BMP4 inhibits proliferation and promotes myocyte differentiation of lung fibroblasts via Smad1 and JNK pathways

Trina K. Jeffery,1 Paul D. Upton,1 Richard C. Trembath,2 and Nicholas W. Morrell1

1Division of Respiratory Medicine, Department of Medicine, University of Cambridge, School of Clinical Medicine, Addenbrooke’s Hospital, Cambridge; and 2Division of Medical Genetics, University of Leicester, Leicester, United Kingdom

Submitted 28 June 2004; accepted in final form 27 October 2004

THE PULMONARY CIRCULATION is a high-flow, low-resistance system. However, in primary pulmonary hypertension (PPH) there remain poorly understood. BMPRII belongs to the transforming growth factor (TGF)-β superfamily of receptors (including TGF-β, BMP, and activin receptors) and is a constitutively active serine/threonine kinase receptor, signaling via formation of heterocomplexes with one of three type I receptors (ALK-3/BMPRIA, ALK-6/BMPRIB, or ALK-2) in response to ligand (BMP2, BMP4, and BMP7). BMPRII phosphorylates the associated type I receptor, thereby activating intracellular signaling via the Smad family of proteins (reviewed in Refs. 20 and 36), although alternative pathways involving the MAPK, including p38MAPK and c-Jun NH2-terminal kinases (JNK), are now recognized (36). The role that BMPRII and its associated ligands play in pulmonary vascular remodeling remains unclear. Thus, to further understand the functional significance of BMPRII in the pulmonary vasculature, we examined the expression of TGF-β superfamily receptors in human fetal lung fibroblasts (HFL) and investigated the role of BMP4 on cell cycle regulation, fibroblast proliferation, and differentiation. Furthermore, signaling pathways involved in these processes were examined. HFL expressed BMPRI and BMPRII mRNA and demonstrated specific BMP binding sites. BMP4 inhibited [3H]thymidine incorporation and proliferation of HFL; protein expression was increased for the cell cycle inhibitor p21 and reduced for the positive regulators cyclin D and cdk2 by BMP4. BMP4 induced differentiation of HFL into a smooth muscle cell phenotype since protein expression of α-smooth muscle actin and smooth muscle myosin was increased. Furthermore, p38MAPK, ERK1/2, JNK, and Smad1 were phosphorylated by BMP4. Using specific MAPK inhibitors, a dominant negative BMPRII construct, and Smad1 siRNA, we found that the antiproliferative and prodifferentiation effects of BMP4 were Smad1 dependent with JNK also contributing to differentiation. Because failure of Smad phosphorylation is a major feature of BMPRII mutations, these results imply that BMPRII mutations may promote the expansion of fibroblasts resistant to the antiproliferative, prodifferentiation effects of BMPs and suggest a mechanism for the vascular obliteration seen in familial PPH.

Bone morphogenetic protein type II receptor; α-smooth muscle actin; mitogen-activated protein kinase; cell cycle; pulmonary vascular remodeling. Thus, to further understand the functional significance of BMPRII in the pulmonary vasculature, we examined the expression of TGF-β superfamily receptors in human fetal lung fibroblasts (HFL) and investigated the role of BMPs on cell cycle regulation, fibroblast proliferation, and differentiation. Furthermore, we examined the signaling pathways involved in these processes. Our results indicate that BMP4 is antiproliferative and induces myocyte differentiation. This requires the activation of Smad1 and for differentiation also involves the phosphorylation of the MAPK JNK.

MATERIALS AND METHODS

Cell culture. HFL (HFL-1; European Collection of Cell Cultures) were grown in Ham’s F-12 media (BioWhittaker) containing 10% FCS, 2 mM glutamine, 1% nonessential amino acids, and antimicrobics/antibiotics at 37°C.

Expression profile of BMP receptors by RT-PCR. Because the characterization of the TGF-β family of receptors and its ligands has not been described previously for HFL, we sought to examine which members were expressed. For these experiments, mRNA expression of TGF-β, BMP, and activin type I and II receptors was determined. In addition, the expression of the reported ligands for BMPRII, BMP2, BMP4, and BMP7 was also examined; for these experiments, mRNA from Saos cells was used as a positive control.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
since these cells have previously been shown to express these ligands (35). Total RNA was isolated from HFL-1 grown in 10% FCS with TRIZol reagent (Life Technologies), and RT-PCR was carried out using the Access RT-PCR System (Promega). Sequences of the primers have been previously described elsewhere (27) and were synthesized by Sigma-Genosys. PCR products were visualized by electrophoresis in agarose (2%) gels stained with ethidium bromide. Control reactions were run without the addition of reverse transcriptase.

Radioligand binding studies. Competitive radioligand binding studies using 125I-BMP4 were performed. Human recombinant BMP4 (R&D Systems) was iodinated using the chloramine T-method, as previously described (10). Cells were grown to confluence in Ham’s F-12 media/10% FCS in 24-well plates and were preincubated with Ham’s F-12 containing 0.5% BSA for 1 h at 37°C. This was followed by incubation at 4°C for 2 h with Ham’s F-12/0.5% BSA containing 125I-BMP4 (15 pM or 0.6 ng/ml; sp act 454–553 Bq/fmol) in the absence or presence of increasing concentrations (0.1–100 ng/ml) of unlabeled BMP2, BMP4, BMP7, TGF-β, and activin A (R&D Systems). Cells were then lysed with 50 mM HEPES (pH 7.4), 10% sample buffer (0.5 M Tris-HCl, 1% Triton, 10% glycerol, 100 mM NaCl, 0.5 μCi/well; Amersham), which was allowed to incorporate for 6 h. The amount of radioactivity in the TCA (10% trichloroacetic acid) insoluble fraction precipitated by 0.2 M NaOH was measured. To determine the effects of BMP4 on cell proliferation, cells were plated as described above and were grown in 5% FCS containing Ham’s F-12 media with or without BMP4 (50 ng/ml); the media was replaced every 2–3 days. Cells were stained with propidium iodide and counted using a hemacytometer.

Flow cytometry. To determine at what phase of the cell cycle BMP4 acts, flow cytometric analysis of propidium iodide-stained cells was carried out. Propidium iodide is a fluorophore that binds to DNA, and hence the amount bound to cells is directly proportional to the amount of DNA in the nucleus. Thus the relative distribution of cells throughout the different phases of the cell cycle can be quantitated for any given cell population. Cells, once 80–90% confluent, were grown to confluence in serum-free Ham’s F-12 media for 48 h. Ham’s F-12 containing 5% FCS with or without BMP4 was then added for 18 h before addition of [methyl-3H]thymidine (0.5 μCi/well; Amersham), which was allowed to incorporate for 6 h. The amount of radioactivity in the TCA (10% trichloroacetic acid) insoluble fraction precipitated by 0.2 M NaOH was measured. To determine the effects of BMP4 on cell proliferation, cells were plated as described above and were grown in 5% FCS containing Ham’s F-12 media with or without BMP4 (50 ng/ml); the media was replaced every 2–3 days. Cells were stained with propidium iodide and counted using a hemacytometer.

Immunoblotting. Protein for cell cycle analysis was harvested by washing cells in cold PBS and scrapping cells into 100 μl of lysis buffer (20 mM Tris·HCl, 1% Triton, 10% glycerol, 100 mM NaCl, and 1 mM EDTA, pH 7.2–7.4) containing Complete Protease Inhibitor Cocktail (Roche), PMSF (1 mM), sodium fluoride (50 mM), and sodium orthovanadate (0.1 mM). Samples were incubated on ice for 30 min and centrifuged at 120,000 g for 15 min, and the supernatant was collected and stored at −20°C. Protein for α-smooth muscle actin (α-SMA), MAPK, and Smad1 was harvested by washing cells with cold PBS and immediately freezing them in an ethanol-dry ice bath. Protein was collected by scraping cells into 40 μl of 2× protein sample buffer (0.5 M Tris·HCl, 4% SDS, 20% glycerol) containing Complete Protease Inhibitor Cocktail. Samples were then boiled for 5 min before centrifugation and stored at −20°C.

Proteins (5–50 μg) were separated by SDS-PAGE (10 or 12%) and transferred to nitrocellulose membranes. For cell cycle proteins, membranes were blocked in 1% milk powder/0.1% Tween in Tris-buffered saline (TBS) for 1 h at room temperature. Blots were then incubated with anti-p21, cyclin D, or cdk2 (1:100; Santa Cruz Biotechnology) in 1% milk powder/0.1% Tween in TBS overnight at 4°C. For all other proteins of interest, membranes were blocked in 5% milk powder/0.1% Tween in TBS for 1 h at room temperature. Blots were incubated with anti-phospho and total p38, p44/42, JNK, and Smad1 antibodies (1:1,000; Cell Signaling), anti-smooth muscle myosin, anti-α-SMA, and anti-β-actin antibodies (1:500, 1:1,000, and 1:2,000, respectively; Sigma) in 5% BSA/0.1% Tween in TBS. For all blots, membranes were washed and then incubated with the appropriate horseradish-conjugated secondary antibody (1:2,000–5,000; Dako and Stressgen) in 1–5% milk powder/0.1% Tween in TBS for 1 h at room temperature. Bands were visualized by chemiluminescence (ECL; Amersham).

Immunofluorescence. Fibroblasts grown on coverslips or chamber slides were quiesced for 48 h in serum-free media and then treated with BMP4 (50 ng/ml). After treatment, cells were washed and fixed in 100% ice-cold methanol for 10 min at 0°C. For α-SMA staining, cells were washed in PBS/0.1% BSA and incubated in primary antibody (Sigma; 1:100 in PBS/0.05% BSA) for 1 h at room temperature, washed, and then incubated with an anti-mouse antibody conjugated to FITC (1:1,000; Dako) for 1 h at room temperature. Immunostaining for phospho-Smad1 was carried out according to the manufacturer’s instructions (1:100, Cell Signaling; anti-rabbit FITC 1:500). Slides were then washed, incubated with Hoechst (1 ng/ml) for 10 min, mounted in PBS/glycerol, and then examined using a fluorescence microscope.

Transfection. Cells were transfected using Lipofectamine 2000 (Invitrogen). Preparation of the transfection reactions was carried out according to the manufacturer’s instructions. The following plasmids were used: pcDNA 3.0 and pcDNA-BMPR2 (D485G). siRNA suppression of p21 and Smad1. p21 small interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology (SC-29427). A pool of siRNA against Smad1 was produced using the Invitrogen Dicer kit, following the manufacturer’s instructions. Fibroblasts were transfected with p21 (100 nM) or Smad1 siRNA (500 ng/ml) using Lipofectamine 2000 according to the manufacturer’s protocol. Western blots for p21 and Smad1 expression were carried out to ensure that the relevant siRNAs caused knockdown of p21 or Smad1 expression.

Statistics. Data were expressed as means ± SE and analyzed with GraphPad Prism version 3.0 (GraphPad Software, San Diego, CA). Comparisons were made by Student’s t-test (2-tailed; paired and unpaired) or one-way ANOVA with Dunnet’s post hoc test, as appropriate. A value of P < 0.05 indicated statistical significance.

RESULTS

BMP receptor and ligand profile in HFL. The expression of the TGF-β family of receptors has not been previously reported for HFL. Using RT-PCR on total RNA extracted from HFL and whole lung, we found that mRNA transcripts for BMP type I (BMPRIA and BMPRIB; also known as ALK-3 and -6, respectively) and type II receptors (Fig. 1A) as well as their ligands BMP2 and BMP4 but not BMP7 (Fig. 1B). In addition, TGF-β type I (ALK-1 and ALK-5) and II receptors were expressed as was the activin type I (Act-R1) and II (Act-R1 and Act-R1B) receptors (Fig. 1A).

Receptor binding studies. Specific binding of 125I-BMP4 was demonstrated in HFL cells and was 58.1 ± 3.6% of total binding. Competition binding curves demonstrated concentration-dependent competition by unlabeled BMP4, and analysis
of the competition profile implied a two-site curve (Fig. 2). The IC₅₀ value for the high-affinity sites was 1.02 ± 0.41 ng/ml, and these sites comprised 44.9 ± 4.3% of the specific binding sites (n = 3 curves). An IC₅₀ value of 44.4 ± 23.3 ng/ml was calculated for the low-affinity sites (n = 3 curves). Binding of ¹²⁵I-BMP4 was inhibited to a similar degree by BMP2, although IC₅₀ values could not be determined as fewer concentrations of BMP2 were used. Moreover, ¹²⁵I-BMP4 binding was only weakly inhibited by BMP7 and was not altered at all in the presence of TGF-β or activin A.

**Effect of BMP4 on DNA synthesis and proliferation.** BMP4 dose dependently inhibited [³H]thymidine incorporation and hence DNA synthesis by HFL cells incubated with 5% FCS (Fig. 3A). The effect of BMP4 on serum-induced proliferation was also examined by cell counting, and BMP4 was found to inhibit the serum-stimulated proliferation of these cells (Fig. 3B).

**Effect of BMP4 on fibroblast differentiation.** In culture, HFL expressed low but detectable levels of α-SMA as seen by immunofluorescence in cell monolayers or by Western blot (Fig. 4). However, when lung fibroblasts were stimulated with BMP4, there was a significant increase in α-SMA protein expression (Fig. 4, A and B). Immunocytochemical techniques showed that α-SMA was organized into filaments (Fig. 4B). To determine whether BMP4 induced the expression of other smooth muscle markers, the expression of myosin was also examined. It was found that BMP4 also increased smooth muscle myosin expression (Fig. 4C). Increases in α-SMA

---

**Fig. 1.** mRNA expression of transforming growth factor (TGF)-β superfamily receptors and bone morphogenetic protein (BMP) ligands. Representative ethidium bromide-stained gels demonstrating expression of mRNA transcripts for activin type I (Act-RI) and II (Act-RII and Act-RIIB) receptors, BMP type I (ALK-3 and ALK-6) and II receptors (BMPRII), and TGF-β type I (ALK-1 and ALK-5) and II (TGFRII) receptors in human whole lung and lung fibroblasts (A) and the BMPRII ligands BMP2, BMP4, and BMP7 in human fetal lung fibroblasts (HFL) and Saos-2 (osteosarcoma cell line) cells (B) generated by RT-PCR.

**Fig. 2.** Characterization of ¹²⁵I-BMP4 binding sites in human lung fibroblasts. Graph shows equilibrium competition binding curves for ¹²⁵I-BMP4 in the presence of increasing concentrations of unlabeled BMP2, BMP4, BMP7, TGF-β, and activin (Act A). Data shown are means ± SE; n = 3.

**Fig. 3.** Effect of BMP4 on human lung fibroblast cell growth and proliferation. A: [³H]thymidine incorporation in human lung fibroblasts in response to 5% serum in the presence or absence of BMP4 (0.1–100 ng/ml). *P < 0.05, **P < 0.01 compared with 0 ng/ml of BMP4, n = 3. Data shown are means ± SE. B: rate of fibroblast proliferation in response to 5% serum in the presence or absence of BMP4 (50 ng/ml) over 7 days. Shown are representative data of n = 3 experiments. **P < 0.01, ***P < 0.001 compared with corresponding day in 5% FCS-treated cells.

---

**Fig. 4.** Effect of BMP4 on fibroblast differentiation. In culture, HFL expressed low but detectable levels of α-SMA as seen by immunofluorescence in cell monolayers or by Western blot
in response to BMP4, was concentration dependent (Fig. 4B). In addition, increased α-SMA expression, occurring in a time-dependent manner, taking 48 h to reach maximum (Fig. 4B). In addition, increased α-SMA expression, in response to BMP4, was concentration dependent (Fig. 4D). In examining the effect of different ligands on HFL, we found that BMP4 and TGF-β induced expression of α-SMA, whereas BMP2 and BMP7 did not (Fig. 4E).

**BMP4 and the cell cycle.** To further investigate the regulation of HFL growth by BMP4, the effect of BMP4 on the cell cycle was examined. First, flow cytometric analysis of propidium iodide-stained cells was used to determine the phase of the cell cycle that BMP4 was acting. After stimulation of cells with 5% FCS, the number of cells in the S phase increased from 0.6 ± 0.18% in serum-free media to 4.4 ± 0.80% (P < 0.05 by paired t-test; n = 6). Addition of BMP4 caused a consistent reduction in the percentage of cells in the S phase (5% + BMP4 = 3.4 ± 0.71%, n = 6; P < 0.05 compared with 5% FCS by paired t-test) and thus inhibited exit of cells from the G0/G1 phase of the cell cycle. The effect of BMP4 on proteins involved in regulating the progression of HFL through the G1 phase of the cell cycle was examined by Western analyses. Growth-arrested cells stimulated with 5% FCS showed a marked reduction in the expression of the cell cycle inhibitor p21<sup>WAF1/CIP1</sup> with a concurrent increase in the cell cycle inducers cyclin D and cdk2 (Fig. 5A). In the presence of BMP4 (50 ng/ml), the expression of the cell cycle inhibitor p21 was markedly increased (Fig. 5A). This was seen after 6–12 h and was maintained after 24 h. A corresponding decrease in the positive regulators of the cell cycle, cyclin D and cdk2, was also observed by 6 h, and this was still maintained after 24 h of stimulation (Fig. 5A). To further examine the role of p21 in BMP4-induced growth inhibition, cells were transfected with p21 siRNA. Transfection of cells with p21 siRNA prevented BMP4’s antiproliferative effect (Fig. 5B).

**Effect of BMP4 on intracellular signaling pathways.** To determine the intracellular signaling pathways utilized by BMP4/BMP2 in lung fibroblasts, we first examined whether the classic pathway involving Smads was involved. Western analyses demonstrated that BMP4 led to phosphorylation of Smad1 after only 15 min, and this was maximal by 60 min, disappearing by 120 min (Fig. 6A). In order for Smad1 to exert its effect on gene expression, it must be able to translocate into the nucleus. Immunostaining for phospho-Smad1 indicated
that indeed BMP4 induced Smad1 to translocate from the cytoplasm to the nucleus (Fig. 6B).

Recent evidence has also pointed toward the involvement of members of the MAPK pathway in BMP signaling. Using Western blot analyses, we also examined the effect of BMP4 on ERK1/2, p38MAPK, and JNK activation in fibroblasts. BMP4 induced phosphorylation and hence activation of all three MAPKs (Fig. 6, A–C). The activation of p38MAPK appeared to occur in a biphasic manner, with an initial increase in protein expression at 5 min and declining after 30 min and a second activation after 90 min (Fig. 6A). In contrast, activation of ERK1/2 occurred at a much later time point, with increased expression of phospho-ERK1/2 observed after 120 min (Fig. 6B). Phosphorylation of JNK occurred rapidly following a 5-min BMP4 stimulation and declining toward baseline levels after 30 min (Fig. 6C).

Role of intracellular pathways in HFL differentiation. We also examined whether Smad1 and/or MAPKs played a role in fibroblast differentiation. For these studies, we used Smad1 siRNA and various MAPK inhibitors. We also attempted to transiently transfect HFL with plasmids containing dominant negative Smad1 and mutant BMPRII; however, transfection of these cells with empty plasmid (pcDNA 3.0) in the absence of BMP4 induced differentiation of HFL (data not shown). In response to BMP4, α-SMA expression was less in cells transfected with Smad1-siRNA compared with untransfected cells; this was supported by both Western blot analyses...
and immunocytochemistry (Fig. 8, A and B). Incubation with an inhibitor of JNK reduced BMP4-induced β-SMA expression, but the inhibitors of p38MAPK and ERK1/2, SB-203580 and PD-98059, respectively, had no effect (Fig. 8 C).

DISCUSSION

The recent discovery that mutations in the gene for BMPRII underlie many cases of familial and sporadic PPH provided the impetus to better understand the role that BMPRII and its ligands play in the pulmonary circulation. Because fibroblasts are key mediators in the remodeling of the pulmonary vasculature associated with PPH, it was important to examine whether this system played a role in pulmonary fibroblast function. This study has demonstrated for the first time that BMP4 functions to inhibit proliferation and promote differentiation of human pulmonary fibroblasts and that these effects are Smad1 dependent with the MAPK and JNK also contributing to differentiation.

Expression profiling of receptors of the TGF-β superfamily by RT-PCR demonstrated expression of a variety of type I and type II receptors. The existence of multiple type I receptors is consistent with previous reports of vascular cells (1, 27). Although the expression of TGF type II receptors is well documented, there is less information available for BMPRII. This study is the first to report the expression of BMPRII in human lung fibroblasts and is consistent with our previous observations in human pulmonary artery smooth muscle and endothelial cells (2, 27). Fibroblasts were also found to express the BMP type I receptors BMPRIA and BMPRIB as well as the ligands for these receptors, namely BMP2 and BMP4. Radio-ligand binding studies demonstrated specific, high-affinity binding sites for BMP2 and BMP4. It is accepted that BMP2 and BMP4 utilize BMPRII in association with either of the type I receptors, BMPRIA and BMPRIB. Not surprisingly,
TGF-β and activin were unable to compete for 125I-BMP4 since these ligands are known to bind to TGFBRII and Act-RI together with TGFBRI and Act-RIB, respectively (44). The finding that BMP7 only weakly competed with 125I-BMP4 is consistent with our previous findings in smooth muscle cells (Upton PD, unpublished observations) and suggests that in pulmonary fibroblasts, BMP7 selectively binds to receptors distinct from those for BMP2 and BMP4. Although there is evidence demonstrating that BMP7 can bind BMPRIA and BMPRIB, albeit weakly, in other cell types (44), there are also reports that BMP7 utilizes Act-RII/IIB and Act-RI (25). Hence, in pulmonary fibroblasts, the weak competition of BMP7 for BMP4 may be due to weak binding of BMP7 to BMPRIA and/or BMPRIB and that its predominant receptor is Act-RI.

In contrast to TGF-β, the effects of BMPs on vascular cell proliferation are not well characterized. Although BMP2, BMP4, and BMP7 have been demonstrated to inhibit aortic and pulmonary vascular smooth muscle cell proliferation (7, 27, 30, 46), there is also evidence that TGF family members can stimulate cell proliferation (e.g., activin) (32). Hence, it was important to characterize the effects of BMP4 on fibroblasts. In this study, we found that BMP4 inhibits lung fibroblast proliferation, thus providing further insight into the function of BMPs in the pulmonary vasculature. Fetal lung fibroblasts were used because they represent a pluripotent lung cell line with the potential to differentiate into myofibroblasts and smooth muscle cells. During lung development, it is likely that these mesenchymal cells contribute to the formation of the media (42). In addition, during the process of vascular remodeling, the interstitial, as well as the adventitial fibroblast, have been implicated in the formation of the neointima and the process of distal muscularization of small arteries (19). Thus the fetal lung fibroblast is a very relevant cell type for these studies, both developmentally and in the response to injury. To better understand the mechanisms involved in BMP4’s antiproliferative effects, we investigated the regulation of the cell cycle by BMP4.

The cell cycle is employed by cells to regulate cell division and hence proliferation. Of the five phases, it is the transition between the G1 and S phases at which most pro- and antiproliferative stimuli act. Progression through the cell cycle depends on the activation and/or inactivation of cyclins, cyclin-dependent kinases, and cdk inhibitors. We found that the cell cycle inhibitor p21 was upregulated in the presence of BMP4. Concomitantly, cdk2 and cyclin D, important for cells to progress into S phase, were downregulated. Further support for the involvement of p21 in BMP4’s antiproliferative activity was provided by the finding that transfection of cells with p21 antisense oligonucleotide attenuated the antiproliferative effect of BMP4. Similarly, in human aortic smooth muscle cells, the antiproliferative effects of BMP2 and BMP7 involve p21 (7, 46). In other nonvascular cell lines, the involvement of p21 in response to BMPs has also been reported (29, 34). Only recently has the role of the cell cycle in the pulmonary vasculature been examined. Fouty et al. (9) reported that in rat pulmonary artery smooth muscle cells, the cdk inhibitor p27 plays an important role in maintaining cells in a quiescent state. In pulmonary artery smooth muscle cells from PPH patients, BMP4 is no longer able to inhibit cell growth (27). One possible explanation for this may be that there is a failure to downregulate the cdk inhibitors, including p21 and p27. Interestingly, in endothelial cells lining the central core of plexiform lesions of PPH patients, the expression of p27 has been reported to be reduced or absent (5). Furthermore, in other diseases characterized by uncontrolled cell proliferation, such as cancer, there is no longer an antiproliferative response to TGF-β as a result of aberrant cell cycle control (31). Whether the antiproliferative response to BMP4 is abrogated in fibroblasts derived from PPH patients remains to be determined.

Differentiation of cells is one well-known function attributed to BMPs and other members of the TGF-β superfamily and has been demonstrated in a variety of vascular and nonvascular cells. Fibroblasts are known to differentiate into a myofibroblast phenotype, which is characterized by the expression of smooth muscle contractile proteins, mainly α-SMA. In this study, BMP4 was shown to upregulate α-SMA expression in lung fibroblasts and indicates that BMP4 can induce pulmonary myofibroblast differentiation. This response was not universal for all TGF superfamily members since BMP2 and BMP7 did not induce fibroblast differentiation. This differential response to ligand has been reported for corneal fibroblasts (48) and may be a function of the signaling pathways utilized or, alternatively, the regulation of different transcriptional repressors or activators.

Myofibroblasts are key participants in a variety of pathological conditions involving tissue remodeling. Indeed, in pulmonary hypertension, fibroblasts undergo a transition in phenotype to a myofibroblast. For example, in plexiform lesions of PPH patients, myofibroblasts have been observed (41, 47). What mediates this change in phenotype is not known, although various stimuli that are thought to play a role in pulmonary hypertension, such as thrombin, interleukins, and hypoxia, have also been demonstrated to induce differentiation of lung fibroblasts into myofibroblasts (3, 13, 40). BMPs therefore may be an important mediator of this process. The functional significance of myofibroblasts and α-SMA expression is unclear, although it may lead to prevention of cell migration and concentrate these cells at the site of injury (37). In addition, they may be involved in branching morphogenesis since myofibroblasts have been observed in developing pulmonary vessels (24). Also, since these cells synthesize extracellular matrix proteins such as collagen, they may contribute to the increased production of these proteins seen in the remodeled pulmonary vasculature of pulmonary hypertension.

The classic intracellular signaling pathway utilized by BMPs involves a specific set of receptor-mediated Smad proteins, Smad1, Smad5, and Smad8, which complex with the common partner Smad4, to allow translocation of the signaling complex into the nucleus and regulate target-gene transcription. In this study, we found that BMP4 induced phosphorylation and hence activation of Smad1 with translocation of the activated Smad1 into the nucleus. There is also increasing evidence that BMPs activate another signaling pathway, the MAPKs, including ERK1/2, p38MAPK, and JNK, in a cell-specific manner. We found that p38MAPK, ERK1/2, and JNK were all activated by BMP4 in lung fibroblasts. The activation of these MAPKs has also been described for BMP2 in a number of nonvascular cells, including rat pheochromocytoma cells, mouse and human osteoblasts, and mouse myoblasts (11, 12, 16, 22, 23). However, this is the first report of activation of MAPK by BMPs in pulmonary fibroblasts.
Having demonstrated that the Smad and MAPK pathways were activated by BMP4 in lung fibroblasts, we then determined the contribution of these pathways to the antiproliferative and prodifferentiation effects of BMP4. We found that prodifferentiation effects were independent of ERK and p38MAPK activation but dependent on Smad1 and JNK. Similarly, TGF-β-induced lung fibroblast differentiation has also been reported to involve JNK but not p38MAPK and ERK1/2 (14), and TGF-β-induced Smad3 activation regulates α-SMA expression (15). The interaction between JNK and Smad1 in myofibroblast differentiation and the question of whether the interaction is a direct or indirect one were not examined in this study. However, in mouse lung epithelial cells where TGF-β led to JNK and Smad3 phosphorylation, it was demonstrated that JNK could phosphorylate Smad3 (8). Other possible roles for ERK and p38MAPK activation observed in this study include regulation of apoptosis and cell migration (21). The antiproliferative response to BMP4 was dependent on Smad1. The possibility that other pathways that have been recently reported to be involved in BMP signaling in other cell types, such as those involving phosphoinositol 3-kinase and protein kinase C (12), contribute to BMP4’s antiproliferative and prodifferentiation effects in lung fibroblasts remains to be examined.

Because Smad1 appears to be the predominant signaling molecule involved in both fibroblast proliferation and differentiation, it is interesting to speculate on the downstream interaction of this protein with its effector genes. Smad1 has been reported to interact with other transcription factors or genes including Sp1, ATF-2, and Kruppel-like growth factor (18, 28, 39). From reports in the literature, it is likely though that Smad1 activates a transcription factor such as Sp1 that in turn activates p21 (28, 33). The regulation of α-SMA by Smad1 may involve Id1 (4). Interestingly, the transcription factor ATF-2 is a common target for Smads and MAPK, JNK, and p38MAPK. Moreover, in a recent report by Monzen and colleagues (26), BMP-induced cardiomyocyte differentiation involved the interaction of JNK and Smad with ATF-2.

Failure of Smad phosphorylation is a major feature of mutations in BMPRII (38). In this study, we demonstrate that intact Smad signaling plays a critical role in growth inhibition and fibroblast differentiation into myocytes. Indeed, dominant negative Smad1 or the introduction of a kinase-deficient BMPRII mutant reversed the growth-suppressive effects of BMPs in HFL cells. Furthermore, knockdown of Smad1 inhibited the ability of BMP to induce SMA expression. Together, these results imply that BMPRII mutations would promote the expansion of fibroblasts resistant to the antiproliferative, prodifferentiation effects of BMPs and suggest a mechanism for the vascular obliteration seen in familial PPH.

**GRANTS**

This work was funded by a C. J. Martin Postdoctoral Fellowship from the National Health and Medical Research Council of Australia (to T. K. Jeffery) and the British Heart Foundation.

**REFERENCES**


