Dysfunctional Smad Signaling Contributes to Abnormal Smooth Muscle Cell Proliferation in Familial Pulmonary Arterial Hypertension

Xudong Yang, Lu Long, Mark Southwood, Nung Rudarakanchana, Paul D. Upton, Trina K. Jeffery, Carl Atkinson, Hailan Chen, Richard C. Trembath, Nicholas W. Morrell

Abstract— Mutations in the bone morphogenetic protein type II receptor gene (BMPR2) are the major genetic cause of familial pulmonary arterial hypertension (FPAH). Although smooth muscle cell proliferation contributes to the vascular remodeling observed in PAH, the role of BMPs in this process and the impact of BMPR2 mutation remains unclear. Studies involving normal human pulmonary artery smooth muscle cells (PASMCs) suggest site-specific responses to BMPs. Thus, BMP-4 inhibited proliferation of PASMCs isolated from proximal pulmonary arteries, but stimulated proliferation of PASMCs from peripheral arteries, and conferred protection from apoptosis. These differences were not caused by differential activation of BMP signaling pathways because exogenous BMP-4 led to phosphorylation of Smad1, p38MAPK, and ERK1/2 in both cell types. However, the proliferative effect of BMP-4 on peripheral PASMCs was found to be p38MAPK/ERK-dependent. Conversely, overexpression of dominant-negative Smad1 converted the response to BMP-4 in proximal PASMCs from inhibitory to proliferative. Furthermore, we confirmed that proximal PASMCs harboring kinase domain mutations in BMPR2 are deficient in Smad signaling and are unresponsive to the growth suppressive effect of BMP-4. Moreover, we show that the pulmonary vasculature of patients with familial and idiopathic PAH are deficient in the activated form of Smad1. We conclude that defective Smad signaling and unopposed p38MAPK/ERK signaling, as a consequence of mutation in BMPR2, underlie the abnormal vascular cell proliferation observed in familial PAH. (Circ Res. 2005;96:1053-1063.)

Key Words: vascular remodeling ■ pulmonary hypertension ■ transforming growth factor-β ■ smooth muscle cells ■ cell signaling

Primary, or idiopathic, pulmonary arterial hypertension (IPAH) is a rare disorder that is progressive and often fatal, leading to death within a median of 3 years from right ventricular failure without treatment.1 The disease is characterized by vascular cell proliferation and obliteration of small pulmonary arteries by smooth muscle cells and myofibroblasts.2 In addition, plexiform lesions comprising endothelial cells and myofibroblasts are found in ∼50% of cases.3 Genetic studies have revealed heterozygous mutations in the BMPR2 gene encoding the type II bone morphogenetic protein receptor (BMPR-II), a member of the TGF-β superfamily of receptors,4,5 underlying the familial form of the disease (FPAH). Subsequently, BMPR2 mutations were found in ∼25% of apparently sporadic cases of IPAH, many of which are examples of familial transmission with low disease gene penetrance.6,7 Although these genetic studies point toward a critical role for the TGF-β superfamily in the regulation of pulmonary vascular cell growth and differentiation, the precise molecular mechanisms leading to disease pathogenesis remain to be elucidated.

Signaling by BMP receptors involves heterodimerization of 2 transmembrane serine/threonine kinases: the constitutively active type II receptor, BMPR-II, and a corresponding type I receptor, BMPR-IA or BMPR-IB. Activated BMPR-I receptors phosphorylate a set of BMP restricted Smad proteins, (Smad1, 5, and 8),8 which then complex with the common partner Smad, Smad4 (Co-Smad), and translocate into the nucleus to regulate transcription of target genes9 in a tissue- and cell-specific manner. Although signaling via Smads is well characterized, there is increasing evidence that mitogen-activated protein kinases (MAPKs), including ERK1/2, JNK, and p38MAPK are activated by BMPs and TGF-βs in certain cell types.10,11 In transfected mouse mammary gland epithelial cells we previously demonstrated that most BMPR-II mutants fail to activate a BMP responsive reporter gene, suggesting a deficiency in Smad mediated
signaling. However, all mutations studied were associated with heightened activation of p38MAPK coupled to increased serum-induced proliferation.

Although the effect of TGF-β on endothelial and smooth muscle cell growth, differentiation, and matrix production has been extensively studied, the effects of bone morphogenetic proteins (BMPs) on vascular cell function are not well characterized. BMPs have been shown to inhibit proliferation of human aortic smooth muscle cells and increase expression of smooth muscle differentiation markers. In addition, BMP-2 inhibits vascular smooth muscle cell proliferation after balloon injury in rats. We have previously shown that BMP-4 inhibits proliferation of PASMCs isolated from the main pulmonary artery of normal subjects but fails to suppress proliferation of PASMCs isolated from patients with IPAH.

In the present study we aimed to determine the major signaling pathways downstream of BMP receptors linked to proliferation and survival, and the impact of BMPR2 mutation on these responses in PASMCs. We demonstrate that Smad1, p38MAPK, and ERK1/2 pathways are activated in normal human PASMCs by BMP-4. However, the stimulation or inhibition of cell proliferation depends on the differential utilization of these pathways. We show that Smad signaling is antiproliferative in PASMCs whereas p38MAPK/ERK is pro-proliferative and antiapoptotic. In addition, we demonstrate that kinase domain mutations in BMPR2 disrupt nuclear translocation of Smads, and that PASMC lines isolated from patients with PAH harboring mutations in BMPR2 are deficient in Smad signaling. Furthermore, we report evidence for deficient Smad1 activation in the pulmonary vasculature of patients with PAH. Our findings suggest that Smad/MAPK signaling exert opposing effects on the proliferation of PASMCs and that abnormal signaling via these pathways as a consequence of mutation in BMPR2 contributes to the abnormal cell proliferation and vascular obliteration that characterize PAH.

Materials and Methods

Culture of Human PASMCs

Proximal and peripheral segments of human pulmonary artery were obtained from unused donors (n=5) for transplantation. The Harefield and Papworth Hospital ethical review committees approved the study, and subjects or relatives gave informed written consent. PASMCs were explanted from proximal lobar arteries and peripheral arteries (<1 to 2 mm external diameter), as previously described. Cells were maintained in 10% fetal bovine serum (FBS)/Dulbecco’s modified Eagle Medium (DMEM) and used between passage 4 and 6. Proximal smooth muscle cell lines were obtained from unused donors (n=10) with or without BMP-4 (50 ng/mL) for up to 90 minutes. After washing and fixing, anti-flag M2 monoclonal antibody (1:500) was added to cells for 1 hour followed by anti-mouse-TRITC (1:500). Finally, the cells were incubated with H-33342 and propidium iodide (PI) staining, and a cellular DNA fragmentation ELISA kit (Roche Molecular Biochemicals, Inc, UK). See online supplement (available at http://circres.ahajournals.org) for further details.

Immunohistochemistry

Lang tissue from patients with pulmonary hypertension and control subjects was obtained from the Papworth Hospital NHS Trust (UK).
tissue bank. Samples were obtained from patients with familial PAH (n=6) and idiopathic PAH (n=6). Patients had received heart-lung transplantation for pulmonary arterial hypertension. DNA extracted from lung tissues was examined for the presence of mutations in the BMPR2 gene, as described previously (supplemental Table I). Control lung (n=6) comprised tissue taken from the uninvolved lobe after pneumonectomy for lung neoplasia or from unused donors. All subjects or their relatives gave informed written consent, and the study had approval from the Local Research Ethics Committee.

Formalin-fixed, wax-embedded lung sections (5 μm) were processed using antigen retrieval techniques, as previously described. Sections were batched and stained with anti–phospho-Smad1 and anti-Smad1 (Upstate, UK). The extent of Smad1 phosphorylation in the smooth muscle of normal and hypertensive arteries (100 to 200 μm diameter) was determined by counting the total number of smooth muscle cell nuclei and the number of nuclei which stained positively for phospho-Smad1, including at least 10 arteries from each case. The percentage of phospho-Smad1 positive nuclei was then calculated for controls, IPAH, and FPAH cases. The percentage of cells whose cytoplasm stained positively for total Smad1 was calculated similarly.

**Statistical Analysis**
Data were expressed as mean±SEM and analyzed with GraphPad Prism version 3.0 (GraphPad Software). Comparisons were made by Student t test or ANOVA with a Tukey post-hoc test, as appropriate. A value of P<0.05 indicated statistical significance.

**Results**
**Differential Effect of BMP-4 on Growth and Apoptosis of PASMCs Derived From Proximal and Peripheral Pulmonary Arteries**
The phenotype of PASMCs derived from proximal and distal pulmonary arteries was similar as assessed by the expression of the smooth muscle markers, α-smooth muscle actin, smooth muscle myosin, vimentin, and fibronectin (Figure 1A through 1H). PASMCs derived from proximal pulmonary artery exhibited inhibition of [3H]-thymidine incorporation in peripheral PASMCs over 24 hours (open bars) but inhibited [3H]-thymidine incorporation in proximal PASMCs (I). Cell counts confirmed a similar differential effect on serum-stimulated cell proliferation (J and K). *P<0.05, **P<0.01 compared with control or baseline.
Peripheral PASMCs exhibited a concentration-dependent increase in [3H]-thymidine incorporation and proliferation in response to BMP-4 (Figure 1I). The absolute rate of [3H]-thymidine incorporation was higher at baseline in distal cells (289 ± 33 cpm) compared with proximal cells (1063 ± 96 cpm), reflecting the increased proliferative capacity of distal versus proximal cells, as previously reported.17 This difference was confirmed by cell counting, though the differential response to BMP-4 was again evident (Figure 1J through 1K). Furthermore, in peripheral but not proximal PASMCs, BMP-4 conferred protection from staurosporine induced apoptosis, as assessed by nuclear morphology and by DNA fragmentation ELISA. Cells incubated in serum free media exhibited 3% to 4% apoptosis rate after 24 hours. In the presence of staurosporine, apoptosis affected 40% of cells. Interestingly, the survival effect of BMP-4 in peripheral PASMCs was greater at lower concentrations of BMP-4, where the effect was similar to that seen with IGF-1 (30 ng/mL) (I). These effects were confirmed using a DNA fragmentation ELISA (J). *P<0.05, **P<0.01, ***P<0.001 compared with staurosporine treated cells, n=5 for proximal and peripheral cells.

BMP-4 Binds to Human PASMCs and Signals via Smad1 and p38MAPK

Proximal and peripheral PASMCs exhibited specific 125I-BMP-4 binding sites and expressed mRNA for all type I (ALK1–6) and type II (BMPR-II, TGF-βRII, ActRII, and ActRIIB) receptors examined (data not shown). Specific binding was ~50% of total binding and unlabeled BMP-4 produced concentration-dependent competition of 125I-BMP-4 binding, with an IC50 of 1.35 ± 4.5 ng/mL (Figure 3A). To determine the presence of intact BMP-4–mediated signal transduction pathways in proximal and peripheral PASMCs, we studied the phosphorylation of Smad1, p38MAPK, and ERK1/2, and the induction of mRNA for inhibitory Smads 6 and 7. Immunoblotting demonstrated that BMP-4 (50 ng/mL) led to phosphorylation of Smad1 and p38MAPK within 1 hour in both cell types (Figure 3B) and activation of ERK1/2 within minutes. Furthermore, BMP-4 led to induction of mRNA for the known BMP-inducible genes, Smad 6 and 7, in both proximal and peripheral cells within 2 hours (Figure 3D).

p38MAPK and ERK1/2 Mediates BMP-4–Stimulated Proliferation in Peripheral PASMCs

Having demonstrated that BMP-4 leads to phosphorylation of p38MAPK and ERK1/2 in proximal and peripheral PASMCs, we investigated the role of these pathways in PASMC proliferation. In peripheral PASMCs, the selective p38MAPK
inhibitor, SB203580 (1 μmol/L), and the ERK1/2 inhibitor, U0126 (1 μmol/L), both inhibited the proliferative response to BMP-4 (Figure 4A). In contrast, SB203580 and U0126 had no effect on the inhibition of [3H]-thymidine incorporation by BMP-4 observed in PASMCs derived from proximal pulmonary arteries (Figure 4B).

Smad1 Mediates Growth Inhibition in PASMCs

We used transient transfection of a dominant-negative Smad1 to determine the role of Smad1 signaling in the growth responses to BMP-4. First we confirmed that PASMCs transfected with Flag-Smad1 exhibited nuclear translocation of Smad1 in response to BMP-4 stimulation (Figure 5A). Transfection efficiency ranged between 40% to 60% based on expression of Flag-Smad1. Next, cotransfection of cells with Flag-Smad1 and DN-Smad1 demonstrated that DN-Smad1 prevented the nuclear accumulation of Smad1 (Figure 5A through 5H). In growth assays, DN-Smad1 reversed the BMP-4–induced inhibition of [3H]-thymidine incorporation observed in proximal PASMCs (Figure 5I). In fact, there was a significant increase in [3H]-thymidine incorporation in response to BMP-4 in proximal PASMCs transfected with DN-Smad1. DN-Smad1 had no effect on the BMP-4–induced proliferation observed in peripheral PASMCs (Figure 5J).

p38MAPK, But Not Smad1, Is Involved in BMP-4 Mediated Survival in PASMCs

The survival effect of BMP-4 on staurosporine-induced apoptosis in peripheral PASMCs was almost completely reversed in the presence of the p38MAPK inhibitor, SB203580.
However, BMP-4 had no effect on survival of proximal PASMCs, and this was not influenced by SB203580. Transient transfection of cells with DN-Smad1 did not influence the survival response to BMP-4, in the presence or absence of SB203580 (Figure 6B).

Mutation in the Kinase Domain of BMPR-II Inhibits Smad Signaling and Promotes BMP-4–Induced Proliferation

PASMCs derived from proximal pulmonary artery were obtained from 3 patients with FPAH. Two of these mutations involved the kinase domain of BMPR-II (R491W and C347 Y), and 1 involved the cytoplasmic tail (N903S). These cells were used to investigate whether naturally occurring disease-causing mutations in BMPR-II disrupt BMP signaling and alter cell function. Comparison of BMP-4–induced phosphorylation of Smad1 and p38MAPK in kinase domain mutants versus control PASMCs demonstrated reduced Smad1 phosphorylation in mutant cells but similar activation of p38MAPK and ERK1/2 (Figure 7A). The cytoplasmic tail domain mutant (N903S) also reduced Smad1 phosphorylation, though to a lesser extent than the kinase domain mutants (Figure 7B).

In addition, using a BMP-responsive luciferase reporter gene construct we show that, whereas control cells exhibited an increase in luciferase activity when stimulated with BMP-4, kinase mutant cells were unable to activate the reporter (Figure 7C). The growth suppressive response to BMP-4 was compared in control cells derived from the proximal pulmonary artery and PASMCs derived from 3 patients with idiopathic PAH, in whom no BMPR2 mutation was detectable, and 3 mutant PASMC isolates (Figure 7D). The growth suppressive response to BMP-4 was markedly attenuated in mutant PASMCs compared with control cells, but also in idiopathic PAH, though the effect was not so marked.

Suppression of Smad1 Activation in PAH Vasculature

Phospho-Smad1 was clearly expressed in normal lung, being localized to pulmonary capillary endothelial cells in the normal lung parenchyma and to the endothelial cells of small peripheral pulmonary arteries, but was also present in smooth muscle cells comprising the media and intima of muscular arteries (Figure 8A through 8C). At higher magnification, much of the phospho-Smad1 staining had a nuclear localization (Figure 8D through 8F). In PAH lungs, both with and without mutations in BMPR2, fewer medial and intimal cells expressed phospho-Smad1 compared with control arteries (Figure 8J). Serial sections were stained with total Smad1, which showed similar levels of staining in controls and cases (Figure 8G through 8I).

Discussion

This study has demonstrated that BMP-4 exerts complex effects on the growth and survival of PASMCs. In these studies we found that BMP-4 caused either inhibition or stimulation of cell proliferation, depending on the site of origin of cells. Thus, normal PASMCs isolated from the proximal (main) pulmonary artery were inhibited, whereas normal PASMCs isolated from peripheral arteries (1 to 2 mm diameter) were stimulated to proliferate in the presence of
BMP-4. These differences allowed us to explore the relative contribution of the major BMP-mediated signaling pathways to the observed responses. Interestingly, we found that BMP-4 activated both the canonical BMP signaling peptide, Smad1, and the Smad-independent p38MAPK and ERK1/2 pathway in proximal and peripheral cells. Nevertheless, inhibition of p38MAPK or ERK1/2 using selective inhibitors blocked the proliferative response to BMP-4 in peripheral PASMCs. Conversely, inhibition of Smad1 nuclear accumulation, using dominant-negative Smad1, reversed the inhibition of proliferation observed in proximal PASMCs, and in fact allowed proliferation of these cells in the presence of BMP-4. Therefore, although both potentially antiproliferative (Smad1) and pro-proliferative (p38MAPK, ERK1/2) pathways are activated in PASMCs from proximal and peripheral arteries, the utilization of these signals appears to differ between cell types.

In general, the response to TGF-β/BMP signaling occurs in a cell type–specific manner, giving rise to the context-specific nature of TGF-β responses.22 Many genes contain Smad-binding elements in their promoter regions.10 Smad access to target genes depends on the presence and recruitment of transcriptional coactivators and corepressors. Some of these cofactors are ubiquitous whereas others are cell type–restricted.9 Thus differences in utilization of Smad signals between proximal and peripheral PASMCs may have been responsible for the differential effects of BMP-4 on growth and survival in these cells, a possibility that warrants further investigation. Interestingly, we observed consistent induction of mRNA for inhibitory Smads 6 and 7 in both cell types studied, suggesting that at least part of the BMP-Smad signaling pathway is conserved. However, it is recognized that induction of the inhibitory Smads by TGF-β/BMP is largely cell type–independent.9 Alternatively, it remains pos-
sible that other signaling pathways downstream of BMP receptors are differentially activated in the 2 PASMC types.

These studies extend our previous observations of phenotypic diversity between PASMCs isolated from the proximal and peripheral human pulmonary circulation, a phenomenon recognized in the bovine lung. It is likely that these differences are a consequence of the different developmental origins of these cells. Whereas the proximal (main) pulmonary artery is mainly derived from neural crest, peripheral pulmonary arteries are formed predominantly by vasculogenesis within the developing lung mesenchyme.

A further novel finding of this study is that primary cultured PASMCs harboring a mutation in the kinase domain of BMPR2 demonstrated defective Smad signaling, as evidenced by reduced activation of phospho-Smad1 and by reduced transcription of a BMP/Smad responsive luciferase reporter gene. Although we have previously shown this in HeLa cells overexpressing mutant protein, this is the first demonstration in primary cultured pulmonary vascular cells harboring a defined mutation in BMPR2. Moreover, mutant proximal PASMCs cells were resistant to the growth suppressive effects of BMP-4. Taken together our results suggest that BMP-mediated Smad signaling exerts a powerful antiproliferative effect in some PASMC populations, and that loss of this effect as a consequence of mutation in BMPR2 could contribute to the abnormal pulmonary vascular cell proliferation observed in PAH. Interestingly, PASMCs harboring a mutation in the cytoplasmic tail (N903S) of BMPR-II were also partially deficient in Smad signaling, though to a lesser extent than the kinase domain mutants. The role of the BMPR-II cytoplasmic tail in the activation of Smads is not known, but we have previously demonstrated that truncation of the cytoplasmic tail reduces the efficiency with which BMPR-II phosphorylates Tctex-1, a light chain of the motor complex dynein.

In this study we included a comparison of the growth suppressive effect of BMP-4 on PASMCs derived from PAH patients in whom no mutation was detected in the coding sequence of BMPR2. It should be noted that the techniques used for mutation analysis may have missed large deletions or noncoding mutants, and the true prevalence of BMPR2 mutation in familial and sporadic disease remains to be determined. PASMCs from idiopathic PAH patients exhibited an attenuated growth suppressive response to BMP-4 compared with control cells, but this was not as marked as in familial PAH cells. We could not demonstrate a consistent reduction on phospho-Smad1 in response to BMP-4 in these cells (data not shown). Nevertheless, our immunohistochemical study did show a reduced proportion of medial and intimal cells expressing phospho-Smad1 in both familial and idiopathic PAH cases. Taken together, these findings suggest that dysfunction of BMP signaling may contribute to idiopathic PAH in the absence of BMPR2 mutations. In support of this, reduced expression of BMP type 1 receptor has been
reported in diverse forms of pulmonary arterial hypertension.27

In previous studies in transfected cells11 we were able to demonstrate that the presence of a mutant BMPR-II receptor led to constitutive activation of p38MAPK even in the absence of ligand stimulation. In the present study we were not able to confirm constitutive activation of p38MAPK in primary cultured PASMCs harboring a mutation in \( \text{BMPR}_2 \). However, our findings suggest that BMP-mediated activation of p38MAPK exerts a pro-proliferative effect in proximal PASMCs when Smad1 signaling is silenced and drives proliferation in peripheral PASMCs. Interruption of p38MAPK signaling may provide a new target for antiproliferative strategies aimed at preventing or reversing vascular remodeling in PPH associated with \( \text{BMPR}_2 \) mutation.

We have previously demonstrated reduced pulmonary vascular expression of BMPR-II protein in the concentric and plexiform lesions of PAH in patients harboring mutations in \( \text{BMPR}_2 \).2 Most striking in that study was the widespread reduction in alveolar capillary BMPR-II expression seen in patients with and without mutations. In the present study we have extended these observations to demonstrate that a reduced proportion of smooth muscle cells express the activated form of Smad1 within media and intima of small arteries in patients with IPAH and FPAH. Our immunohistochemical studies strongly support the contention that dysregulation of Smad1 may be critical in promoting lesion formation in PAH.

The effects of TGF-\( \beta \)/BMPs on apoptosis are highly cell type-specific. During development, BMP-4 signaling is necessary for apoptosis during mouse limb bud development.28 In contrast, BMP-4 prevents apoptosis in the developing mouse metanephric mesenchyme.29 A recent report found that BMP-2 and BMP-7 induced apoptosis in normal human PASMCs, and that apoptosis was suppressed in PASMCs isolated from patients with PAH.30 In our study, BMP-4 clearly protected against staurosporine-induced apoptosis in peripheral PASMCs, whereas BMP-4 had no significant effect on apoptosis in PASMCs derived from the proximal pulmonary artery. The survival effect of BMP-4, similar to the effect of BMP-4 on proliferation, was mediated by p38MAPK and was independent of Smad1.

How can the apparently opposite effects of BMP-4 on the proliferation of PASMCs from proximal and peripheral pul-

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**Figure 7.** Mutant (R491W) cells demonstrated reduced phosphorylation of Smad1 compared with control cells, but no difference in the phosphorylation of p38MAPK or ERK1/2 was observed (A). Activation of Smad1 in response to 1 ng/mL BMP-4 was reduced in all mutant cell preparations compared with control cells studied, though this was more evident in the kinase domain than in the cytoplasmic tail mutant (B). Mutant cells showed reduced BMP-4–stimulated transcriptional activity of a BMP/Smad responsive luciferase reporter gene (C). Inability of BMP-4 to inhibit proliferation of mutant PASMCs compared with control cells (D). Cells from IPAH patients with no detectable mutation in \( \text{BMPR}_2 \) also exhibited a reduced response to BMP-4, though to a lesser extent than mutant cells. Results represent the mean of 3 separate experiments (C and D), or are representative of at least 3 experiments (A and B).
monary artery be contributing to the pathogenesis of the occlusive vascular lesions observed in patients with PAH? The effect of BMPR2 mutation in proximal cells will be to counteract the normal inhibitory effect of BMP-4 on cell proliferation, an effect that we have confirmed in mutant cells derived from proximal artery. However, we were unable to study the effects of BMP-4 on the proliferation of PASMCs derived from the peripheral pulmonary circulation and harboring a mutation in BMPR2, because of the lack of availability of these cells. Peripheral cells exhibit p38MAPK-dependent proliferation and survival in response to BMP-4. It is conceivable that mutant BMPR-II in these cells further promotes exaggerated proliferation/survival by abnormal activation of p38MAPK and suppression of Smad signaling. Thus, BMPR2 mutation would favor abnormal proliferation of both proximal and peripheral cells.

In summary, this study has shown that BMP exerts complex effects on the growth and survival of PASMCs. However, we describe a predominant role for Smad1 in growth suppression, and p38MAPK and ERK1/2 as a mitogenic pathway in PASMCs in response to exposure to BMP-4. Furthermore we have provided evidence that dysfunction of Smad1, as a consequence of BMPR2 mutation in primary cultured cells, contributes to the failure to suppress cell proliferation, an effect that may be amplified by abnormal activation of p38MAPK. These observations may generate novel potential targets for the treatment of familial PAH aimed at inhibition of abnormal vascular cell proliferation.

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References


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METHODS

Apoptosis assays
PASMCs were plated at $10^4$/well in 8-well glass chamber slides (Merck, UK) and grown to a near-confluence. Staurosporine (SP, 50nM) in serum-free DMEM for 6 hr was used to induce apoptosis, in the presence or absence of BMP-4 (1-100 ng/ml). The smooth muscle cell survival factor, IGF-I (30 ng/ml), was used as a positive control. After 6 hr, adherent cells were stained with a mixture of nuclear chromatin stain H-33342 (1µg/ml) and propidium iodide (PI; 5µg/ml) for 15 min. One hundred nuclei from random fields were analysed for each treatment. Apoptotic cells were expressed as percentage of the condensed nuclei/total number of nuclei.

In addition, a cellular DNA fragmentation ELISA kit was used for detection of bromodeoxyuridine (BrdU)-labelled DNA fragments in cells, following the manufacturer’s instructions.

$^{125}$I-BMP-4 competition binding studies
For binding assays cells were initially washed twice for 5 min in binding buffer (DMEM/0.5% BSA + 23mM HEPES), and then incubated in the binding buffer for 1 hr at 37°C. Cells were then incubated with binding buffer containing $^{125}$I-BMP-4 ligand in the presence or absence of unlabelled BMP-4 (0.03-300 ng/ml) at 4°C for 2 hr with gentle rocking. Then cells were then washed 3 times with ice-cold binding buffer and lysed (0.2M NaOH with 0.1% SDS). The lysates were counted in a gamma-counter.
Reverse transcription-polymerase chain reaction

The primers were: Smad6 (5’), 5’-CCC CCG GCT ACT CCA TCA AGG TGT-3’; Smad6 (3’), 5’-GTC CGT GGG GGC TGT GTC TCT GG-3’; Smad7 (5’), 5’-GTG GGG AGG CTC TAC TGT GTC-3’; Smad7 (3’), 5’-GTC GAA AGC CTT GAT GGA GAA ACC-3’. The amount of starting material and the number of cycles were selected so that amplified product signal was semi-quantitatively related to the input of RNA. β-actin (sense primer: 5’-ATG AAG TGT GAC GTT GAC ATC CG-3’ and antisense primer: 5’-GCT TGC TGA TCC ACA TCT GCT G-3’) was used as an internal control. PCR products were visualised by electrophoresis in 2% agarose gels stained with ethidium bromide. Control reactions were run without the addition of reverse transcriptase. The identity of PCR products was confirmed by direct sequencing. In additional RT-PCR studies we examined the expression of TGF-β/BMP type 1 receptors, ALK1-6, and type II receptors using primers previously described 1,2.

Immunoblotting for BMP signalling pathways

PASMCs were seeded at 5x10^5 in 10cm dishes and grown to ~95% confluence. After 48 hr quiescence, cells were treated with or without BMP-4 (1-100ng/ml) in 0.1% FBS/DMEM for up to 90 min. At specified time points cells were lysed in buffer as described previously 3. Samples (100µg) were electrophoresed by 10% SDS-PAGE, and then transferred to nitrocellulose membranes by semi-dry transfer at 100mA for 1 hr. Following incubation with the appropriate HRP-conjugated secondary antibody blots were treated with ECL-reagents and exposed to radiographic film.
REFERENCES


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PAP indicates pulmonary arterial pressure; VSD, ventricular septal defect; ASD, atrial septal defect; FPAH, familial PAH; IPAH, idiopathic PAH; NA, not available; nd, not detected.
Online supplementary information

METHODS

Apoptosis assays
PASMCs were plated at 10^4/well in 8-well glass chamber slides (Merck, UK) and grown to a near-confluence. Staurosporine (SP, 50nM) in serum-free DMEM for 6 hr was used to induce apoptosis, in the presence or absence of BMP-4 (1-100 ng/ml). The smooth muscle cell survival factor, IGF-I (30 ng/ml), was used as a positive control. After 6 hr, adherent cells were stained with a mixture of nuclear chromatin stain H-33342 (1µg/ml) and propidium iodide (PI; 5µg/ml) for 15 min. One hundred nuclei from random fields were analysed for each treatment. Apoptotic cells were expressed as percentage of the condensed nuclei/total number of nuclei.

In addition, a cellular DNA fragmentation ELISA kit was used for detection of bromodeoxyuridine (BrdU)-labelled DNA fragments in cells, following the manufacturer’s instructions.

125I-BMP-4 competition binding studies
For binding assays cells were initially washed twice for 5 min in binding buffer (DMEM/0.5% BSA + 23mM HEPES), and then incubated in the binding buffer for 1 hr at 37°C. Cells were then incubated with binding buffer containing 125I-BMP-4 ligand in the presence or absence of unlabelled BMP-4 (0.03-300 ng/ml) at 4°C for 2 hr with gentle rocking. Then cells were then washed 3 times with ice-cold binding buffer and lysed (0.2M NaOH with 0.1% SDS). The lysates were counted in a gamma-counter.
**Reverse transcription-polymerase chain reaction**

The primers were: Smad6 (5’), 5’-CCC CCG GCT ACT CCA TCA AGG TGT-3’; Smad6 (3’), 5’-GTC CGT GGG GGC TGT GTC TCT GG-3’; Smad7 (5’), 5’-GTG GGG AGG CTC TAC TGT GTC-3’; Smad7 (3’), 5’-GTC GAA AGC CTT GAT GGA GAA ACC-3’. The amount of starting material and the number of cycles were selected so that amplified product signal was semi-quantitatively related to the input of RNA. β-actin (sense primer: 5’-ATG AAG TGT GAC GTT GAC ATC CG-3’ and antisense primer: 5’-GCT TGC TGA TCC ACA TCT GCT G-3’) was used as an internal control. PCR products were visualised by electrophoresis in 2% agarose gels stained with ethidium bromide. Control reactions were run without the addition of reverse transcriptase. The identity of PCR products was confirmed by direct sequencing. In additional RT-PCR studies we examined the expression of TGF-β/BMP type 1 receptors, ALK1-6, and type II receptors using primers previously described \(^1\)\(^2\).

**Immunoblotting for BMP signalling pathways**

PASMCs were seeded at 5x10^5 in 10cm dishes and grown to ~95% confluence. After 48 hr quiescence, cells were treated with or without BMP-4 (1-100ng/ml) in 0.1% FBS/DMEM for up to 90 min. At specified time points cells were lysed in buffer as described previously\(^3\). Samples (100μg) were electrophoresed by 10% SDS-PAGE, and then transferred to nitrocellulose membranes by semi-dry transfer at 100mA for 1 hr. Following incubation with the appropriate HRP-conjugated secondary antibody blots were treated with ECL-reagents and exposed to radiographic film.
REFERENCES


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