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Characterization of the Bone Morphogenetic Protein (BMP) System in Human Pulmonary Arterial Smooth Muscle Cells Isolated from a Sporadic Case of Primary Pulmonary Hypertension: Roles of BMP Type IB Receptor (Activin Receptor-Like Kinase-6) in the Mitotic Action

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The functional involvement of bone morphogenetic protein (BMP) system in primary pulmonary hypertension (PPH) remains unclear. Here we demonstrate a crucial role of the BMP type IB receptor, activin receptor-like kinase (ALK)-6 for pulmonary arterial smooth muscle cell (pphPASMC) mitosis isolated from a sporadic PPH patient bearing no mutations in *BMPR2* gene. A striking increase in the levels of ALK-6 mRNA was revealed in pphPASMC compared with control PASMCs, in which ALK-6 transcripts were hardly detectable. BMP-2 and -7 stimulated the mitosis of pphPASMCs, which was opposite to their suppressive effects on the mitosis of the control PASMCs. BMP-4 and -6 and activin inhibited pphPASMC mitosis, whereas these did not affect control PASMCs. The presence of BMP signaling machinery in pphPASMCs was elucidated based on the analysis on Id-1 transcription and Smad-reporter genes. Overexpression of a dominant-negative ALK-6 construct revealed that ALK-6 plays a key role in the mitosis as well as intracellular BMP signaling of pphPASMCs. Gene silencing of ALK-6 using small interfering RNA also reduced DNA synthesis as well as Id-1 transcription in pphPASMCs regardless of BMP-2 stimulation. Although Id-1 response was not stimulated by BMP-2 in control PASMCs, the gene delivery of wild-type ALK-6 caused significant increase in the Id-1 transcripts in response to BMP-2. Additionally, inhibitors of ERK and p38 MAPK pathways suppressed pphPASMC mitosis induced by BMP-2, implying that the mitotic action is in part MAPK dependent. Thus, the BMP system is strongly involved in pphPASMC mitosis through ALK-6, which possibly leads to activation of Smad and MAPK, resulting in the progression of vascular remodeling of pulmonary arteries in PPH. (*Endocrinology* 145: 4344–4354, 2004)

PRIMARY PULMONARY HYPERTENSION (PPH) is a life-threatening disease that has prevalence of one to two occurrences per 1 million individuals and is twice as common in women as men (1). This disease is characterized histologically by excessive proliferation of vascular endothelium and smooth muscle cells, causing thickening the walls of pulmonary arterioles and the formation of plexiform lesions that eventually occlude the vascular lumen (2). This remodeling of pulmonary arterioles increases vascular resistance and reduces vascular dilatation, which ultimately results in right ventricular failure. Therefore, the median survival of PPH patients is, if untreated, less than 3 yr.

Approximately 6% of the PPH patients possess an autosomal dominant pattern of inheritance. Recent research has uncovered the basis of a genetic predisposition to familial PPH (3, 4). Studies involving the screening of the locus of the gene for PPH, mapped to chromosome 2q33 (*PPH1*) have unveiled mutations in the *BMPR2* gene encoding the bone morphogenetic protein (BMP) type II receptor (BMPRII) receptor in nine of 19 (5), or seven of eight families (6). These mutations were not detected in 350 nonaffected controls. Furthermore, the mutation in the *BMPR2* gene can be detected in at least a quarter of apparently sporadic PPH cases (7).

BMP ligands transduce their signals via two types of serine/threonine kinase receptors, type I and II receptors, both of which are required for signal transduction (8, 9). Receptors for BMPs include three different type II receptors,

Abbreviations: ActRI, Activin type I receptor; ActRII, activin type II receptor; ALK, activin receptor-like kinase; Ang II, angiotensin II acetate salt; BMP, bone morphogenetic protein; BMPRI, BMP type I receptor; BMPRII, BMP type II receptor; BtcAMP, N^6,$O^{2'}$-dibutyryl cAMP, monosodium salt; cPASMC, control pulmonary arterial smooth muscle cell; DN, dominant negative; FCS, fetal calf serum; FSK, forskolin; β-gal, β-galactosidase; HA, hemagglutinin; PAI, plasminogen activator inhibitor; PASMC, pulmonary artery smooth muscle cell; pCMV, cytomegalovirus plasmid; PDGF, platelet-derived growth factor; PPH, primary pulmonary hypertension; pphPASMC, PPH PASMC; siRNA, small interfering RNA; Smad, mothers against decapentaplegic homolog; Wt, wild-type.

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i.e. activin type II receptors, ActRIIA and ActRIIB, and BMPRII; and three different type I receptors activin type I (ActRI), BMP type IA (BMPRIA) and BMP type IB (BMPRIB), also called activin receptor-like kinase (ALK)-2, -3, and -6, respectively. On binding of BMPs, type II receptors phosphorylate corresponding type I receptors, which in turn phosphorylate intracellular signal-transducing molecules mothers against decapentaplegic homolog (Smad)1, -5, and -8. BMP signaling is appeared to be involved in the regulation of proliferation of human pulmonary smooth muscle cells; however, it has been unknown whether the pathogenesis of PPH-carrying *BMPR2* gene mutations is caused by perturbation of the BMP signaling. Indeed, the *BMPR2* mutations are distributed throughout the coding regions of the *BMPR2* gene (5, 10), suggesting a heterogeneity of their contributions to the pathogenesis of PPH. In addition, there are still many familiar and sporadic PPH patients who do not have detectable mutations in the *BMPR2* gene.

As of yet, specific BMP ligands that are involved in the aberrant signaling caused by the mutations in the BMPRII receptor in PPH patients have not been elucidated. Additionally, because it is well established that in the TGFβ superfamily, cooperation between the type II and type I receptors is required for signal transduction; the roles and involvement of BMP type I receptors in the proliferation of PPH smooth muscle cells is also an important unresolved question. In the present study, we used isolated primary pulmonary arterial smooth muscle cells (PASMCs) that we had the opportunity to collect from a patient who had developed severe PPH to attempt to elucidate the effects of specific BMP ligands on PASMC mitosis as well as to identify the critical roles of individual type I receptors.

Materials and Methods

Reagents and supplies

DMEM, penicillin-streptomycin solution, angiotensin II acetate salt (Ang II), adrenocorticotropic hormone human fragment 1–24 (1–24 ACTH), and forskolin (FSK) were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO). Recombinant human BMP-2, -4, -6, and -7 were purchased from R&D Systems (Minneapolis, MN), human platelet-derived growth factor (PDGF)-BB, and TGFβ1 were from PeproTech EC Ltd. (London, UK), and human activin-A and follistatin were a kind gift from Dr. Shunichi Shimasaki (University of California, San Diego, San Diego, CA). N^6,$O^{2\prime}$-dibutyryl cAMP, monosodium salt (BtcAMP) from Yamasa-shoyu (Tokyo, Japan). U0126 and SB203580 were from Promega Corp. (Madison, WI). Plasmids of Tlx2-Luc were from Dr. Jeff Wrana (Samuel Lunenfeld Research Institute, Toronto, Ontario, Canada), and 3TP-Luc and pc3-ALK6(Wt)-hemagglutinin (HA) from Dr. Kohei Miyazono (Tokyo University, Tokyo, Japan). Plasmid of Xvent2-Luc and expression plasmids for dominant-negative (DN)-BMP receptors including pActRII-DN, BMPRII-DN, pALK-3DN and pALK-6DN were kindly provided from Dr. Shunichi Shimasaki.

Isolation of human PASMCs and establishment of cell culture

The human primary pulmonary hypertension PASMCs (pphPASMCs) were isolated from a 13-yr-old female patient diagnosed with PPH when surgical procedure of lung transplantation was performed. She had developing dyspnea, severe hypoxia, and right ventricular heart failure without any particular family history. As control studies, normal pulmonary arterial smooth muscle cells (cPASMCs) were isolated from the normal lung tissues resected from three female patients aged 28, 30, and 73 yr for medical reasons including lung cancer or lung donor. The sections of the resected lung tissues were examined by hematoxylin-eosin staining and then the PASMCs were explanted, isolated, and cultured in DMEM containing 10% fetal calf serum (FCS) and 1% penicillin-streptomycin solution as we have previously reported (11). Until scheduled experiments, the cells were cultured in 75-cm^2 flasks at 37 C under a humid atmosphere of 95% air/5% CO_2. Cells were used for the scheduled experiments at the passages of 5–7. All human subject protocols were approved by our institutional committee, and written permission from each individual regarding the experimental use of the tissues was obtained in advance of the surgery.

Total cellular RNA extraction and RT-PCR

The PASMCs were grown in six-well plates to approximately 80% confluence, and after the treatment indicated, the medium was removed and total cellular RNA was extracted by isothiocyanate-acidphenol-chloroform methods using TRIzol (Invitrogen Corp., Carlsbad, CA) and quantified by measuring absorbance at 260 nm and stored at −80 C until assay. The expression of BMP/activin receptors and follistatin mRNAs were detected by RT-PCR analysis. Oligonucleotides used for PCR were custom ordered from Kurabo Biomedical Co. (Osaka, Japan). PCR primer pairs for BMPRII were selected from the sequence published by Deng *et al.* (5), and the other primer sets were selected from different exons of the corresponding genes to discriminate PCR products that might arise from possible chromosome DNA contaminants as we have earlier reported (12). The extracted RNA (1 μg) was subjected to a reverse transcription reaction using first-strand cDNA synthesis system (Invitrogen Corp.) with random hexamer (2 ng/μl), reverse transcriptase (200 U), and deoxynucleotide triphosphate (0.5 mM) at 42 C for 50 min and 70 C for 10 min. Subsequently, hot-start PCR was performed using $MgCl_2$ (1.5 mM), deoxynucleotide triphosphate (0.2 mM), and 2.5 U *Taq* DNA polymerase (Invitrogen Corp.) under the conditions we have reported. Aliquots of PCR products were electrophoresed on 1.5% agarose gels, visualized after ethidium bromide staining, photographed, and scanned.

Genomic DNA extraction and sequence determination of BMPR2 gene

Genomic DNA isolated were extracted from peripheral leukocytes of the PPH patient using a genomic DNA isolation kit (Gentra Systems Inc., Minneapolis, MN), and each part that contains 12 exons were PCR amplified using corresponding primers that can recognize each exon of *BMPR2* gene as reported by Deng *et al.* (5). The sequences of the PCR products, which include each exon of *BMPR2* gene, were directly determined by sequencer (ABI Prism 310, Applied Biosystems, Foster City, CA).

Thymidine incorporation assay

PASMCs (30×10^3/well) were precultured in 12-well human fibronectin-coated plates (Biocoat; BD-Falcon, Bedford, MA) containing 1 ml culture medium. After 48 h, medium was replaced with fresh medium containing 1% FCS and indicated growth factors were added. After 24 h, 0.5 μCi/well of [methyl-^{3}H] thymidine (Amersham Pharmacia, Piscataway, NJ) was added and incubated for 3 h at 37 C. The incorporated thymidine was detected as we previously reported (13). Cells were then washed with PBS, incubated with 10% ice-cold trichloroacetic acid for 30 min at 4 C, solubilized in 0.5 M NaOH, and its radioactivity determined by liquid scintillation counter (TRI-CARB 2300TR; Packard Co., Meriden, CT).

Quantitative real-time RT-PCR analysis

For the quantification of Id-1, plasminogen activator inhibitor (PAI)-1, and L19 mRNA levels, quantitative real-time PCR was performed using LightCycler-FastStart DNA master SYBR Green I system (Roche Diagnostic Co., Tokyo, Japan) under the condition of annealing at 60 C with 4 mM $MgCl_2$ following the manufacturer's protocol. Accumulated levels of fluorescence during 65-cycle amplification were analyzed by the second-derivative method after the melting-curve analysis (Roche Diagnostic), and then the expression levels of Id-1 and PAI-1 were standardized by L19 level in each sample.

Transient transfection and luciferase assay

After 24 h preculture in 12-well human fibronectin-coated plates (Biocoat, BD-Falcon), cells (~70% confluency) were transiently transfected with 1.0 μg of each luciferase reporter plasmid (Xvent2-Luc, 3TP-Luc, and Tlx2-Luc) and 0.1 μg of cytomegalovirus (pCMV)-β-galactosidase plasmid (β-gal) using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) for 24 h. The cells were then treated with indicated concentrations of growth factors in DMEM containing 1% FCS for 24 h. The cells were washed with PBS and lysed with cell culture lysis reagent (Toyobo, Osaka, Japan). Luciferase activity and β-gal activity of the cell lysate were measured by a TD-4000 luminometer (Turner Designs, Sunnyvale, CA) and the data were shown as the ratio of luciferase to β-gal activity as we have previously reported (14).

DN receptor study and overexpression of wild-type ALK-6

For a DN receptor study, extracellular domains of BMP receptors were independently overexpressed on the PASMC using expression plasmids to produce DN models of BMP receptors by PASMCs. After 24 h preculture in 12-well human fibronectin-coated plates (Biocoat, BD-Falcon), cells (~70% confluency) were transiently transfected with pBUD-CE4.1 (Mock), pActRII-DN (containing extracellular domain of ActRII), pBMPRII-DN (containing extracellular domain of BMPRII), pALK3-DN (containing extracellular domain of BMPRIA/ALK-3) or pALK6-DN (containing extracellular domain of BMPRIB/ALK-6) at 0.5 pmol using FuGENE 6 (Roche Molecular Biochemicals) for 24 h. For a wild-type (Wt)-ALK-6 study, control PASMCs were transfected with an expression plasmid of wt-ALK-6 receptor construct pc3-ALK6(Wt)-HA, which encompasses normal mouse ALK-6 coding sequence. The cell preparation and gene transfection protocol was the same as performed in the preceding DN study. The transfected cells were subsequently used for thymidine assays or total cellular RNA extractions for quantification of Id-1 transcripts by real-time RT-PCR as mentioned above.

Silencing of ALK-6 mRNA

Gene silencing of ALK-6 was performed by a small interfering RNA (siRNA) method established by Elbashir *et al.* (15, 16). Based on their method, the targeted region for ALK-6 was selected from the cDNA sequence [863–885 (NM_001203)] and the siRNA duplex was custom ordered from Proligo Co. (Kyoto, Japan). After 24 h preculture in 12-well human fibronectin-coated plates (Biocoat, BD-Falcon), cells (~70% confluency) were transiently transfected with 100 nM siRNA duplex targeting a part of ALK-6 sequence using oligofectamine reagent (Invitrogen). For RT-PCR analysis of ALK-6 mRNA expression, total cellular RNAs were extracted as mentioned above at 12–48 h after the transfection with ALK-6-siRNA. After determination of the maximal effect of ALK-6 mRNA inhibition at 24 h, thymidine assay was subsequently performed as mentioned above.

Statistical analysis

All results shown are mean ± SEM of at least three separate experiments, with triplicate determinations for each treatment. Differences between groups were analyzed for statistical significance using ANOVA (StatView 5.0 software; Abacus Concepts, Inc., Berkeley, CA). $P < 0.05$ was accepted as statistically significant.

Results

We first characterized the pathological and biological features of PASMCs from normal cPASMCs and a pphPASMC. The cPASMCs were isolated from non-PPH patients (28F, 30F, and 73F) with histologically normal pulmonary arteries (30F; Fig. 1A) and the pphPASMCs were isolated from a PPH patient who developed severe plexiform lesions in the pulmonary arteries (13F; Fig. 1B). Cultured pphPASMCs exhibited readily observable morphological and functional differences, compared with cPASMCs (Fig. 1, C and D). As shown in Fig. 1D, pphPASMCs appeared spindle shaped and divided at a rapid rate. The mitotic activity of PASMCs was quantified by a thymidine incorporation assay (Fig. 1E). The DNA synthesis by the isolated pphPASMCs was 5- to 20-fold

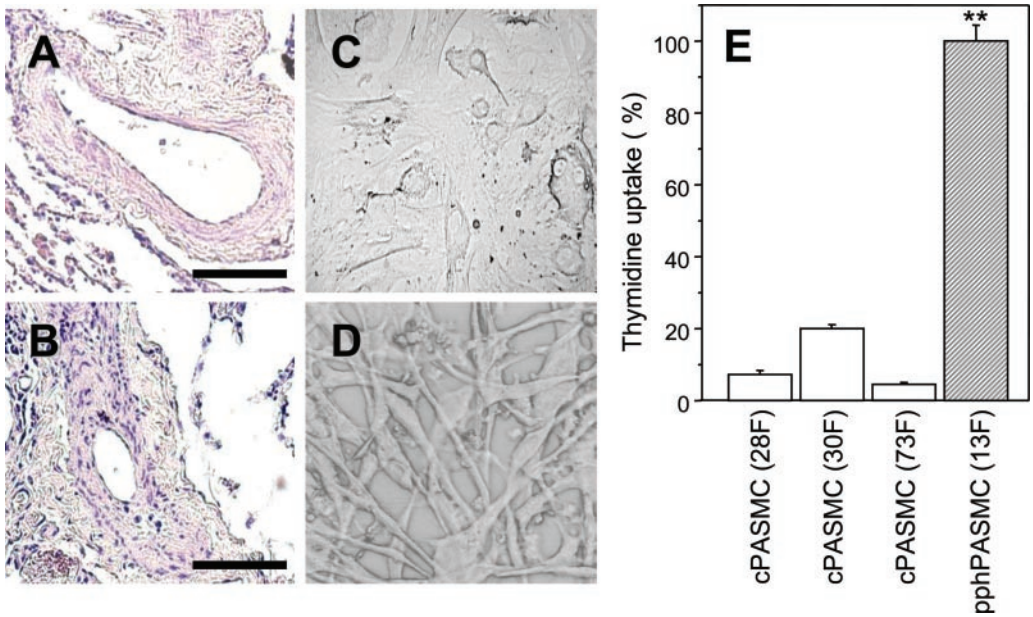

FIG. 1. Isolation of PASMCs from the pulmonary arteries of the lung tissues. Representative sections of pulmonary arteries from control (A) and PPH lungs (B) were presented (hematoxylin-eosin staining; *bars*, 100 μm). Pulmonary arteries obtained from the PPH patient exhibited concentric proliferation of endothelial and smooth muscle cells (B). Compared with the normal cPASMCs (C, ×200), the isolated pphPASMCs were spindle shaped and massively proliferating (D, ×200). Basal levels of DNA synthesis were determined by thymidine uptake analysis. Isolated pphPASMCs (13F) and cPASMCs (28F, 30F, and 73F) were cultured in DMEM containing 1% FCS followed by a 4-h pulse exposure of 0.5 μCi/well of thymidine, in which the thymidine level incorporated into the cells was counted (E). Results show the mean ± SEM of data from three separate experiments, each performed with triplicate cultures; **, $P < 0.01$ *vs.* cPASMCs.

higher than that of the cPASMCs isolated from three different normal females under serum-deprived (1%) conditions.

To investigate whether the isolated pphPASMCs express all the key exons of *BMPR2* gene, the mRNA expression of each exon in the extracellular and N-terminal region of transmembrane domains were examined (Fig. 2A). This shows that the first seven exons of BMPRII are clearly expressed in the pphPASMCs as well as cPASMCs. The direct sequence of PCR products of all the 12 exons of the *BMPR2* gene was performed following the method reported by Deng *et al.* (5). As a result, no *BMPR2* mutations were found in the genomic DNA of the patient from which we collected the pphPASMCs, and in the pphPASMCs, BMPRII mRNA, including the N terminus region that encodes the extracellular and transmembrane domains, was effectively expressed.

To characterize the expression of fundamental components of the BMP system in PASMCs, we also examined the mRNA expression of ActRII, BMP type I receptors (ALK-2, -3, and -6), and the activin/BMP-binding protein, follistatin. As shown in Fig. 2B, the pphPASMCs expressed all the BMP receptors including ActRII, ALK-2, -3, -6, and follistatin in addition to BMPRII. Notably, the expression of ALK-6 mRNA was not detected by RT-PCR in cPASMCs. Quantification using real-time PCR revealed that the ALK-6 expression in pphPASMCs was more than 10-fold higher that in cPASMCs (Fig. 2C), suggesting that a fundamental difference between c- and pphPASMCs may be the abnormal expression of ALK-6.

To characterize the responsiveness of pphPASMCs to exogenous stimuli, cells were treated with PDGF-BB, which is known to be a potent growth factor for PASMCs (17) (Fig. 3A). PDGF-BB increased DNA synthesis of the pphPASMCs up to approximately 2-fold in a concentration-dependent manner. Other hormonal factors including a stress-induced hormone, ACTH, and a vasoconstrictive agent, Ang II, did not alter the DNA synthesis by pphPASMCs, whereas cAMP donors, BtcAMP and FSK, exhibited potent growth-suppressive properties on pphPASMCs (Fig. 3B).

Because the BMP system was found to be present in the pphPASMCs, we next examined whether this receptor system is functional. To evaluate the effect of each BMP ligand, pphPASMCs were cultured under the presence of various BMP ligands. BMP-2 and -4 bind predominantly to BMPRII and type I receptors including ALK-3 and -6, which subsequently activate Smad1/5/8 signaling pathway (9, 18–20). BMP-6 and -7 can bind to either BMPRII or ActRII and type I receptors including ALK-2 and -6, which lead to Smad1/5 activation. In contrast, activin and TGFβ1 bind their specific type II receptors (ActRII and TGFβRII, respectively), which are distinct to BMPRII and activates the Smad2/3 pathway after recruiting type I components (9, 18–20). As shown in Fig. 3, C and D, activin-A showed an inhibitory effect on cell mitosis, whereas TGFβ1 showed a biphasic effect on DNA synthesis in pphPASMCs. In particular, TGFβ1 showed a peak stimulatory effect of DNA synthesis at 10 ng/ml, but the higher doses of TGFβ1 rather suppressed the cell mitosis. It is notable that there were remarkable differences on the effects of individual BMP ligands in regulating pphPASMC mitosis with BMP-4 and -6 inhibiting thymidine incorporation and BMP-2 and -7 acting as stimulators of mitosis (Fig. 3, E–H). Thus, BMP ligands, which all stimulate a common Smad1/5/8 pathway, show divergent actions on DNA synthesis by pphPASMCs. In comparison with pphPASMCs, the BMP effects on the cPASMC mitosis were also evaluated using the saturating doses of each ligand (Fig. 4). In contrast to the response shown in the pphPASMCs, both BMP-2 and BMP-7 significantly and commonly suppressed the mitogenic effect of three different cPASMCs. BMP-4, -6, and ac-

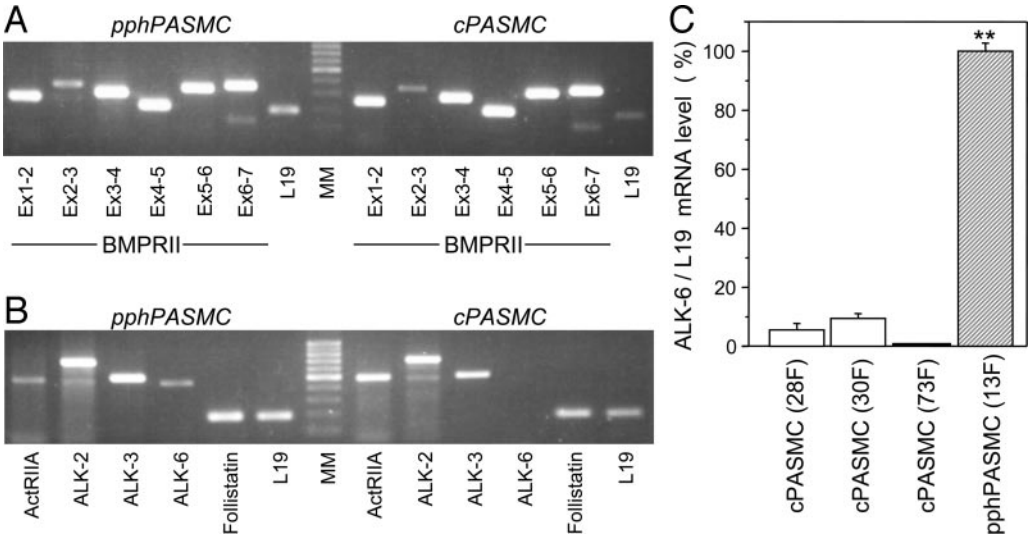

FIG. 2. Expression of BMPRs in PASMCs. Total cellular RNA was collected from cultured pphPASMCs (13F) and cPASMCs (28F, 30F, and 73F). Expressions of the BMPRII exons encoding extracellular and N-terminal part of transmembrane domains and a housekeeping gene L19 were determined by RT-PCR (A). Expressions of ActRII, ALK-2, -3, and -6 and follistatin were also evaluated in both cells (B). Aliquots of PCR products were electrophoresed on 1.5% agarose gel, visualized by ethidium bromide staining, and shown as representative of those obtained from three independent experiments. MM, Molecular weight marker. The mRNA levels of ALK-6 in pphPASMCs and cPASMCs were determined by quantitative real-time RT-PCR analysis (C). Results show the mean ± SEM of data from three separate experiments, each performed with triplicate cultures; **, $P < 0.01$ *vs.* cPASMCs.

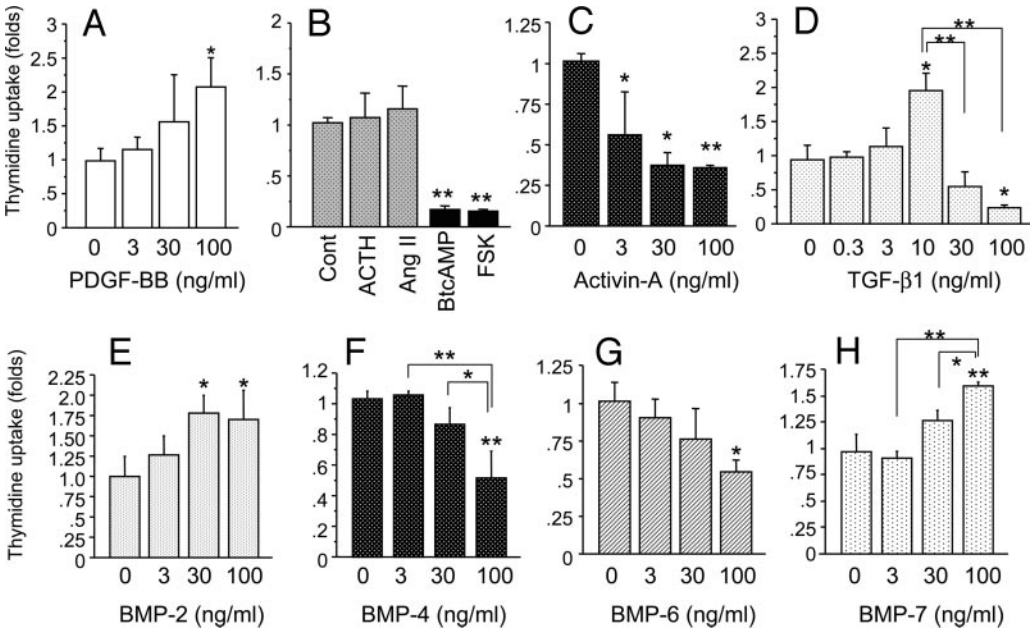

FIG. 3. Effects of BMPs on proliferation of pphPASMCs. The pphPASMC were cultured in DMEM containing 1% FCS and treated with indicated concentrations of PDGF-BB (A); hormones (B) including ACTH (100 ng/ml), Ang II (10 nM), BtcAMP (1 mM), and FSK (10 μM); activin-A (C); TGFβ1 (D); and BMP-2, -4, -6, and -7 (E–H) for 24 h followed by a 4-h pulse exposure of 0.5 μCi/well of thymidine. The thymidine level incorporated into the cells was counted. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate cultures; *, $P < 0.05$ and **, $P < 0.01$ *vs.* control or between the indicated groups.

tivin-A failed to affect the mitogenicity of the control PASMCs, although PDGF-BB exhibited stimulatory effects on this control cell mitosis as shown in the pphPASMCs.

Because we detected the cell response to exogenous BMP ligands, we further sought whether the effects of BMPs occur through Smad signaling. First, we determined the expression levels of the target genes for TGFβ and BMPs (PAI-1 and Id-1, respectively) by quantitative real-time RT-PCR analysis. Indeed, BMP-2 increased the expression of Id-1 but not PAI-1, whereas TGFβ1 increased PAI-1 but not Id-1 (Fig. 5A). The Smad signaling induced by BMP-2 or activin-A was also evaluated using reporter gene assays for the Smad-responsive elements. Xvent2-Luc (21) and Tlx2-Luc (22) are specifically responsive to the BMP-Smad1/5/8 pathway, whereas 3TP-Luc (23) can work for the response to activin/TGFβ-Smad2/3 and weakly to BMP-Smad1/5/8 as well. As shown in Fig. 5B, BMP-2 but not activin-A potently activated both Xvent-Luc and Tlx2-Luc, whereas BMP-2 and activin-A showed a weak increase in the 3TP-luc activity. These findings demonstrate that pphPASMCs carry both of the Smad pathways activated by BMPs as well as activin/TGFβs.

Cell mitosis in most smooth muscle cell types is associated with activation of the MAPK pathway. Recently cross-talk between BMP/Smad signaling and the MAPK pathway has been recognized in many types of cells (24). To investigate the involvement of MAPK signaling in our pphPASMCs, specific inhibitors of ERK and p38 pathway of MAPK system, U0126 and SB203580, respectively, were used. Treatment with U0126 and SB203580 potently suppressed pphPASMC mitosis in a dose-dependent manner (Fig. 6A), with showing IC_{50} of U0126 and SB203580 at approximately 1 and approximately 0.3 μM, respectively. Effects of these MAPK inhibitors on pphPASMC mitosis were further evaluated under the presence of potent mitogens. The mitotic action induced by either PDGF-BB or BMP-2 was totally abolished by either treatment of U0126 or SB203580 at each dose of IC_{50} (Fig. 6B). Thus, the mitotic effects induced by BMP-2 or PDGF-BB appeared to occur, at least in part, through activating the MAPK pathway in the pphPASMCs.

To investigate the contribution of BMPs endogenously produced by the cells to the mitotic actions of pphPASMCs, cells were treated with follistatin, a binding protein of activins as well as BMPs. Follistatin treatment did not significantly alter the DNA synthesis by pphPASMCs (Fig. 7), suggesting that mitotic activity due to activin and BMPs may become static when endogenous BMP/activin actions are nonspecifically perturbed in the pphPASMCs by adding exogenous follistatin.

We therefore attempted to eliminate the specific actions of BMP receptors by using DN constructs for the BMP receptors. The pphPASMCs were transiently transfected with each BMP receptor-DN plasmid that can overexpress only the extracellular domain of the BMP receptor, and then the DNA synthesis activity was subsequently evaluated by thymidine assays. As shown in Fig. 8A, treatments with type II BMP receptor-DNs including ActRII-DN and BMPRII-DN did not affect the DNA synthesis by pphPASMCs. However, transfection with the ALK-6/BMPRIB-DN potently inhibited the DNA synthesis by pphPASMCs, although ALK-3/BMPRIA-DN did not affect the DNA synthesis. To confirm whether this inhibitory effect of ALK-6-DN on pphPASMC mitosis is due to the suppression of BMP signaling, the Id-1 (a known BMP target gene) expression level was also evaluated by real-time RT-PCR. Consistent with our earlier data, BMP-2 increased the Id-1 mRNA level, which was abolished by

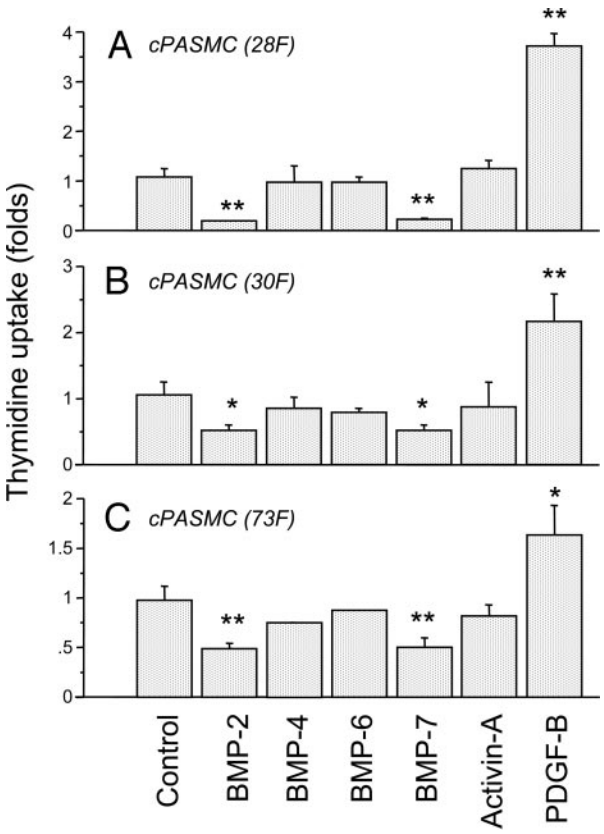

FIG. 4. Effects of BMPs on control PASMC mitosis. The three different cPASMCs (A, B, and C; 28F, 30F, and 73F, respectively) were cultured in DMEM containing 1% FCS and treated with 100 ng/ml BMP-2, -4, -6, -7, activin-A, or PDGF-BB for 24 h followed by a 4-h pulse exposure of 0.5 μCi/well of thymidine. The thymidine level incorporated into the cells was counted. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate cultures; *, $P < 0.05$ and **, $P < 0.01$ *vs.* control.

the overexpression of ALK-6-DN, although basal levels of Id-1 expression was marginally suppressed by ALK-6-DN (Fig. 8B). Thus, the interference of ALK-6 signaling by ALK-6-DN is critical for the mitotic action of pphPASMCs.

To further investigate the role of ALK-6 in pphPASMC mitosis, ALK-6 transcription was interfered by ALK-6-specific siRNA. After the optimization of ALK-6 mRNA reduction by siRNA (Fig. 9A), mitotic activities of pphPASMCs were evaluated by thymidine assay. As a result, 24-h treatment with ALK-6-siRNA markedly suppressed the mitotic activity up to approximately 40%, compared with the control (Fig. 9B). Hence, it appeared that ALK-6 is necessary for the spontaneous mitosis of pphPASMCs. The silencing effect of ALK-6 was further confirmed by quantifying the Id-1 mRNA level. As shown in Fig. 9C, ALK-6-siRNA inhibited steady-state levels of Id-1 mRNA as well as BMP-2-induced an increase in the Id-1 expression. Thus, the silencing of ALK-6 transcription resulted in suppression of the BMP signaling as well as pphPASMC mitosis, regardless of the presence of BMP-2 stimulation. Additionally, we tested the growth-inhibitory effects of ALK-6-DN and ALK-6-siRNA on two other ALK-6-positive PASMCs that were isolated from different sporadic PPH patients: case 2: 13-yr-old female and case 3: 31-yr-old female. As shown in Fig. 10, the DNA synthesis was reduced by either ALK-6-DN or ALK-6-siRNA treatment in both of the PASMCs, although the effect of ALK-6-siRNA was not statistically significant in the PASMCs of case 2.

To further confirm the roles of ALK-6 in PASMC signaling, increasing amounts (0–300 ng) of wt-ALK-6 plasmid was transfected into the cPASMCs (Fig. 11). In contrast to the results obtained from pphPASMCs (Fig. 5A), BMP-2 (100 ng/ml) failed to increase the Id-1 mRNA level in the absence of wt-ALK-6 induction, suggesting the impairment of ALK-6 action in these cells. However, it is of

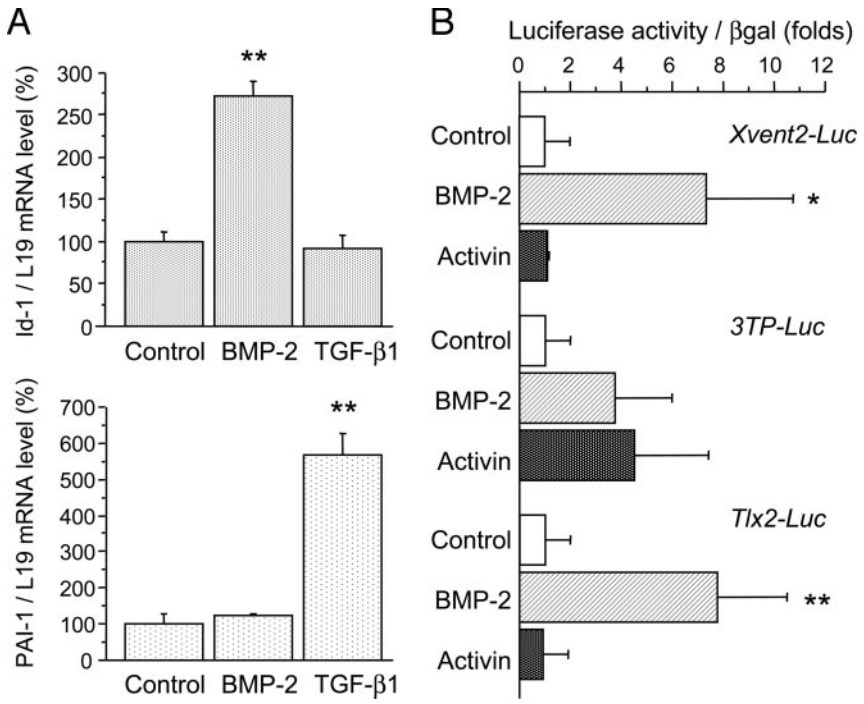

FIG. 5. Effects of BMPs on the target gene response and the cell signaling in pphPASMCs. A, Effect of BMPs on the Id-1/PAI-1 expression in pphPASMCs. The pphPASMCs were cultured in DMEM containing 1% FCS and treated with BMP-2 (100 ng/ml) or TGFβ1 (100 ng/ml) for 24 h. Then the total cellular RNA was extracted and the mRNA levels of target genes Id-1 and PAI-1 for BMP and TGFβ1, respectively, were determined by quantitative real-time RT-PCR analysis. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate cultures; **, $P < 0.01$ *vs.* control. B, Effect of BMPs on Smad signaling in pphPASMCs. For the assessment of Smad-signaling pathway, pphPASMC was transfected with 1.0 μg of each luciferase reporter plasmid (Xvent2-Luc, 3TP-Luc, and Tlx2-Luc) and 0.1 μg of pCMV-βgal for 24 h and then treated with BMP-2 (100 ng/ml) or activin-A (100 ng/ml) in DMEM containing 1% FCS for 24 h. The cells were washed with PBS, lysed, and the luciferase activity and β-gal activity measured by luminometer. Data were shown as the ratio of luciferase to β-gal. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate cultures; *, $P < 0.05$ and **, $P < 0.01$ *vs.* control.

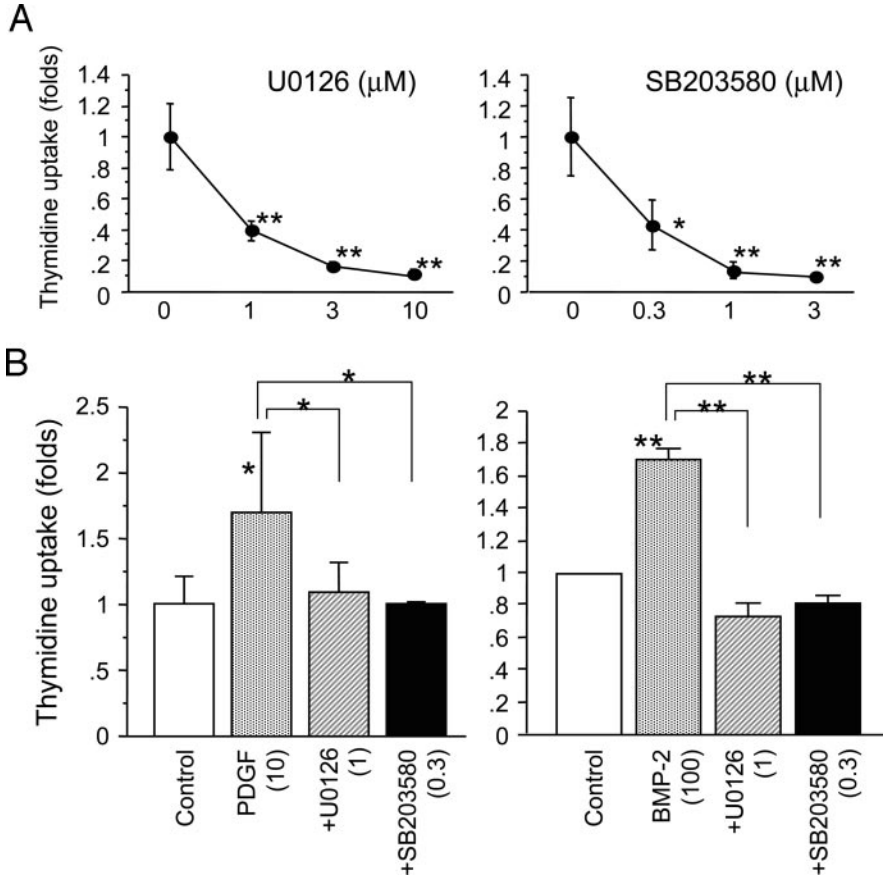

FIG. 6. Effects of MAPK inhibitors on pphPASMC mitosis. A, The pphPASMCs were cultured in DMEM containing 1% FCS and treated with increasing concentrations of U0126 or SB203580 for 24 h followed by a 4-h pulse exposure of 0.5 μCi/well of thymidine. The thymidine level incorporated into the cells was counted. B, The pphPASMCs were treated with an IC_{50} concentration of U0126 or SB203580 for 24 h in combination with PDGF-B (10 ng/ml) or BMP-2 (100 ng/ml) followed by a 4-h pulse exposure of 0.5 μCi/well of thymidine. The thymidine level incorporated into the cells was counted. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate cultures; *, $P < 0.05$ and **, $P < 0.01$ *vs.* control or between the indicated groups.

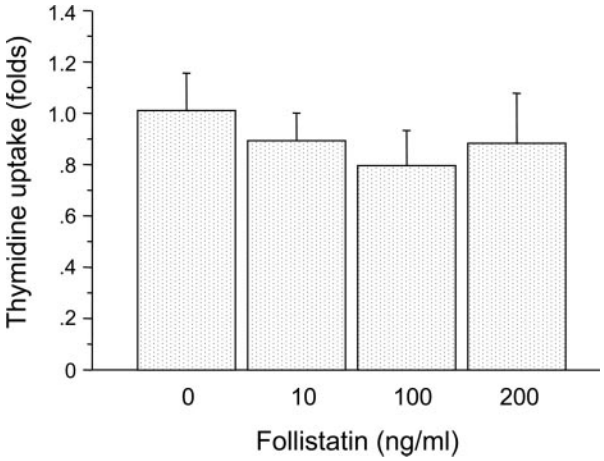

FIG. 7. Effects of follistatin on pphPASMC mitosis. The pphPASMCs were cultured in DMEM containing 1% FCS and treated with indicated concentrations of follistatin for 24 h followed by a 4-h pulse exposure of 0.5 μCi/well of thymidine. The thymidine level incorporated into the cells was counted. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate cultures.

interest that wt-ALK-6 induction dose-responsively augmented the Id-1 transcript level induced by BMP-2 stimulation. Collectively, ALK-6 is highly required for the mitogenesis of pphPASMCs, and the ALK-6 action is likely to be a key factor distinguishing a mitogenic difference between pphPASMCs and cPASMCs.

Discussion

The identification of heterozygous germline mutations in the *BMPR2* gene in familiar and sporadic cases of PPH has proven to be a crucial breakthrough in elucidating underlying pathogenesis of PPH (3). Subsequent functional studies have provided compelling evidence that PPH cells harboring the *BMPR2* mutations exhibit aberrant function of BMPRII and disrupted BMP signaling (17, 25). However, given that almost half of PPH patients do not bear the *BMPR2* mutation, it is still difficult to explain the entire mechanism of PASMC mitogenesis in PPH lungs.

In normal lung tissue, BMPRII is predominantly expressed by endothelial cells in the pulmonary circulation and, to lesser extents, vascular smooth muscle (26). In PPH patients BMPRII is expressed in the characteristic lesions of PPH lungs, specifically by endothelial cells of plexiform lesions and by endothelial and myofibroblast cells in the intimal lesions (26). The cellular localization of BMPRII implies that mutations in BMPRII most likely play key roles in the development of PPH lesions. BMPRII protein, however, is reduced in the lungs of patients with severe pulmonary hypertension, including those with secondary pulmonary hypertension. This suggests that the reduction of BMPRII signaling is implicated in the PPH pathogenesis, including in patients that are BMPRII mutation negative. In the present study, we investigated the roles of BMP ligands and type I receptors in PASMC physiology, both of which must interact with BMPRII for the propagation of BMPRII signaling.

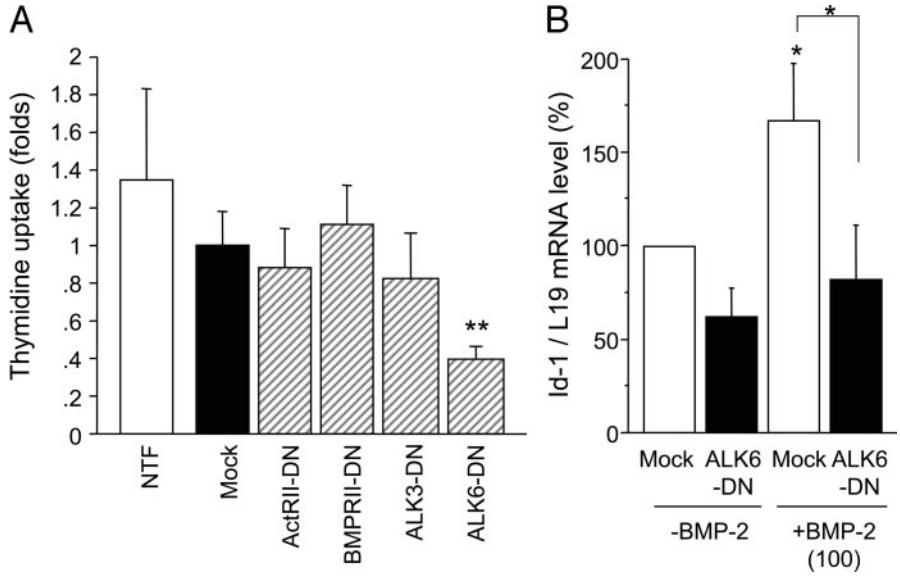

FIG. 8. Effects of BMPR DNs on pphPASMC mitosis. A, The pphPASMCs were transfected with Mock (pBUD-CE4.1), pActRII-DN, BMPRII-DN, pALK3-DN, or pALK6-DN at 0.5 pmol and cultured in DMEM containing 1% FCS for 24 h followed by a 4-h pulse exposure of 0.5 μCi/well of thymidine. The thymidine level incorporated into the cells was counted. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate cultures. B, Sixteen hours after the transfection of pALK6-DN, the pphPASMC was treated for 24 h with or without BMP-2 (100 ng/ml) in DMEM containing 1% FCS, and the total cellular RNA was extracted. The mRNA levels of Id-1 of pphPASMCs were then evaluated using quantitative real-time RT-PCR analysis. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate cultures; *, $P < 0.05$ and **, $P < 0.01$ *vs.* Mock control or between the indicated groups. NTF, nontransfection control.

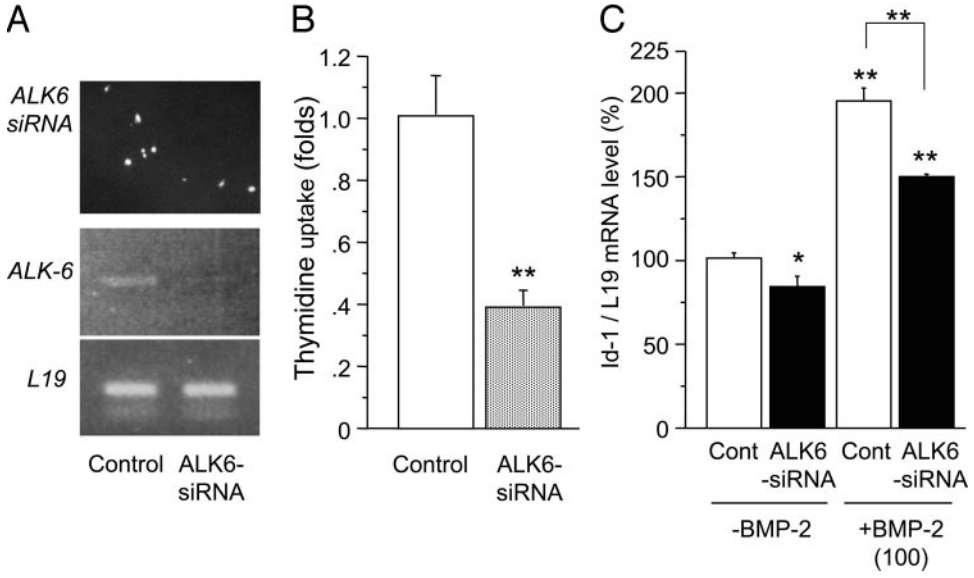

FIG. 9. Silencing effect of ALK-6 mRNA on pphPASMC mitosis. A, The pphPASMC was transiently transfected with 100 nM of fluorescein isothiocyanate-labeled siRNA duplex targeting a part of ALK-6 sequence (*upper panel*). ALK-6 mRNA levels were examined by semiquantitative RT-PCR for 48 h, and a 24 h-treatment with siRNA clearly suppressed the ALK-6 mRNA levels, compared with L19 control (*lower panel*). B, Effects of gene silencing of ALK-6 on pphPASMC mitosis were evaluated by thymidine uptake assay. After 24 h transfection with ALK-6-siRNA, the cells were cultured in DMEM containing 1% FCS followed by a 4-h pulse exposure of 0.5 μCi/well of thymidine. The thymidine level incorporated into the cells was counted. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate cultures. C, Sixteen hours after the transfection of ALK-6-siRNA, the pphPASMC was treated for 24 h with or without BMP-2 (100 ng/ml) in DMEM containing 1% FCS, and the total cellular RNA was extracted. The mRNA levels of Id-1 of pphPASMC were then evaluated using quantitative real-time RT-PCR analysis. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate cultures; *, $P < 0.05$ and **, $P < 0.01$ *vs.* control or between the indicated groups.

Here we first determined the growth-promoting effects of different BMP ligands in pphPASMC cells from a patient who did not carry *BMPR2* gene mutations. Furthermore, the pphPASMCs expressed all the key domains of BMPRII mRNA, similar to that of the cPASMCs. Of the components of the BMP system that we studied, the remarkable difference between the c- and pphPASMCs was revealed to be the expression levels of ALK-6. The present pphPASMCs exhibited at least approximately 10-fold higher level of ALK-6 mRNA, compared with three different cPASMCs, although the slight difference of ALK-6 expression among the control cells could be partly due to the conditions of cell passage or its isolation. Given the findings that the ALK-6 mRNA was not expressed or hardly detected in cPASMCs and that induction of wt-ALK-6 could restore the BMP signaling in the cPASMCs, a key factor of PASMC mitosis is likely to be the level of ALK-6 expression. However, the mechanism by which ALK-6 is up-regulated in our pphPASMCs has yet to be elucidated in a future study.

Among the BMPs we examined, BMP-2 and -7 showed

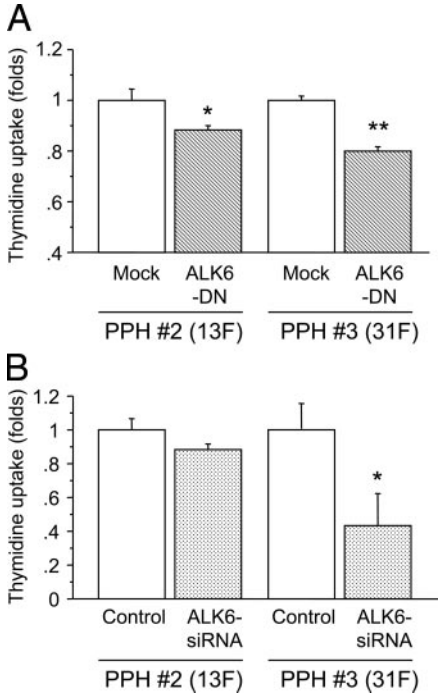

FIG. 10. Effects of ALK-6 blockage on different PASMCs isolated from sporadic PPH patients. A, Effect of ALK-6 DNs on PASMC mitosis. The pphPASMCs, cases 2 (13F) and 3 (31F), were transfected with Mock (pBUD-CE4.1) or pALK6-DN at 0.5 pmol and cultured in DMEM containing 1% FCS for 24 h followed by a 4-h pulse exposure of 0.5 μCi/well of thymidine. B, Effect of ALK-6-siRNA on PASMC mitosis. The pphPASMCs, cases 2 and 3, were transiently transfected with 100 nM fluorescein isothiocyanate-labeled siRNA duplex targeting a part of ALK-6 sequence and evaluated by thymidine uptake assay. After 24 h transfection with ALK-6-siRNA, the cells were cultured in DMEM containing 1% FCS followed by a 4-h pulse exposure of 0.5 μCi/well of thymidine. The thymidine level incorporated into the cells was counted. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate cultures. *, $P < 0.05$ and **, $P < 0.01$ *vs.* control.

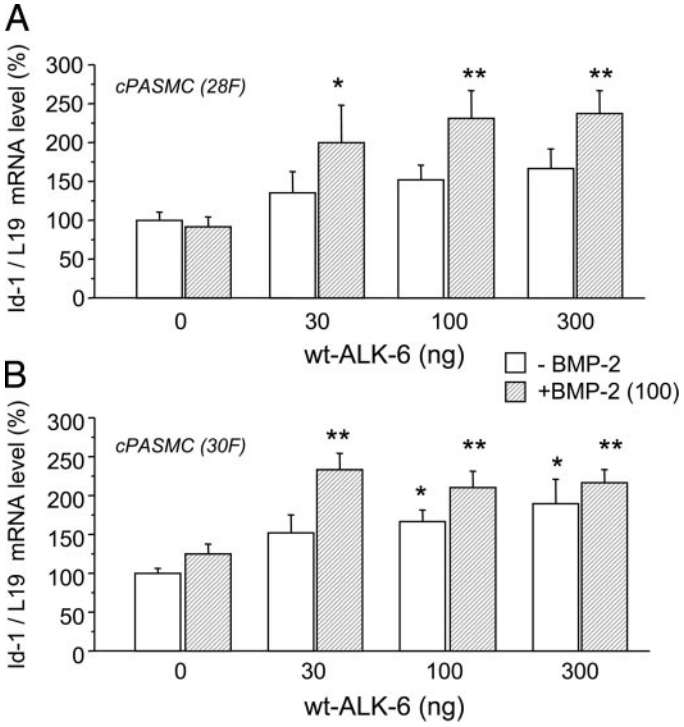

FIG. 11. Effects of wild-type ALK-6 overexpression on BMP signaling by PASMCs. Control PASMCs (A and B; 28F and 30F, respectively) were transfected with expression plasmid of wt ALK-6 receptor. Sixteen hours after the transfection of wt-ALK-6 construct, cPASMCs were treated for 24 h with or without BMP-2 (100 ng/ml) in DMEM containing 1% FCS, and the total cellular RNA was extracted. The mRNA levels of Id-1 of pphPASMCs were then evaluated using quantitative real-time RT-PCR analysis. Results show the mean ± SEM of data from three separate experiments, each performed with triplicate cultures; *, $P < 0.05$ and **, $P < 0.01$ *vs.* control (without ALK-6 and BMP-2).

stimulatory effects in DNA synthesis, whereas BMP-4 and -6 and activin-A showed a growth-suppressive effect in pphPASMCs. Thus, the reaction of pphPASMCs in response to exogenous BMPs was variable; however, it was clearly different from that of cPASMCs, which commonly exhibited a growth-suppressive effect by BMP-2 and -7. Taking into consideration that the experimental blockage of endogenous ALK-6 by a DN technique and siRNA abrogates the mitotic action and BMP signaling of pphPASMCs, a crucial role of ALK-6 in developing cell proliferation of pphPASMCs can be recognized. Furthermore, given that gene induction of full-structure ALK-6 into normal PASMCs enabled the enhancement of BMP signaling Id-1 in response to BMP-2, the impairment or defect of ALK-6 activation is most likely to be a critical difference between the PASMCs from normal and PPH lung in our study.

It is of interest that follistatin does not inhibit the mitotic activity of pphPASMCs. Follistatin was originally isolated from ovarian follicular fluid as an inhibitor of pituitary gonadotropin secretion and then found to be a potent binding protein for activins (27). The functions of follistatin were extended to include the regulation of the activities of BMP-4, -7, and -4/7 heterodimers and BMP-15 (28–30). Therefore, it is not clear whether a given *in vivo* effect of follistatin is caused by the inhibition of the activins and/or BMPs. A recent study showed that follistatin rather augments certain BMP-7 biological actions while inhibiting other BMP-7 biological activities (31). Thus, it is likely that the follistatin effect that nonspecifically neutralizes the actions of endogenous activins/BMPs is not critical to alter the mitotic activity on pphPASMCs. This finding may support the diverse effects of each activin/BMP ligand on the pphPASMC mitosis.

It has been difficult to determine the involvement of BMP type I receptors in the signal transduction of a particular BMP ligand because a specific BMP response can be mediated by different type I receptors, depending on the cell type or cell conditions (32). Nonetheless, some general preferences for the combination of BMP ligands and receptors have been recognized to date, *e.g.* BMP-2 and -4 and growth and differentiation factor-5 preferentially bind to ALK-3 and/or ALK-6; BMP-6 and -7 most readily bind to ALK-2 and/or ALK-6 (28, 33–35); and BMP-15 efficiently binds to ALK-6 with very much lower affinity for ALK-3 (21). Among the BMP ligands tested, our present data showed that BMP-2 and -7 are stimulatory factors for pphPASMC mitosis, which were completely different from the effects in the normal PASMCs. The receptor pair of ALK-6/BMPRII is recognized

to be the common functional complex for BMP-2 and -7 (8, 9, 28, 35). Because the BMP effects including its signaling and mitogenicity were abolished by the inhibition of ALK-6 activation through DN and siRNA techniques in the present results, the underlying mechanism by which BMP regulates cell mitosis would include the interaction between BMP ligands and ALK-6 in the pphPASMCs.

BMP ligands show higher affinity for type I receptors than type II receptors when independently overexpressed (36–38). Because the affinity of BMP ligands for type II receptors is enhanced when type II receptors are expressed together with the appropriate type I receptor (33, 36–38), type II and the appropriate type I receptors may act together to form a high-affinity complex. It can also be proposed that BMP ligands first bind to the type I subunits, and then type II receptors are secondarily recruited (21, 39), opposite of the pattern for activin/TGFβs. This concept may fit our present results. In particular, treatment with ALK-6-DN effectively inhibits BMP signaling as well as the spontaneous PPH cell mitosis. It is therefore possible that not only the oligomerization inhibition of given ALK-6 and BMPRII but also the inhibition of the binding between BMP ligands and endogenous ALK-6 is involved in the mechanism that suppresses pphPASMC mitosis by overexpressing ALK-6-DN. The oligomerization process of ALK-6 and BMPRII may be varied according to the conditions of *BMPR2* mutations. To characterize a complex of BMP ligands and ALK-6/BMPRII, oligomer may lead to clarifying the crucial difference between the PASMCs of familiar and sporadic PPH.

Morrell *et al.* (17) demonstrated interesting data on the effect of BMP ligands on PASMC mitosis. The PASMCs from PPH patients exhibited altered growth responses to exogenous BMP ligands, *i.e.* BMP-2, -4, and -7 failed to inhibit the PPH cell mitosis, unlike the PASMCs from normal control or secondary pulmonary hypertension (17). This result raised the possibility that PASMCs of PPH is resistant to the antiproliferative effects of TGFβ1 and BMPs. The abnormality of PASMC proliferation may result directly from the mutation or dysfunction in BMPRII and BMPRII-related signaling. However, a recent study by Zhang *et al.* (40) suggested the importance of apoptosis induced by BMPs in the PPH lung. In their study, BMP-2- and -7-induced apoptosis was significantly inhibited in PASMCs derived from PPH, compared with those from secondary pulmonary hypertension. The antiproliferative effects of BMPs, which are partially due to induction of apoptosis, may be an indispensable factor to manage the PASMC growth in normal pulmonary arteries.

The roles of *BMPR2* mutations in BMP signaling were carefully investigated by Nishihara *et al.* (10). They attempted to determine the biological activities of the BMPRII mutants reported in PPH patients. They demonstrated that missense mutations within the extracellular and kinase domains of BMPRII abrogate signal transduction, which seems to be caused in part by the altered localization these receptor domains. In contrast, the BMPRII mutants with the truncated cytoplasmic tails retained the ability to transduce normal BMP signaling, suggesting that BMPRII cannot be a single factor for the PPH pathogenesis. These findings raised the possible involvement of additional genetic and/or somatic factors to trigger the development of PPH lesions and further implied that causal factors for the PPH development are heterogeneous.

In this regard, Rindermann *et al.* (41) reported compelling evidence showing the genetic heterogeneity of PPH. In their study, 10 families of PPH patients were screened, and results found that two families showed a mutation in the *BMPR2* gene locus on chromosome 2q33. However, three families with no *BMPR2* mutation showed the new linkage to a more proximal location on 2q31, which was designated *PPH2*. Taking into consideration the existence of complication of hemorrhagic hereditary teleangiectasia, which possesses the *ALK-1* gene mutation in addition to PPH (42), environmental and genetic triggers other than *BMPR2* gene mutations may be necessary for the onset of PPH. As a possible candidate gene of *PPH2* region, activating transcription factor-2 transcription factor has been strongly postulated (41). Given the fact that activating transcription factor-2 plays a role in the downstream p38 kinase pathway (43), the involvement of MAPK signaling in the PPH pathogenesis has now emerged.

In the present study, we revealed that mitotic effects of BMP-2 and PDGF-BB are, at least in part, MAPK (ERK and p38) dependent. Whereas the majority of research on BMP signaling has focused on the Smad pathway, there is increasing evidence that other signaling pathways may also be involved in mediating BMP actions. Examples include TGFβ-activated kinase-1, a member of the MAPK kinase kinase family (44), which is activated by BMP-4 or TGFβ (44, 45), and the Ras/Rac families of small GTP-binding proteins, which are also implicated in the TGFβ signaling. Moreover, the ERK-1 and -2, and stress-activated protein kinase/c-Jun N-terminal kinase are linked to TGFβ signal transduction in some cell types (46, 47). Thus, there can be cross-talk in the BMP signaling pathway between Smads and MAPK signaling molecules (21, 24, 48). Our data here provided the first evidence that the MAPK pathway is functionally involved in the BMP system mainly composed of BMP-2 and ALK-6 in the pphPASMC mitosis.

Collectively, the present data imply the significant roles of a BMP type I receptor, ALK-6, in regulating PASMC cell mitosis in the PPH lung. In addition to the aberrant signaling through the mutated BMPRII, the significance of ALK-6 is now recognized in the PASMC mitosis of PPH. The present finding may provide further understanding with regard to the molecular pathogenesis of PPH and the novel therapeutic prospect that controls ALK-6 activity and/or MAPK activity for the prevention of excessive mitosis of pphPASMCs.

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References

1. **Rubin LJ** 1997 Primary pulmonary hypertension. N Engl J Med 336:111–117
2. **Palevsky HI, Schloo B, Pietra GG, Weber KT, Janicki JS, Rubin E, Fishman AP** 1989 Primary pulmonary hypertension: vascular structure, morphometry, and responsiveness to vasodilator agents. Circulation 80:1207–1221
3. **Wilkins MR, Gibbs JS, Shovlin CL** 2000 A gene for primary pulmonary hypertension. Lancet 356:1207–1208
4. **Nichols WC, Koller DL, Slovis B, Foroud T, Terry VH, Arnold ND, Siemieniak DR, Wheeler L, Phillips JA, Newman JH, Conneally PM, Ginsburg D, Loyd JE** 1997 Localization of the gene for familial primary pulmonary hypertension to chromosome 2q31–32. Nat Genet 15:277–280
5. **Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, Kalachikov S, Cayanis E, Fischer SG, Barst RJ, Hodge SE, Knowles JA** 2000 Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. Am J Hum Genet 67:737–744
6. **Lane KB, Machado RD, Pauciulo MW, Thomson JR, Phillips 3rd JA, Loyd JE, Nichols WC, Trembath RC** 2000 Heterozygous germline mutations in BMPR2, encoding a TGF-β receptor, cause familial primary pulmonary hypertension. The International PPH Consortium. Nat Genet 26:81–84
7. **Thomson JR, Machado RD, Pauciulo MW, Morgan NV, Humbert M, Elliott GC, Ward K, Yacoub M, Mikhail G, Rogers P, Newman J, Wheeler L, Higenbottam T, Gibbs JS, Egan J, Crozier A, Peacock A, Allcock R, Corris P, Loyd JE, Trembath RC, Nichols WC** 2000 Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding BMPR-II, a receptor member of the TGF-β family. J Med Genet 37:741–745
8. **Massague J** 1998 TGF-β signal transduction. Annu Rev Biochem 67:753–791
9. **Miyazono K, Kusanagi K, Inoue H** 2001 Divergence and convergence of TGF-β/BMP signaling. J Cell Physiol 187:265–276
10. **Nishihara A, Watabe T, Imamura T, Miyazono K** 2002 Functional heterogeneity of bone morphogenetic protein receptor-II mutants found in patients with primary pulmonary hypertension. Mol Biol Cell 13:3055–3063
11. **Kouchi H, Nakamura K, Fushimi K, Sakaguchi M, Miyazaki M, Ohe T, Namba M** 1999 Manumycin A, inhibitor of ras farnesyltransferase, inhibits proliferation and migration of rat vascular smooth muscle cells. Biochem Biophys Res Commun 264:915–920
12. **Takeda M, Otsuka F, Suzuki J, Kishida M, Ogura T, Tamiya T, Makino H** 2003 Involvement of activin/BMP system in development of human pituitary gonadotropinomas and nonfunctioning adenomas. Biochem Biophys Res Commun 306:812–818
13. **Otsuka F, Shimasaki S** 2002 A negative feedback system between oocyte bone morphogenetic protein 15 and granulosa cell kit ligand: its role in regulating granulosa cell mitosis. Proc Natl Acad Sci USA 99:8060–8065
14. **Otsuka F, Shimasaki S** 2002 A novel function of bone morphogenetic protein-15 in the pituitary: selective synthesis and secretion of FSH by gonadotropes. Endocrinology 143:4938–4941
15. **Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T** 2001 Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411:494–498
16. **Elbashir SM, Lendeckel W, Tuschl T** 2001 RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 15:188–200
17. **Morrell NW, Yang X, Upton PD, Jourdan KB, Morgan N, Sheares KK, Trembath RC** 2001 Altered growth responses of pulmonary artery smooth muscle cells from patients with primary pulmonary hypertension to transforming growth factor-β(1) and bone morphogenetic proteins. Circulation 104:790–795
18. **Kawabata M, Imamura T, Miyazono K** 1998 Signal transduction by bone morphogenetic proteins. Cytokine Growth Factor Rev 9:49–61
19. **Miyazono K** 2000 TGF-β signaling by Smad proteins. Cytokine Growth Factor Rev 11:15–22
20. **Miyazawa K, Shinozaki M, Hara T, Furuya T, Miyazono K** 2002 Two major Smad pathways in TGF-β superfamily signalling. Genes Cells 7:1191–1204
21. **Moore RK, Otsuka F, Shimasaki S** 2003 Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. J Biol Chem 278:304–310
22. **Tang SJ, Hoodless PA, Lu Z, Breitman ML, McInnes RR, Wrana JL, Buchwald M** 1998 The Tlx-2 homeobox gene is a downstream target of BMP signalling and is required for mouse mesoderm development. Development 125:1877–1887
23. **Rosenzweig BL, Imamura T, Okadome T, Cox GN, Yamashita H, ten Dijke P, Heldin C-H, Miyazono K** 1995 Cloning and characterization of a human type II receptor for bone morphogenetic proteins. Proc Natl Acad Sci USA 92:7632–7636
24. **Von Bubnoff A, Cho KWY** 2001 Intracellular BMP signaling regulation in vertebrates: pathway or network? Dev Biol 239:1–14
25. **Rudarakanchana N, Flanagan JA, Chen H, Upton PD, Machado R, Patel D, Trembath RC, Morrell NW** 2002 Functional analysis of bone morphogenetic protein type II receptor mutations underlying primary pulmonary hypertension. Hum Mol Genet 11:1517–1525
26. **Atkinson C, Stewart S, Upton PD, Machado R, Thomson JR, Trembath RC, Morrell NW** 2002 Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. Circulation 105:1672–1678
27. **DePaolo LV, Bicsak TA, Erickson GF, Shimasaki S, Ling N** 1991 Follistatin and activin: a potential intrinsic regulatory system within diverse tissues. Proc Soc Exp Biol Med 198:500–512
28. **Yamashita H, ten Dijke P, Huylebroeck D, Sampath TK, Andries M, Smith JC, Heldin C-H, Miyazono K** 1995 Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. J Cell Biol 130:217–226
29. **Iemura S, Yamamoto TS, Takagi C, Uchiyama H, Natsume T, Shimasaki S, Sugino H, Ueno N** 1998 Direct binding of follistatin to a complex of bone morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early *Xenopus* embryo. Proc Natl Acad Sci USA 95:9337–9342
30. **Otsuka F, Moore RK, Iemura S-I, Ueno N, Shimasaki S** 2001 Follistatin inhibits the function of the oocyte-derived factor BMP-15. Biochem Biophys Res Commun 289:961–966
31. **Amthor H, Christ B, Rashid-Doubell F, Kemp CF, Lang E, Patel K** 2002 Follistatin regulates bone morphogenetic protein-7 (BMP-7) activity to stimulate embryonic muscle growth. Dev Biol 243:115–127
32. **Shimasaki S, Moore RK, Otsuka F, Erickson GF** 2004 The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 25:72–101
33. **ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin C-H, Miyazono K** 1994 Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. J Biol Chem 269:16985–16988
34. **Ebisawa T, Tada K, Kitajima I, Tojo K, Sampath TK, Kawabata M, Miyazono K, Imamura T** 1999 Characterization of bone morphogenetic protein-6 signaling pathways in osteoblast differentiation. J Cell Sci 112:3519–3527
35. **Aoki H, Fujii M, Imamura T, Yagi K, Takehara K, Kato M, Miyazono K** 2001 Synergistic effects of different bone morphogenetic protein type I receptors on alkaline phosphatase induction. J Cell Sci 114:1483–1489
36. **Nohno T, Ishikawa T, Saito T, Hosokawa K, Noji S, Wolsing DH, Rosenbaum JS** 1995 Identification of a human type II receptor for bone morphogenetic protein-4 that forms differential heteromeric complexes with bone morphogenetic protein type I receptors. J Biol Chem 270:22522–22526
37. **Natsume T, Tomita S, Iemura S, Kinto N, Yamaguchi A, Ueno N** 1997 Interaction between soluble type I receptor for bone morphogenetic protein and bone morphogenetic protein-4. J Biol Chem 272:11535–11540
38. **Gilboa L, Nohe A, Geissendorfer T, Sebald W, Henis YI, Knaus P** 2000 Bone morphogenetic protein receptor complexes on the surface of live cells: a new oligomerization mode for serine/threonine kinase receptors. Mol Biol Cell 11:1023–1035
39. **Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, Knaus P** 2002 The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. J Biol Chem 277:5330–5338
40. **Zhang S, Fantozzi I, Tigno DD, Yi ES, Platoshyn O, Thistlethwaite PA, Kriett JM, Yung G, Rubin LJ, Yuan JX** 2003 Bone morphogenetic proteins induce apoptosis in human pulmonary vascular smooth muscle cells. Am J Physiol 285:L740–L754
41. **Rindermann M, Grunig E, von Hippel A, Koehler R, Miltenberger-Miltenyi G, Mereles D, Arnold K, Pauciulo M, Nichols W, Olschewski H, Hoeper MM, Winkler J, Katus HA, Kubler W, Bartram CR, Janssen B** 2003 Primary pulmonary hypertension may be a heterogeneous disease with a second locus on chromosome 2q31. J Am Coll Cardiol 18:2237–2244
42. **Trembath RC, Thomson JR, Machado RD, Morgan NV, Atkinson C, Winship I, Simonneau G, Galie N, Loyd JE, Humbert M, Nichols WC, Morrell NW, Berg J, Manes A, McGaughran J, Pauciulo M, Wheeler L** 2001 Clinical and molecular genetic features of pulmonary hypertension in patients with hereditary hemorrhagic telangiectasia. N Engl J Med 345:325–334
43. **Cocolakis E, Lemay S, Ali S, Lebrun JJ** 2001 The p38 MAPK pathway is required for cell growth inhibition of human breast cancer cells in response to activin. J Biol Chem 276:18430–18436
44. **Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, Matsumoto K** 1995 Identification of a member of the MAPKKK family as a potential mediator of TGF-β signal transduction. Science 270:2008–2011
45. **Yamaguchi K, Nagai S, Ninomiya-Tsuji J, Nishita M, Tamai K, Irie K, Ueno N, Nishida E, Shibuya H, Matsumoto K** 1999 XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway. EMBO J 18:179–187
46. **Nakamura K, Shirai T, Morishita S, Uchida S, Saeki-Miura K, Makishima F** 1999 p38 Mitogen-activated protein kinase functionally contributes to chondrogenesis induced by growth/differentiation factor-5 in ATDC5 cells. Exp Cell Res 250:351–363
47. **Piek E, Heldin CH, Ten Dijke P** 1999 Specificity, diversity, and regulation in TGF-β superfamily signaling. FASEB J 13:2105–2124
48. **Suzuki J, Otsuka F, Inagaki K, Takeda M, Ogura T, Makino H** 2004 Novel action of activin and bone morphogenetic protein in regulating aldosterone production by human adrenocortical cells. Endocrinology 145:639–649

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