (A, B, C), cultured pulmonary artery smooth muscle cells (PASCs; D, E, F) and pulmonary microvascular endothelial cells (PECs; G, H, I) from controls (white bars), PAH patients without (black bars; idiopathic PAH) and with *BMPR-2* mutations (dashed bars; heritable PAH) were assessed by RTQ-PCR as described in **METHODS**. Results are expressed as mean \pm S.E.M. Statistical differences are assessed by Mann-Whitney's test: * $P < 0.05$, ** $P < 0.001$ vs control conditions; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.001$ idiopathic PAH vs heritable PAH conditions.

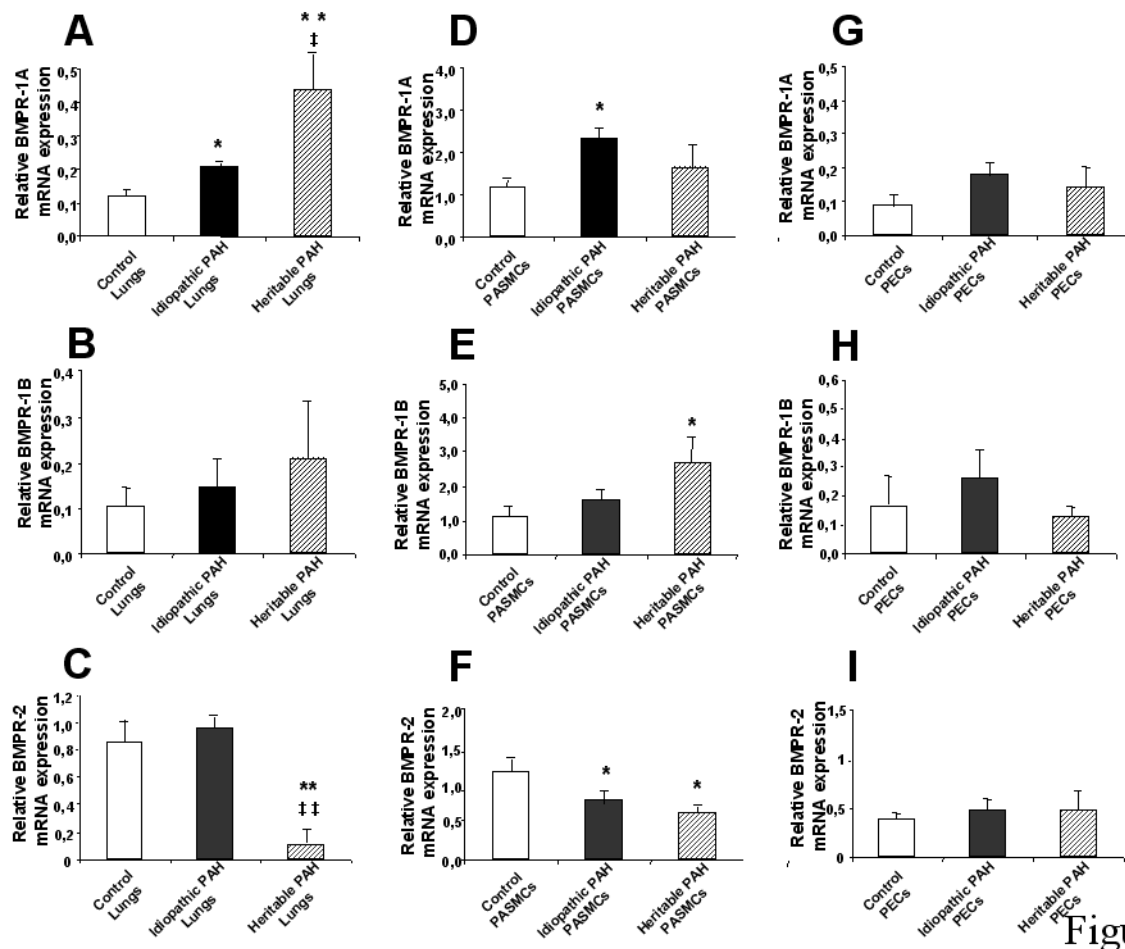


Figure 2

Figure 3. Relative BMPR-1A (A), BMPR-1B (B) and BMPR-2 (C) mRNA expression in PASMCS isolated from heritable PAH patients with naturally occurring *BMPR-2* mutations (Δ exon1, *G/A=W16X*, *G/A=W70X*, *C/G=S107X*, *G/T=E195X*, 22bp del, *T/C=S301P*, *C/T=R491W*, *C/T=Q495X*, *G/A=W70X*, *C/G=S107X*, *G/T=E195X*, 22bp del, *T/C=S301P*, *C/T=R491W*, *C/T=Q495X*). Results are assessed by RTQPCR as described in **METHODS**.

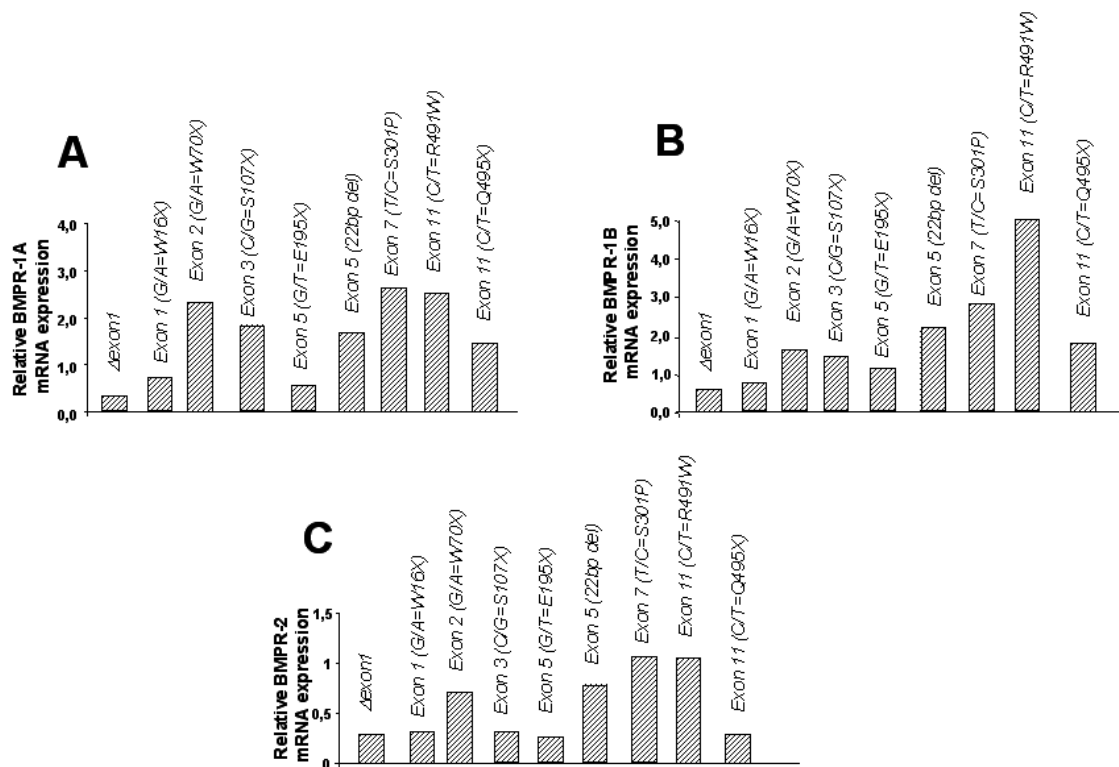


Figure 3

Figure 4. Relative BMPR-1A (A), BMPR-1B (B) and BMPR-2 (C) protein expression in whole lung tissue samples from controls (white bars), PAH patients without idiopathic PAH) and with *BMPR-2* mutations (dashed bars; heritable PAH) were assessed by Western blotting as described in **METHODS**. Results are expressed as mean \pm S.E.M. Statistical differences are assessed by Mann-Whitney's test: * $P < 0.05$ vs control conditions, † $P < 0.05$ idiopathic PAH vs heritable PAH conditions.

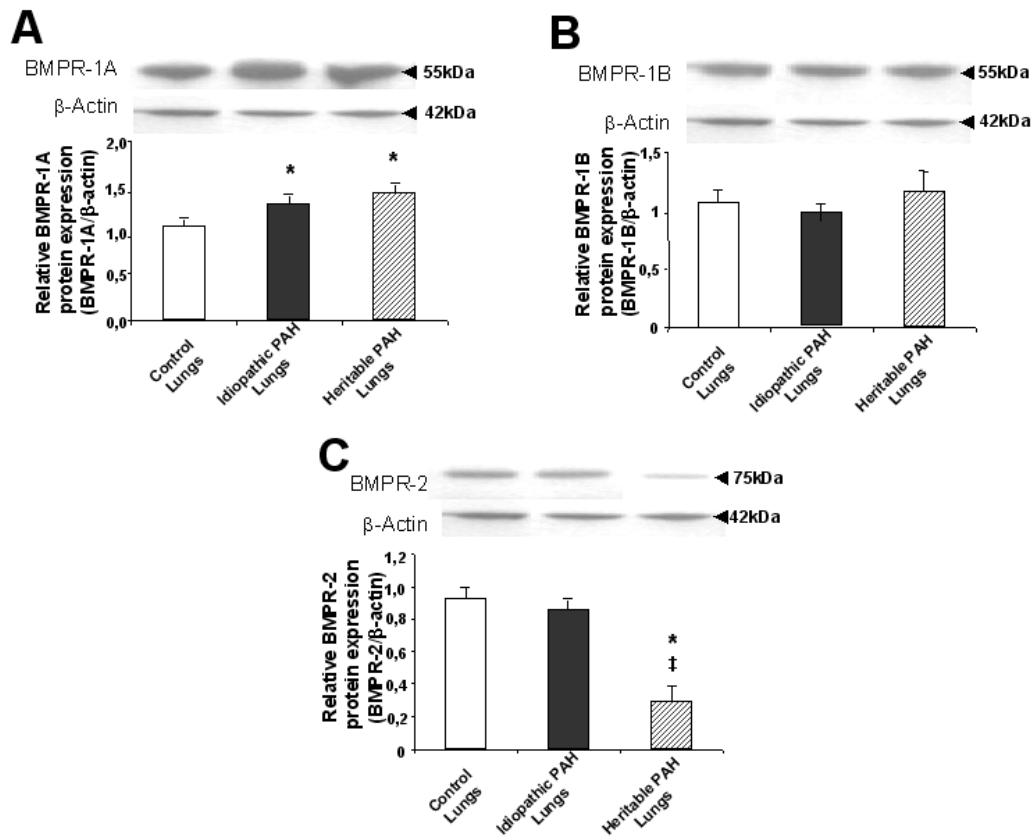


Figure 4

Figure 5. Smad 1/5/8 phosphorylation induced by BMP4. 90% confluent PSMCs isolated from controls (n=10), PAH patients without (idiopathic PAH; n=10) and without *BMPR-2* mutations (heritable PAH; n=9) were stimulated with 100 ng/mL BMP4 for 20 minutes, followed by lysis for total protein. (A) Representative Western blots for phospho-Smad 1/5/8 and for total Smad 1/5/8 to show equal loading. (B) Densitometry of phospho-Smad 1/5/8 and total Smad 1/5/8 bands from Western blots of control (white bars), idiopathic PAH (black bars) and heritable PAH (dashed bars) PSMCs. Results are presented as relative protein expression ratio of phospho-Smad1/5/8 and total Smad1/5/8 band intensity. Data are presented as mean \pm SEM. ** $P < 0,001$ vs basal conditions in nonstimulated PSMCs; [‡] $P < 0,001$ vs values for PSMCs isolated from controls stimulated by BMP4; [†] $P < 0,05$ vs

values for PASMCs isolated from idiopathic PAH patients stimulated by BMP4. (C) Relative densitometry ratio of phospho-Smad 1/5/8 in PASMCs isolated from defined naturally occurring *BMPR-2* mutated patients (Δ exon1, G/A=W16X, G/A=W70X, C/G=S107X, G/T=E195X, 22bp del, T/C=S301P, C/T=R491W, C/T=Q495X).

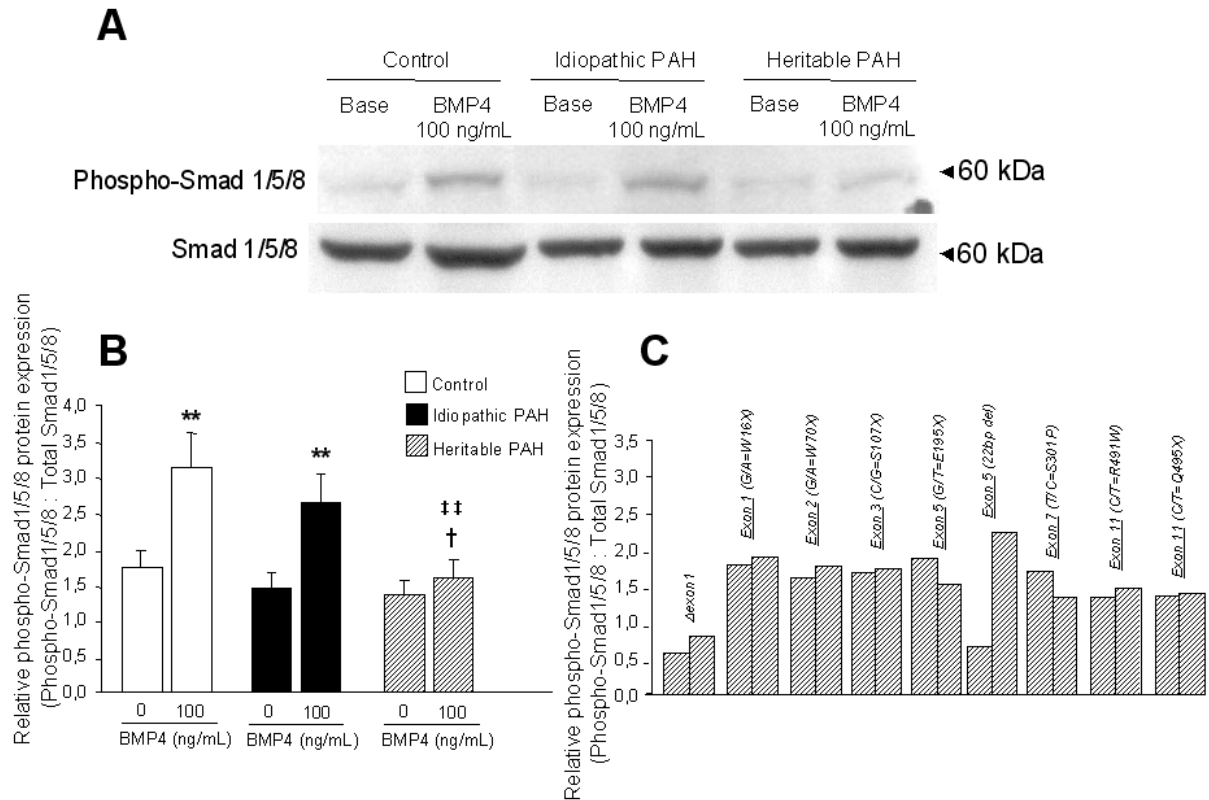


Figure 5

Figure 6. p38MAPK phosphorylation induced by BMP4. 90% confluent PASMCs isolated from controls (n=10), PAH patients without (idiopathic PAH; n=10) and with *BMPR-2* mutations (heritable PAH, n=9) were stimulated with 100 ng/mL BMP4 for 20 minutes, followed by lysis for total protein. (A) Representative Western blots for phospho-p38 and for β -actin to show equal loading. (B) Densitometry of phospho-p38 and β -actin bands from Western blots of control (white bars), idiopathic PAH (black bars) and heritable PAH (dashed bars) PASMCs. Results are presented as relative protein expression ratio of phospho-p38 and

β -actin band intensity. Data are presented as mean \pm SEM. ** $P < 0,001$ vs basal conditions in nonstimulated PSMCs; ‡ $P < 0,001$ vs values for PSMCs isolated from controls stimulated by BMP4; † $P < 0,05$ vs values for PSMCs isolated from idiopathic PAH patients stimulated by BMP4. (C) Relative densitometry ratio of phospho-p38MAPK in PSMCs isolated from defined naturally occurring *BMPR-2* mutated patients (Δ exon1, G/A=W16X, G/A=W70X, C/G=S107X, G/T=E195X, 22bp del, T/C=S301P, C/T=R491W, C/T=Q495X).

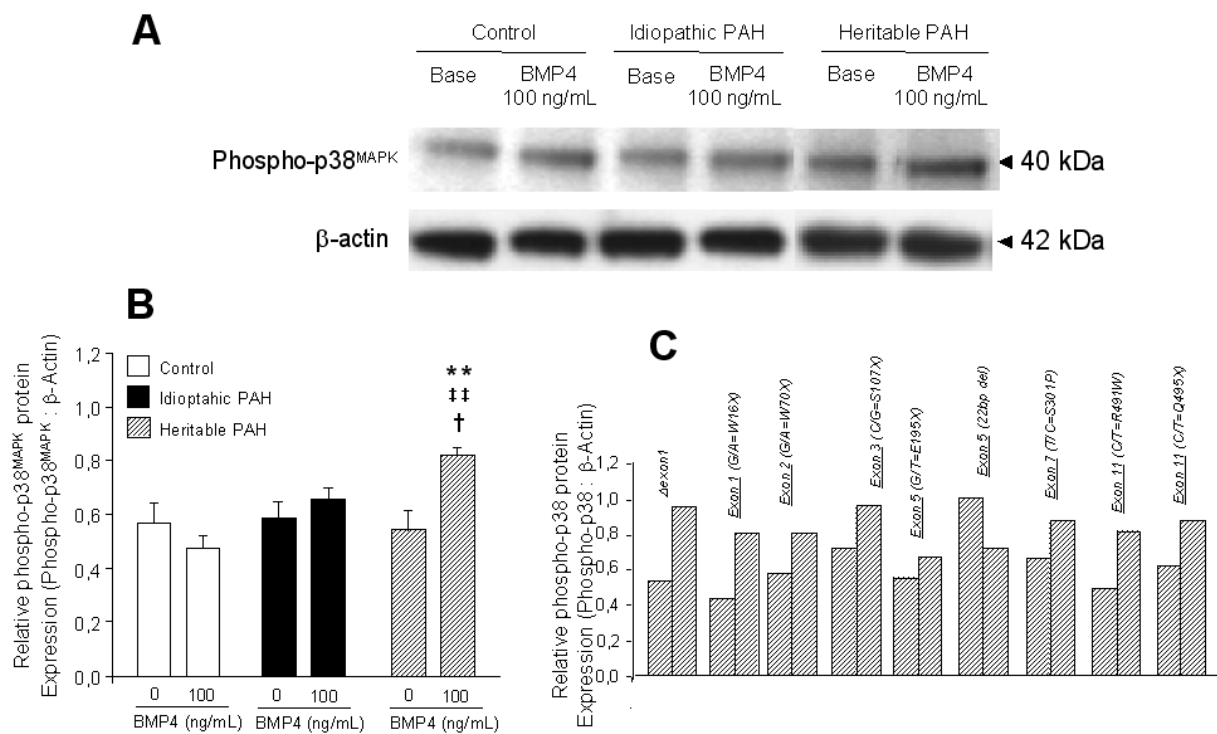


Figure 6

Figure 7. Basal [³H] thymidine incorporation in PSMCs derived from controls (white bars; n=10), PAH patients without (black bars; idiopathic PAH, n=19) and with *BMPR-2* mutations (dashed bars; heritable PAH, n=9) in response to incubation with or without BMP4 (100 ng/mL), in presence of FCS 10% or PDGF (10 ng/mL). Data are presented as mean \pm SEM. *

$P < 0.01$, ** $P < 0.001$ vs PASCs isolated from controls; † $P < 0.05$, †† $P < 0.001$ vs basal conditions in BMP4-nonstimulated PASCs.

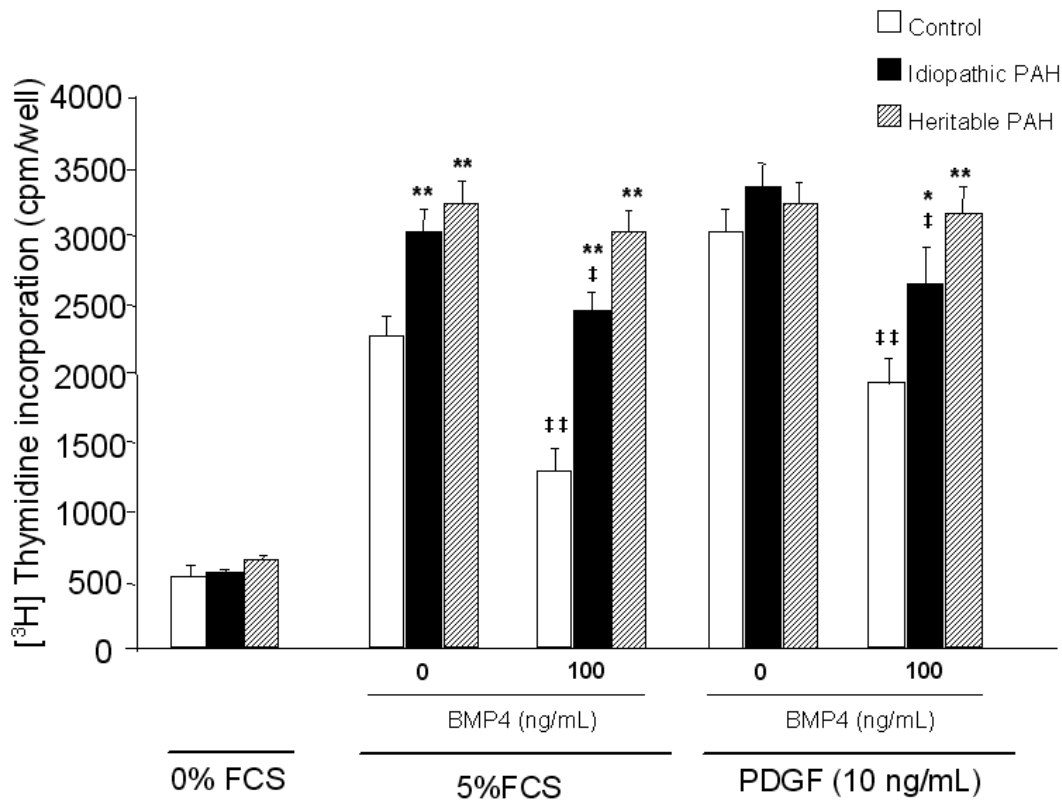


Figure 7

Figure 8. BMP4-induced apoptosis of PASCs isolated from controls (n=10), PAH patients without (idiopathic PAH, n=10) and with *BMPR-2* mutations (heritable PAH, n=9). (A) Flow cytometric analysis of propidium iodide stained control (white bars), idiopathic PAH (black bars) and heritable PAH (dashed bars) PASCs treated with 10 and 100 ng/mL BMP4 to evaluate the distribution of cells in different phases of cell cycle. Apoptotic cells are evaluated as % sub-G0/G1 phase of cell population. (B) Apoptotic index (Bax/Bcl-2 ratio) of BMP4 (100 ng/mL)-treated PASCs isolated from controls (white bars), PAH patients without (idiopathic PAH, black bars) and with *BMPR-2* mutations (heritable PAH, dashed bars).

PASMCs were seeded, synchronized for 48 hours and treated with BMP4 (100 ng/mL). Relative mRNA expression (Bax, Bcl-2) ratio was determined by RTQ-PCR. Data are presented as mean \pm SEM. * $P < 0.05$, ** $P < 0.001$ vs similarly treated PASMCs isolated from controls; ‡ $P < 0.05$, †† $P < 0.001$ vs basal conditions in BMP4-nonstimulated PASMCs.

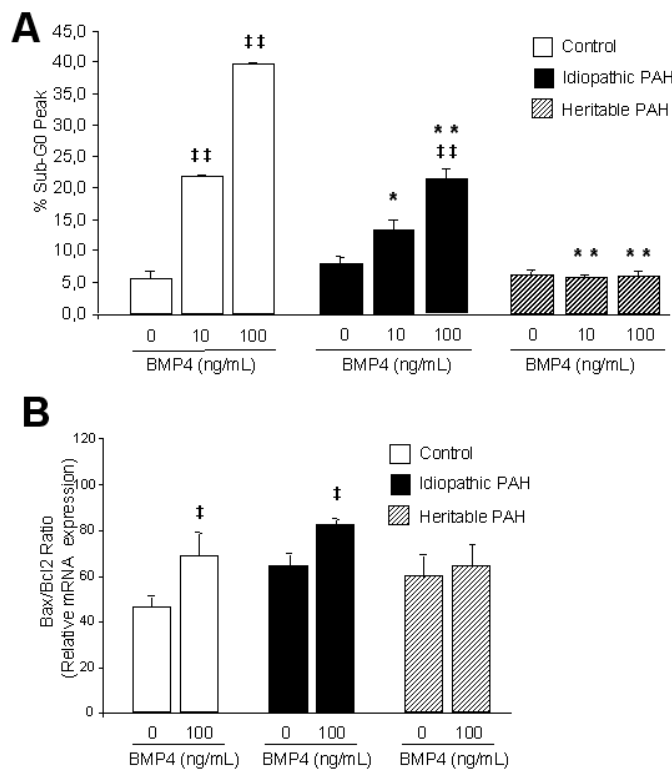


Figure 8