

## ORIGINAL ARTICLE

# Involvement of the bone morphogenetic protein system in endothelin- and aldosterone-induced cell proliferation of pulmonary arterial smooth muscle cells isolated from human patients with pulmonary arterial hypertension

Ryutaro Yamanaka<sup>1</sup>, Fumio Otsuka<sup>1</sup>, Kazufumi Nakamura<sup>2</sup>, Misuzu Yamashita<sup>1</sup>, Hiroyuki Otani<sup>1</sup>, Masaya Takeda<sup>1</sup>, Yoshinori Matsumoto<sup>1</sup>, Kengo F Kusano<sup>2</sup>, Hiroshi Ito<sup>2</sup> and Hirofumi Makino<sup>1</sup>

Recent genetic studies have uncovered a link between familial and idiopathic pulmonary arterial hypertension (PAH) and germline mutations in the bone morphogenetic protein type-II receptor (BMPRII). The pathology of PAH is characterized by remodeling of the pulmonary arteries due to pulmonary artery smooth muscle cell (PASMC) hyperproliferation. Although increased endothelial injury and impaired suppression of PASMC proliferation are both critical for the cellular pathogenesis of PAH, a detailed molecular mechanism underlying PAH has yet to be elucidated. In the present study, we investigated the roles of the BMP system and other vasoactive factors associated with PAH (including endothelin (ET), angiotensin II (Ang II) and aldosterone) in the mitotic actions of PASMCs isolated from idiopathic and secondary PAH lungs. ET1 and aldosterone stimulated PASMC proliferation of idiopathic PAH more effectively than secondary PAH, whereas Ang II and ET3 failed to activate mitosis in either of the PASMC cell type. The effects of ET1 and aldosterone were blocked by bosentan, an ET type-A/B receptor (ETA/BR) antagonist, and eplerenone, a selective mineralocorticoid receptor (MR) blocker, respectively. Among the BMP ligands examined, BMP-2 and BMP-7, but not BMP-4 or BMP-6, significantly increased cell mitosis in both PASMC cell types. Notably, ET1- and aldosterone-induced mitosis and mitogen-activated protein kinase phosphorylation were significantly increased in the presence of BMP-2 and BMP-7 in PASMCs isolated from idiopathic PAH, although additive effects were not observed in PASMCs isolated from secondary PAH. Inhibition of extracellular signal-regulated kinase 1 (ERK1)/ERK2 signaling suppressed basal-, ET1- and aldosterone-induced PASMC mitosis more potently than that of stress-activated protein kinase/c-Jun NH2-terminal kinase inhibition. Given the fact that BMP-2 and BMP-7 upregulated ETA/BR and MR expression and that BMP-2 decreased 11 $\beta$ HSD2 (11 $\beta$ -hydroxysteroid dehydrogenase type 2) levels in PASMCs isolated from idiopathic PAH, BMPR-Smad signaling may have a key role in amplifying the ETA/BR and/or MR-ERK signaling in PASMCs of the PAH lung. Collectively, the functional link between BMP and ET and/or the MR system may be involved in the progress of PASMC mitosis, ultimately leading to the development of clinical PAH.

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## INTRODUCTION

Pulmonary arterial hypertension (PAH) is a life-threatening disease that has a prevalence of one to two patients per one million individuals, with a higher frequency in women.<sup>1</sup> This disease is histologically characterized by excessive proliferation of vascular endothelium and smooth muscle cells, causing thickening of pulmonary arteriole walls and formation of plexiform lesions that eventually occlude the vascular lumen.<sup>2</sup> The remodeling of pulmonary arterioles

increases vascular resistance and reduces vascular dilatation, resulting in right ventricular failure.

Approximately six percent of PAH cases have an autosomal-dominant pattern of inheritance. Recent research has uncovered a genetic predisposition to familial PAH.<sup>3,4</sup> Two studies involving the screening of the locus of the gene for PAH have mapped it to chromosome 2q33 (*PPH1*) and found mutations in the *BMPR2* gene, which encodes bone morphogenetic protein type-II receptor (BMPRII), in 9 out of

<sup>1</sup>Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan and <sup>2</sup>Department of Cardiovascular Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

Correspondence: Dr F Otsuka, Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kitaku, Okayama 700-8558, Japan.

E-mail: fumiotsu@md.okayama-u.ac.jp

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19<sup>5</sup> or 7 out of 8 families.<sup>6</sup> Furthermore, the *BMP2* mutation can be detected in at least one quarter of sporadic PAH cases.<sup>7</sup>

Hence, the identification of heterozygous germline mutations in the *BMP2* gene in familial and sporadic cases of PAH has proven to be a crucial breakthrough in elucidating the underlying pathogenesis of PAH.<sup>3</sup> Subsequent functional studies have provided compelling evidence that PAH cells that harbor the *BMP2* mutations exhibit aberrant BMPRII function and disrupted BMP signaling.<sup>8,9</sup> However, given the fact that almost half of all PAH patients do not have the *BMP2* mutation, the entire mechanism of pulmonary artery smooth muscle cell (PASMC) mitogenesis in PAH lungs is still unclear. BMP ligands that are involved in the aberrant signaling caused by the BMPRII mutations in PAH patients have also not been identified.

Pulmonary vascular wall thickening due to inappropriate proliferation of PASMCs is a central cause of elevated pulmonary vascular resistance and arterial pressure in PAH patients.<sup>10,11</sup> Unbalanced proliferation and apoptosis of smooth muscle cells contributes to vascular remodeling in pulmonary hypertension.<sup>12</sup> Disequilibrium in vasoactive mediators in the lungs, including increased thromboxane and endothelin-1 (ET1) and decreased prostacyclin and nitric oxide, leads to vasoconstriction and proliferation of smooth muscle cells.<sup>13,14</sup> Studies using various ET receptor antagonists have confirmed that ET1 is a critical factor in the pathogenesis of vascular remodeling in experimental models of chronic pulmonary hypertension and clinical observation of pulmonary hypertension.<sup>15,16</sup> In addition to the role of ET1 as a potent vasoconstrictor, it also acts directly to modulate the growth of human pulmonary vascular smooth muscle cells as an autocrine/paracrine mitogen.<sup>17,18</sup>

Additional factors, such as serotonin, platelet-derived growth factor (PDGF) and angiotensins, have been postulated to contribute to pulmonary vasoconstriction, increased smooth muscle growth and increased matrix deposition. A polymorphism in the angiotensin-converting enzyme gene is known to be associated with the manifestation of pulmonary hypertension.<sup>19</sup> Increased angiotensin-converting enzyme expression is observed in plexiform lesions in PAH,<sup>20</sup> and angiotensin-converting enzyme inhibition seems to delay pulmonary vascular neointimal formation.<sup>21</sup> Therefore, the renin-angiotensin-aldosterone system (RAAS) represents a group of candidate genes that could modify disease severity and/or age at diagnosis of individuals predisposed to develop PAH. Angiotensin II (Ang II) binds primarily to the Ang II type-1 receptor (AT1R) to promote vascular smooth muscle constriction. Genetic variants or polymorphisms in genes within AT1R are known to enhance RAAS activation and/or receptor function, which may be linked to modifications in the risk of developing PAH.<sup>22</sup> Ang II also acts on the adrenal cortex to promote aldosterone production by CYP11B2, which encodes P450 aldosterone synthase. However, the significance of aldosterone action in the pathogenesis of PAH has yet to be elucidated. There is also accumulating evidence indicating that aldosterone exerts physiologically and/or pathophysiologically relevant effects on the cardiovascular system, kidney and brain, in addition to its well-established role in regulating body electrolytes and water homeostasis.

In the present study, we attempted to elucidate the underlying mechanism of abnormal PASMC proliferation and the functional link between the BMP system and other vasoactive factors for PAH, including ET, Ang II and aldosterone, in the mitotic actions of PASMCs isolated from primary and secondary PAH lungs.

## METHODS

### Reagents and supplies

Dulbecco's modified Eagle's medium, penicillin-streptomycin solution, angiotensin II acetate salt (Ang II), d-aldosterone, dimethylsulfoxide (DMSO) and

human ET1 and ET3 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human BMP-2, BMP-4, BMP-6 and BMP-7 and mouse noggin were purchased from R&D Systems (Minneapolis, MN, USA) and human PDGF-BB was from PeproTech EC (London, UK). U0126 and SB203580 were purchased from Promega (Madison, WI, USA) and SP600125 was purchased from Biomol Lab (Plymouth Meeting, PA, USA). Bosentan, candesartan (CV-11974) and eplerenone were kindly provided by Actelion Pharmaceuticals (Allschwil, Switzerland), Takeda Chemical Industries (Osaka, Japan) and Pfizer (New York, NY, USA), respectively.

### Isolation of human PASMCs and establishment of primary PASMC culture

Human PASMCs were isolated during lung transplant surgery from patients diagnosed with idiopathic pulmonary arterial hypertension (PAH) and secondary pulmonary hypertension (SPH). The PAH patient (29-year-old male) had developed dyspnea, severe hypoxia and right ventricular heart failure without any family history. The SPH patient (16-year-old female) suffered from general fatigue, hypoxia and right ventricular heart failure due to a progressive atrial septal defect. As controls, normal pulmonary arterial smooth muscle cells (Nor-PASMCs) were isolated from the histologically normal pulmonary arteries of lung tissues resected from a patient (58-year-old male) with lung cancer. The PAH- and SPH-PASMCs were manually isolated from lung tissues that had developed severe plexiform lesions in the pulmonary arteries. The sections of the resected lung tissues were examined by hematoxylin-eosin staining and the PASMCs were then explanted, isolated and cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 1% penicillin-streptomycin solution, as previously described.<sup>23,24</sup> Until the scheduled experiments were carried out, the cells were cultured in 75 cm<sup>2</sup> flasks at 37°C in a humidified atmosphere of 95% air/5% CO<sub>2</sub>. Cells were used for the scheduled experiments at passages 5–7. All human subject protocols were approved by the Ethics Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, and a written informed consent was obtained from all patients before the procedure. The investigation also conforms to the principles outlined in the Declaration of Helsinki.

### Total cellular RNA extraction and reverse transcriptase-PCR

The PASMCs were grown in six-well plates to ~80% confluence, and after the treatment indicated, the medium was removed and total cellular RNA was extracted by the isothiocyanate-acid-phenol-chloroform method using TRIzol (Invitrogen, Carlsbad, CA, USA) and quantified by measuring absorbance at 260 nm. The RNA was stored at -80°C until assays were performed. The expression of BMP/activin receptors, Smads, follistatin and noggin mRNAs were detected by reverse transcriptase (RT)-PCR. Oligonucleotides used for PCR were custom ordered from Invitrogen. PCR primer pairs for human BMP receptors, Smads and a house-keeping gene, ribosomal protein-L19, were selected from different exons of the corresponding genes to discriminate PCR products that might arise from possible chromosome DNA contaminants, as reported earlier.<sup>25</sup> For AT1R, ET type-A (ETAR) and type-B receptors (ETBR), mineralocorticoid receptor (MR), noggin, activin receptor-like kinase (ALK)-1, Id-1 and 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ HSD2), the following sequences were used: AT1R, 34–53 and 376–395 from GenBank accession number M93394; ETAR, 275–295 and 483–503 from L06622; ETBR, 931–951 and 1172–1192 from S44866; MR, 2730–2751 and 2971–2992 from M16801; noggin, 541–561 and 750–770 from NM\_005450; ALK-1, 1160–1180 and 1501–1521 from NM\_001077401; Id-1, 211–230 and 350–369 from X77956; and 11 $\beta$ HSD2, 611–631 and 882–902 from U27317. The extracted RNA (1  $\mu$ g) was subjected to a RT reaction using First-Strand cDNA Synthesis System (Invitrogen) with random hexamers (2 ng  $\mu$ l<sup>-1</sup>), reverse transcriptase (200 U) and deoxynucleotide triphosphate (dNTP; 0.5 mM) at 42°C for 50 min and then at 70°C for 10 min. Hot-start PCR was then performed using MgCl<sub>2</sub> (1.5 mM), dNTP (0.2 mM) and 2.5 U of Taq DNA polymerase (Invitrogen) under the conditions as we have reported previously.<sup>25</sup> Aliquots of the PCR products were electrophoresed on 1.5% agarose gels, visualized after ethidium bromide staining, photographed and scanned.

### Quantitative real-time RT-PCR analysis

For the quantification of Id-1, ETAR, ETBR, MR and 11 $\beta$ HSD2 mRNA levels, real-time PCR was performed using the LightCycler-FastStart DNA master SYBR Green I system (Roche Diagnostic, Tokyo, Japan) under the optimized condition of annealing at 60°C with 4 mM MgCl<sub>2</sub>, following the manufacturers protocol. Accumulated levels of fluorescence for each product were analyzed by the second derivative method after the melting-curve analysis (Roche Diagnostic). Then, following assay validation by calculating the efficiency of each amplification reaction, the expression levels of target genes were quantified based on standard curve analysis for each product and normalized to the ribosomal protein-L19 level in each target.

### Genomic DNA extraction and sequence determination of BMPR2 gene

Genomic DNA was isolated from peripheral leukocytes of PAH patients using a genomic DNA isolation kit (Gentra Systems, Minneapolis, MN, USA) and each of the 12 exons of the *BMPR2* gene were PCR amplified using corresponding primers that recognize each exon.<sup>5</sup> The PCR products were then directly sequenced (ABI Prism 310, Applied Biosystems, Foster City, CA, USA).

### Thymidine incorporation assay

The PASCs (1 $\times$ 10<sup>4</sup> viable cells per well) were precultured in 12-well human fibronectin-coated plates (BD Biosciences, Bedford, MA, USA) containing 1 ml culture medium. After the preculture period, the medium was replaced with fresh medium containing 1% fetal calf serum and the indicated concentrations of hormones and/or growth factors were added. After 24 h of treatment, 0.5  $\mu$ Ci per well of [methyl-<sup>3</sup>H] thymidine (Amersham Pharmacia, Piscataway, NJ, USA) was added and incubated for 3 h at 37°C. The incorporated thymidine was detected as previously reported.<sup>26</sup> Cells were then washed with PBS, incubated with 10% ice-cold trichloroacetic acid for 30 min at 4°C and solubilized in 0.5 M NaOH, and their radioactivity was determined by a liquid scintillation counter (TRI-CARB 2300TR, Packard, Meriden, CT, USA).

### Western immunoblot analysis

The PASCs (1 $\times$ 10<sup>4</sup> viable cells per well) were precultured in 12-well plates in serum-free Dulbecco's modified Eagle's medium. After 24 h of preculture, the indicated concentrations of ET and aldosterone were added to the culture medium either alone or in combination with BMP-2 and BMP-7. After 15 or 60 min stimulation with hormones and/or growth factors, cells were solubilized in 100  $\mu$ l RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY, USA) containing 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 2% sodium dodecyl sulfate and 4%  $\beta$ -mercaptoethanol. The cell lysates were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis/immunoblotting analysis using anti-phospho- and anti-total-extracellular signal-regulated kinase (ERK) 1/2 mitogen-activated protein kinase (MAPK) antibody, anti-phospho- and anti-total-p38 MAPK antibody, anti-phospho- and anti-total-stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) MAPK antibody, anti-phospho- and anti-total-Akt antibody and anti-phospho-Smad1/5/8 and anti-Smad5 antibody (Cell Signaling Technology, Beverly, MA, USA). The relative integrated density of each protein band was digitized by NIH image J 1.34s software.

### Statistical analysis

All results are shown as mean  $\pm$  s.e.m. of data from at least three separate experiments, each performed in triplicate. Differences between groups were analyzed for statistical significance using analysis of variance (ANOVA) with Fisher's protected least significant difference test (StatView 5.0 software, Abacus Concepts, Berkeley, CA, USA). *P*-values <0.05 were accepted as statistically significant.

## RESULTS

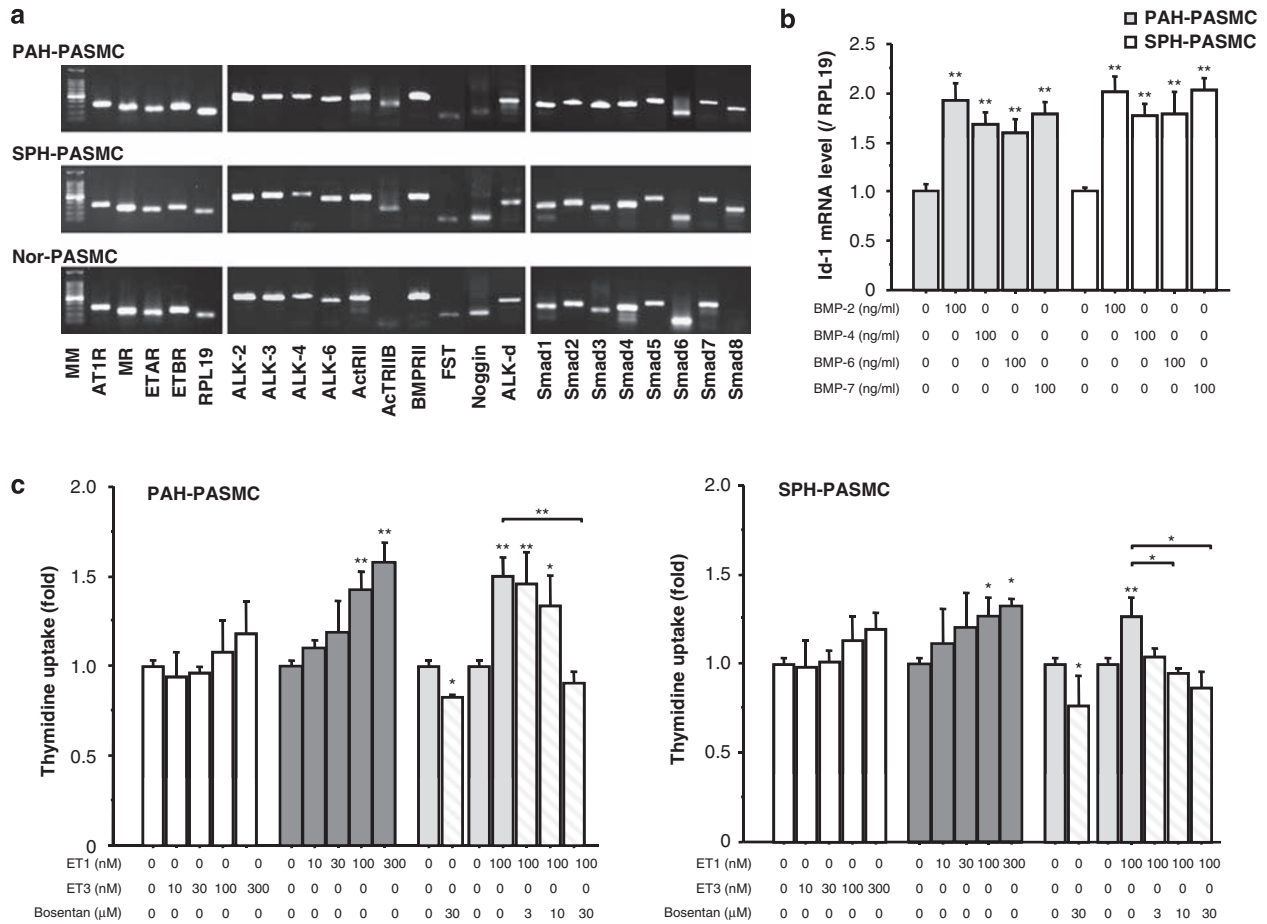
We first characterized the expression profile of key receptors, including Ang II, aldosterone and ET, and the BMP system molecules, including BMP type-I (ALK-2, ALK-3, ALK-4 and ALK-6) and type-II (ActRII, ActRIIB and BMPRII) receptors, binding proteins including follistatin and noggin, a TGF- $\beta$  type-I receptor (ALK-1), Smads1–8 in PASCs

isolated from normal controls (Nor-PASCs), idiopathic pulmonary arterial hypertension (PAH-PASCs) and secondary pulmonary arterial hypertension (SPH-PASCs) (Figure 1). As shown in Figure 1a, AT1R, MR, ETAR and ETBR were expressed in PAH-, SPH- and Nor-PASCs as seen by RT-PCR. All of the type-I receptors examined were expressed in PAH-, SPH- and Nor-PASCs. BMPRII and ActRII were expressed in PAH-, SPH- and Nor-PASCs, although ActRIIB was not detected in Nor-PASCs. Follistatin and noggin were also detected in the three types of PASCs, with noggin expression in PAH-PASCs being comparably lower than that of SPH- and Nor-PASCs. PAH- and SPH-PASCs expressed receptor-specific Smads1, 2, 3, 5 and 8, a common Smad4 and inhibitory Smads6 and 7, whereas Nor-PASCs lacked Smad8 expression. Direct sequencing of PCR products from all 12 exons of the *BMPR2* gene<sup>5</sup> found no *BMPR2* mutations in the genomic DNA of the patient from whom we collected the PAH-PASCs. BMPRII mRNA, including the N-terminus region, which encodes the extracellular and transmembrane domains, was expressed in all three types of PASCs. To compare the BMPR signal activities in PAH- and SPH-PASCs, the transcription of a BMP target gene (Id-1) was examined in PASCs treated with BMP-2, BMP-4, BMP-6 and BMP-7 for 24 h. As shown in Figure 1b, BMP-2, BMP-4, BMP-6 and BMP-7 all increased Id-1 mRNA levels in PAH- and SPH-PASCs by the same amount.

We next examined the effects of ET on mitosis of PASCs isolated from idiopathic and secondary PAH patients. The mitotic activity of PASCs was quantified by a thymidine incorporation assay. As shown in Figure 1c, ET1 induced cell proliferation of PAH- and SPH-PASCs in a concentration-dependent manner for 24 h, in which the maximal effects of ET1 on PAH-PASCs were higher compared with the effects on SPH-PASCs. In contrast to ET1, ET3 treatment failed to induce significant mitotic activity in both PAH- and SPH-PASCs. Bosentan, a competitive ET1 antagonist that inhibits binding to both ETAR and ETBR, reduced basal mitotic activity in both PASC types. Bosentan (3–30  $\mu$ M) treatment reversed ET1-induced cell proliferation, and 30  $\mu$ M bosentan abolished the effect of ET1 on mitosis of both PASC types.

Next, to investigate the contribution of RAAS to PASC mitosis, cells were treated with Ang II for 24 h (Figure 2). As shown in Figure 2a and b, Ang II (30–300 nM) had no significant effects on the mitosis of either PASC type. In addition, candesartan, an AT1R antagonist, at a concentration of 1–10 nM, failed to reverse ET1-induced cell proliferation in these PASCs. Notably, aldosterone (1–300 nM) elicited potent mitotic activity in PAH-PASCs in a concentration-dependent manner (Figure 2c). The effects of aldosterone on PASC mitosis were prominent in PAH-PASCs compared with SPH-PASCs (Figure 2d). An MR antagonist, eplerenone (100–300 nM), abolished aldosterone-induced mitotic activity, although eplerenone alone had no significant suppression of basal cell proliferation in both types of PASCs (Figures 2c and d).

Because the BMP system, including type-I and -II receptors and Smads, was found to be present in both PASC types, we next examined whether this receptor system is functional. To evaluate the effect of each BMP ligand, cells were cultured in the presence of four BMP ligands (Figure 3). As we have previously shown, the effects of each individual BMP ligands on PASC mitosis differed, with BMP-2 and BMP-7 acting as stimulators of mitosis of PAH-PASCs.<sup>26</sup> As shown in Figures 3a and c, BMP-2 and BMP-7 (10–100 ng ml<sup>-1</sup>) induced mitosis in both PAH- and SPH-PASCs. In contrast, BMP-4 and BMP-6 had no effect on PASC mitogenicity. PDGF-BB (100 ng ml<sup>-1</sup>), which is known to be a potent PASC growth factor,<sup>8</sup> increased DNA synthesis by both PASC types by up to



**Figure 1** Characterization of BMP, endothelin and the angiotensin–aldosterone system in isolated PASCs. **(a)** Total cellular RNA was collected from cultured PAH-, SPH- and Nor-PASCs. Expression of mRNAs encoding angiotensin II type-1 receptor (AT1R), mineralocorticoid receptor (MR), endothelin type-A and type-B receptor (ETAR and ETBR), BMP/activin/TGF- $\beta$  type-2 (ALK-1, ALK-2, ALK-3, ALK-4 and ALK-6) and BMP/activin type-2<sup>2</sup> receptors (ActR<sup>22</sup>, ActR<sup>22</sup>B and BMPR<sup>22</sup>), BMP/activin-binding proteins (follistatin; FST and noggin) and the Smad signaling molecules (Smad1–8) were examined by RT-PCR in PAH-, SPH- and Nor-PASCs. MM indicates the molecular weight marker. **(b)** PASCs were treated with BMP-2, BMP-4, BMP-6 and BMP-7 (100 ng ml<sup>-1</sup>) for 24 h. Total cellular RNA was extracted and the Id-1 mRNA level was examined by quantitative real-time PCR. The expression level of Id-1 was standardized by ribosomal protein L19 (RPL19) level in each sample. **(c)** PAH- and SPH-PASCs were cultured in DMEM containing 1% FCS and treated with the indicated concentrations of endothelin-1 (ET1; 10–300 nM) and endothelin-3 (ET3; 10–300 nM) in combination with bosentan (3–30  $\mu$ M) for 24 h followed by a 3 h pulse exposure of 0.5  $\mu$ Ci per well of thymidine. The thymidine level incorporated into the cells was counted. Results are shown as the mean  $\pm$  s.e.m. of data from three separate experiments, each performed in triplicate; \* $P$ <0.05 and \*\* $P$ <0.01 vs. control groups and between the indicated groups.

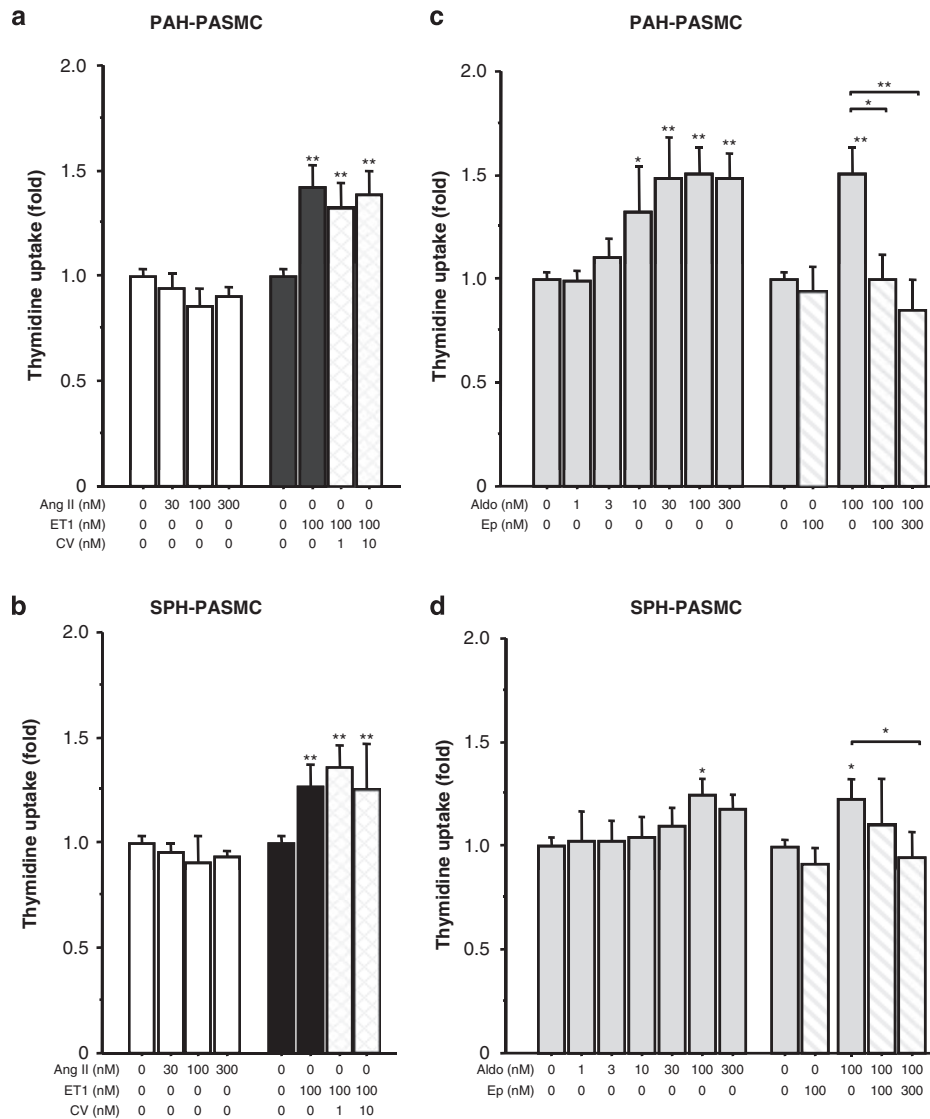
approximately twofold (Figure 3). We further examined the functional interrelationship between the BMP system and ET1 or aldosterone in PASC proliferation. As shown in Figures 3b and d, cells were treated with BMP-2 and BMP-7 (10–100 ng ml<sup>-1</sup>) in combination with either ET1 (10–100 nM) or aldosterone (10–100 nM). In PAH-PASCs, ET1 and aldosterone additively enhanced BMP-2- and BMP-7-induced cell mitosis (Figure 3b). However, in SPH-PASCs, neither ET1 nor aldosterone had any significant effect on BMP-2- and BMP-7-induced cell mitosis (Figure 3d).

To investigate the mechanism by which ET1 and aldosterone augment BMP actions in PAH-PASCs, we examined key signaling pathways, including MAPK and AKT, in PASCs. Cell mitosis in most smooth muscle cell types is generally associated with activation of the MAPK pathway. Recent studies have shown crosstalk between BMP-Smad signaling and the MAPK pathway in various cell types.<sup>27</sup> As shown in Figure 4a, PASCs were stimulated with aldosterone, Ang II, ET1, ET3 and PDGF-BB in serum-free conditions for 15 min. ET1 (100 nM) stimulated ERK1/ERK2 and SAPK/JNK phosphorylation in PAH-PASCs, whereas the ET1 effects on ERK1/ERK2 signal

were less potent in SPH-PASCs (Figure 4a). ET3 also activated ERK1/ERK2 and SAPK/JNK phosphorylation in both PASC types; however, the effects of ET3 were much less potent than that of ET1. On the contrary, ET1 did not affect P38-MAPK and AKT signaling in either PASC type. In addition, the effects of aldosterone and Ang II (100 nM) alone were negligible with regard to MAPK and AKT activation in both PAH- and SPH-PASCs (Figure 4a).

To investigate the involvement of MAPK signaling in PASCs, we used specific inhibitors of ERK1/ERK2 and SAPK/JNK pathways, U0126 and SP600125, respectively. As shown in Figure 4b, treatment with U0126 (0.3–3  $\mu$ M) suppressed basal mitotic activity of both PASC types, whereas the effects of SP600125 (0.3–3  $\mu$ M) were less potent. The effects of these MAPK inhibitors on PASC mitosis were further evaluated in the presence of ET1 and aldosterone. The mitotic action induced by either ET1 or aldosterone was restored by MAPK inhibition, with the effects of U0126 significantly more effective than those of SP600125 (Figure 4b). Thus, the mitotic effects induced by ET1 and aldosterone seemed to occur, at least in part, through activation of the MAPK pathway in both PAH- and SPH-PASCs.

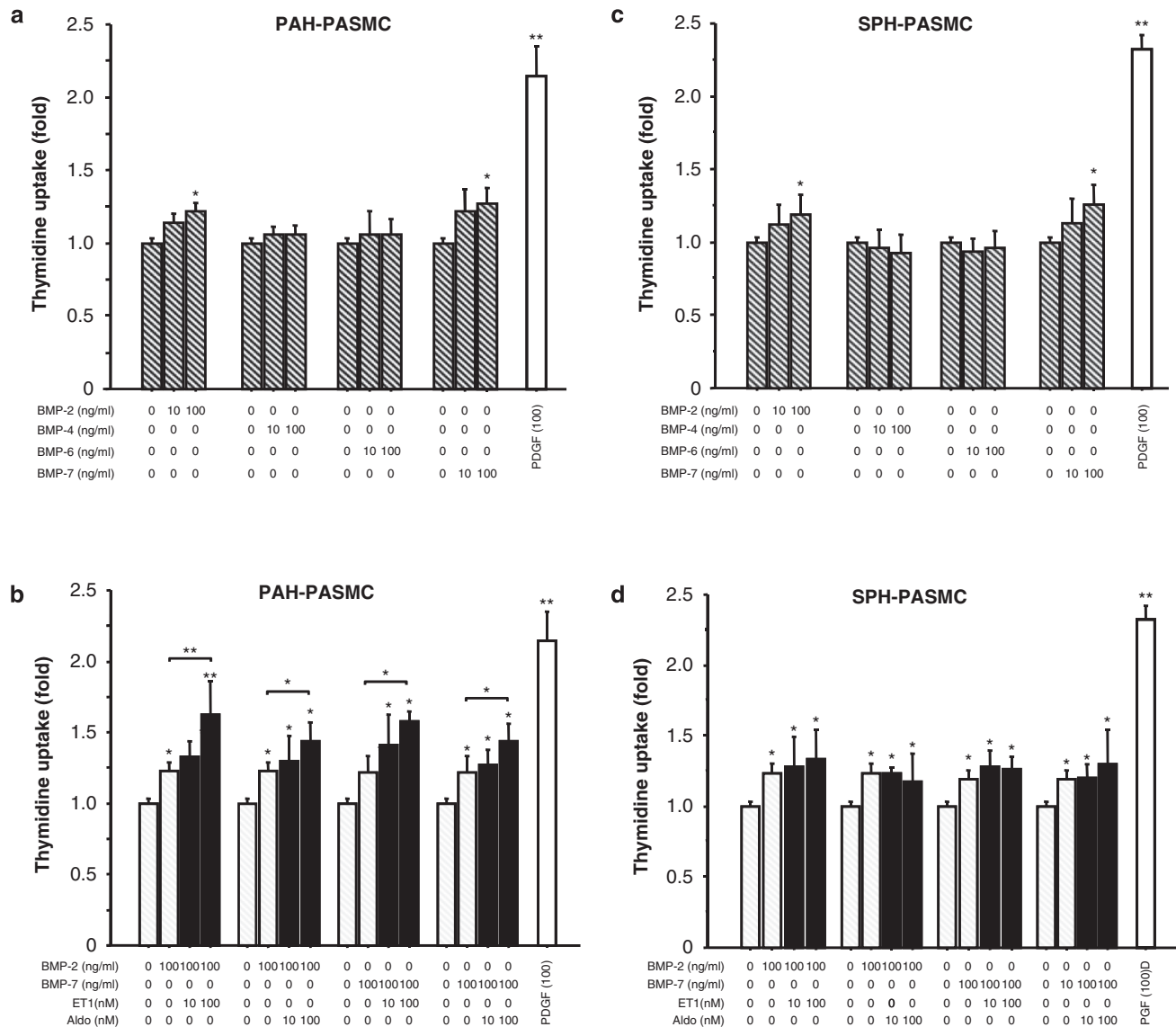




**Figure 2** Effects of angiotensin II (Ang II) and aldosterone on PASM mitosis. PAH- and SPH-PASMCs were cultured in DMEM containing 1% FCS and treated with Ang II (30–300 nM) and ET1 (100 nM) in combination with Ang II type-1 receptor (AT1R) antagonist, candesartan (CV; 1–10 nM) (a, b); or treated with aldosterone (Aldo; 1–300 nM) in combination with MR antagonist, eplerenone (Ep; 100–300 nM) (c, d) for 24 h followed by a 3 h pulse exposure of 0.5  $\mu$ Ci per well of thymidine. The thymidine level incorporated into cells was counted. Results show the mean  $\pm$  s.e.m. of data from three separate experiments, each performed in triplicate; \* $P$ <0.05 and \*\* $P$ <0.01 vs. control groups and between the indicated groups.

To further estimate the signal crosstalk between BMP and MAPK in PASCs, cells were cotreated with BMP-2 or BMP-7 in combination with ET1 and aldosterone. As shown in Figure 5, BMP-2 and BMP-7, which stimulated cell mitosis in both PASC types through Smad1/5/8 phosphorylation, had no effect on MAPK activation (including ERK1/ERK2 and SAPK/JNK). Notably, ET1-induced phosphorylation of ERK1/ERK2 and SAPK/JNK was enhanced in the presence of BMP-2 and BMP-7 in PAH-PASCs (Figure 5a). BMP-2 and BMP-7 also upregulated ERK1/ERK2 and SAPK/JNK phosphorylation in the presence of aldosterone in PAH-PASCs, although aldosterone alone did not activate the ERK1/ERK2 and SAPK/JNK pathways (Figure 5b). In the case of SPH-PASCs, the combined effects of ET1 and either BMP-2 or BMP-7 on MAPK activation were only marginally detected (Figure 5a). No additive effects of aldosterone and either BMP-2 or BMP-7 on MAPK were observed in SPH-PASCs (Figure 5b).

To characterize the differences of ET1 and aldosterone sensitivities on PASC mitosis between PAH- and SPH-PASCs, the expression levels of ETAR, ETBR, MR and 11 $\beta$ HSD2 were examined by real-time PCR (Figure 6). As shown in Figure 6a, basal levels of ETAR and ETBR mRNA were relatively high, but the level of 11 $\beta$ HSD2 mRNA was lower in PAH-PASCs compared with SPH-PASCs. We next examined the effects of endogenous BMPs on ET receptors and MR expression in PASCs using the BMP-binding protein, noggin, which neutralizes broad BMP actions. Noggin treatment (30 ng ml<sup>-1</sup>) suppressed the expression levels of ETAR and ETBR in PAH-PASCs for 24 h, whereas it increased ETAR and ETBR expression in SPH-PASCs (Figure 6b), suggesting that endogenous BMPs act to upregulate ET receptors in PAH-PASCs and downregulate those of SPH-PASCs. MR expression was not affected by noggin in either PASC type. Furthermore, BMP-2 and BMP-7 upregulated ETAR and ETBR mRNA expression, whereas ET1 had no effect on ET



**Figure 3** Combined effects of BMP, endothelin and aldosterone on PASC mitosis. PAH- and SPH-PASCs were cultured in DMEM containing 1% FCS and treated with BMP-2, BMP-4, BMP-6 and BMP-7 ( $10$ – $100$  ng ml<sup>-1</sup>) and PDGF-BB ( $100$  ng ml<sup>-1</sup>) (a, c); or treated with endothelin-1 (ET1;  $10$  and  $100$  nM) and aldosterone (Aldo;  $10$  and  $100$  nM) in combination with BMP-2 and BMP-7 ( $100$  ng ml<sup>-1</sup>) (b, d) for 24 h followed by a 3 h pulse exposure of  $0.5$   $\mu$ Ci per well of thymidine. The thymidine level incorporated into cells was counted. Results show the mean  $\pm$  s.e.m. of data from three separate experiments performed in triplicate; \* $P$  < 0.05 and \*\* $P$  < 0.01 vs. control groups and between the indicated groups.

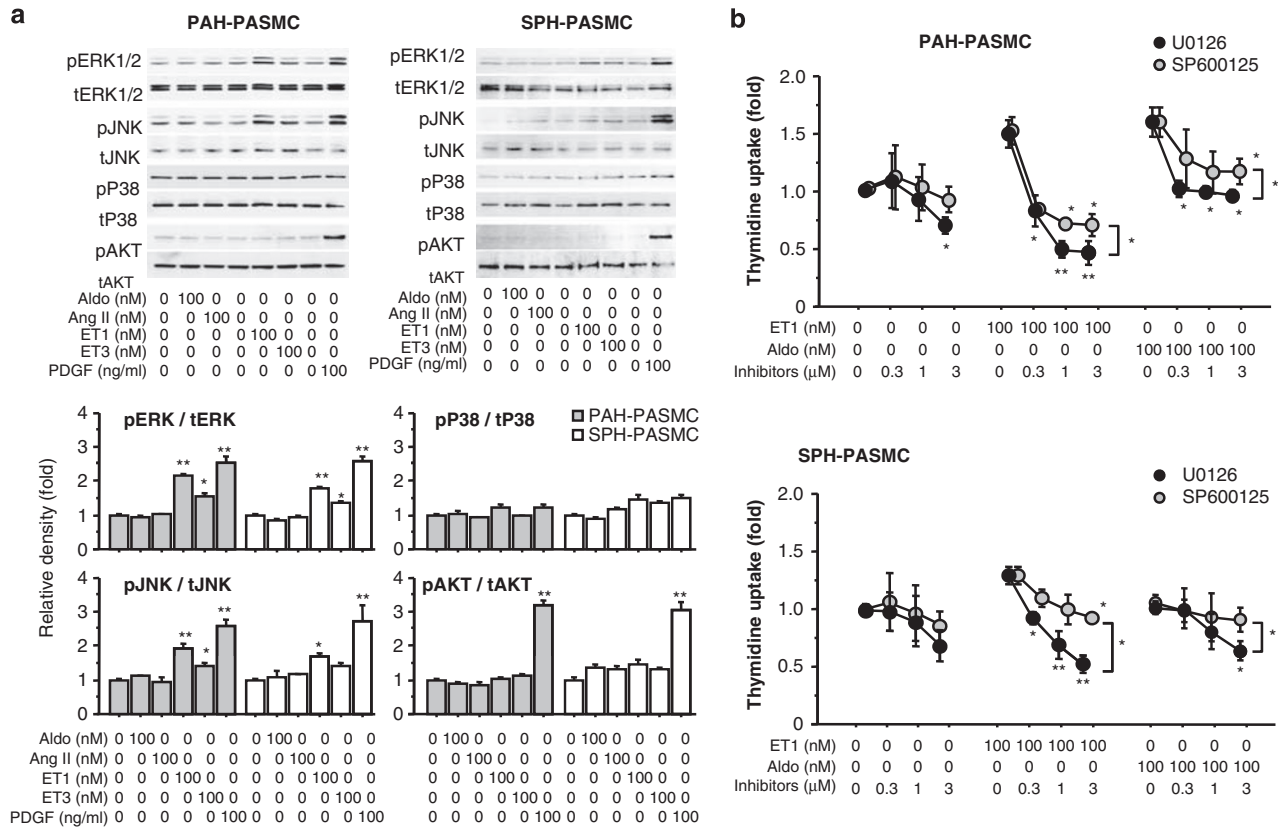
receptor expression in PAH-PASCs (Figure 6c). MR expression was also upregulated by BMP-2 and BMP-7, whereas  $11\beta$ HSD2 mRNA level was decreased by BMP-2 in PAH-PASCs (Figure 6c). In contrast to PAH-PASCs, ETBR and MR expression was decreased by treatment with BMP-2, BMP-7 and ET1, and the expression of  $11\beta$ HSD2 was reduced by BMP-2 and BMP-7 in SPH-PASCs (Figure 6c).

## DISCUSSION

In this study, we have shown a novel interaction between BMP signaling and other mitogenic actions (Figure 7), namely, (1) PAH-PASCs had higher mitotic activity through MAPK in response to ET1 compared with SPH-PASCs, in which the mitotic activity induced by ET1 was significantly inhibited by bosentan; (2) PAH- but not SPH-PASCs had mitotic sensitivity in response to not only ET1 but also aldosterone; (3) ET1 and aldosterone elicited additive

effects on PAH-PASC mitosis and MAPK stimulation, preferentially in the presence of BMP-2 and BMP-7, in which ERK was functionally linked to the mitogenesis of PASCs; and (4) ET receptor expression was relatively high in PAH-PASCs, and ET receptors and MR were upregulated by BMP-2 and BMP-7.

It is well known that increased production of mitogenic agonists, such as ET1 and PDGF, in circulation and lung tissues is linked to the development of pulmonary vascular remodeling.<sup>10,11,28</sup> Enhanced local production of ET1 by pulmonary vascular endothelial cells correlates with the severity of pulmonary vascular remodeling, suggesting a causal role for ET1 in the development of PAH.<sup>28,29</sup> ET1 is a potent pulmonary vasoconstrictor as well as a PASC mitogen,<sup>17,18,30</sup> and ET1 also has antiapoptotic effects on vascular cells, including endothelial cells, fibroblasts and systemic smooth muscle cells.<sup>31,32</sup> The mechanism of ET1 action on PASC involves binding to ET receptors with subsequent phospholipase C activation, which causes hydrolysis



**Figure 4** Effects of aldosterone, angiotensin II and endothelin on MAPK pathway in PASCs. (a) PASCs were precultured in serum-free DMEM for 24 h and stimulated with the indicated concentrations of aldosterone (Aldo), angiotensin II (Ang II), endothelin-1 and endothelin-3 (ET1 and ET3) and PDGF-BB. After a 15 min culture, cells were lysed and subjected to SDS-PAGE/immunoblot analysis using anti-phospho-ERK1/2 (pERK1/2), anti-total-ERK1/2 (tERK1/2), anti-phospho-SAPK/JNK (pJNK), anti-total-SAPK/JNK (tJNK), anti-phospho-P38 (pP38), anti-total-P38 (tP38), anti-phospho-AKT (pAKT) and anti-total-AKT (tAKT) antibodies. The results shown are representative of those obtained from three independent experiments. The relative integrated density of each protein band was digitized by NIH image J 1.34s software (lower panel). (b) PAH- and SPH-PASCs were cultured in DMEM containing 1% FCS and treated with indicated concentrations of U0126 (0.3–3 μM) and SP600125 (0.3–3 μM) in the absence or presence of endothelin-1 (ET1; 100 nM) or aldosterone (Aldo; 100 nM) for 24 h followed by a 3 h pulse exposure of 0.5 μCi per well of thymidine. The amount of thymidine incorporated into cells was counted. Results show the mean ± s.e.m. of data from three separate experiments each performed in triplicate; \**P*<0.05 and \*\**P*<0.01 vs. control groups and between the indicated groups.

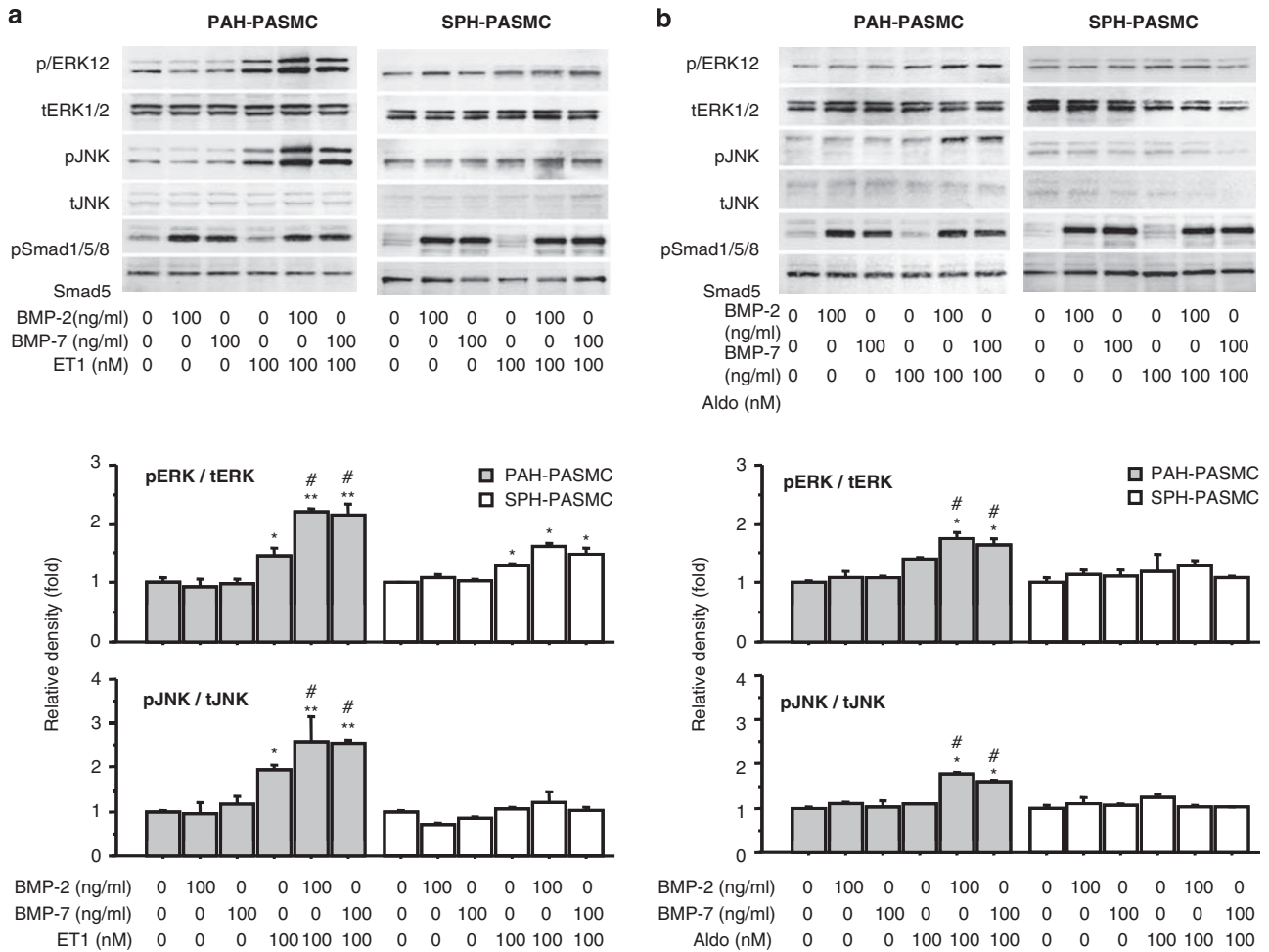
of phosphatidylinositol and generation of cytosolic inositol trisphosphate, leading to an increase of Ca<sup>2+</sup> influx and the induction of cell contraction and membrane-bound diacylglycerol production. Diacylglycerol then activates PKC and triggers intracellular mechanisms that promote cell proliferation and migration through MAPK.<sup>33</sup> Thus, ET receptor signaling is indirectly, but functionally, linked to the MAPK cascade.

Bone morphogenetic protein ligands transduce their signals through two types of serine/threonine kinase receptors, type-I and -II receptors, both of which are required for signal transduction.<sup>34,35</sup> On binding of BMPs, type-II receptors phosphorylate the corresponding type-I receptors, which in turn phosphorylate the intracellular signal-transducing molecules Smad1/5/8. Among the BMP ligands examined, we show that BMP-2 and BMP-7 are stimulatory factors for PAH-PASC mitosis. The ALK-6/BMPRII receptor pair is recognized to be the common functional complex for BMP-2 and BMP-7.<sup>35,36</sup> Earlier, we showed the roles of BMP ligands and type-I receptors in PASC physiology, both of which must interact with BMPRII for the propagation of BMPRII signaling, as BMP effects including its signaling and mitogenicity were abolished by the inhibition of ALK-6 with dominant-negative and siRNA techniques.<sup>26</sup> Thus, the underlying mechanism by which BMP regulates cell mitosis also includes the

interaction between BMP ligands and type-I receptors in PAH-PASCs.

Identification of increased ET1 levels has led to the development of ET receptor antagonists, which have improved the course of the disease for many PAH patients.<sup>37,38</sup> Star *et al.* showed an interesting finding that BMP-2 and BMP-7, but not BMP-4, increased ET1 production by human pulmonary microvascular endothelial cells, with increased phosphorylation of Smad1/5 levels.<sup>39</sup> In our present data, basal expression of ETAR and ETBR in PASCs was relatively high in PAH lungs compared with SPH, in which expression of both ET receptor types was decreased by treatment with the BMP-binding protein noggin, whereas it was increased by BMP-2 and BMP-7 in PAH-PASCs. Thus, the BMP and ET systems synergistically facilitate PASC proliferation in PAH lungs through the enhancement of ET1-induced MAPK activation with upregulation of ET receptors.

As for the roles of RAAS in the pathogenesis of PAH, we showed that aldosterone is also functionally involved in PASC mitosis. Generally, in epithelial cells, MR is protected from excessive cortisol by coexpression of 11βHSD2, which converts active cortisol to inactive cortisone *in vivo*.<sup>40,41</sup> In contrast, in nonepithelial tissues that do not express 11βHSD2, MR is generally understood to be overwhelmingly occupied by existing cortisol rather than aldosterone *in vivo*.



**Figure 5** Effects of BMP on endothelin- and aldosterone-induced MAPK activation in PASCs. PASCs were precultured in serum-free DMEM for 24 h and stimulated with BMP-2 (100 ng ml<sup>-1</sup>) and BMP-7 (100 ng ml<sup>-1</sup>) in the absence or presence of (a) endothelin-1 (ET1; 100 nM) and (b) aldosterone (Aldo; 100 nM). After a 60 min culture, cells were lysed and subjected to SDS-PAGE/immunoblot analysis using anti-pERK1/2, tERK1/2, pJNK, tJNK, pSmad1/5/8 and Smad5 antibodies. The results shown are representative of those obtained from three independent experiments. The relative integrated density of each protein band was digitized by NIH image J 1.34s software (lower panel). Results are shown as mean ± s.e.m. of data from at least three separate experiments, each performed in triplicate; \*P<0.05 and \*\*P<0.01 vs. control groups; #P<0.05 vs. ET1- or Aldo-treated groups.

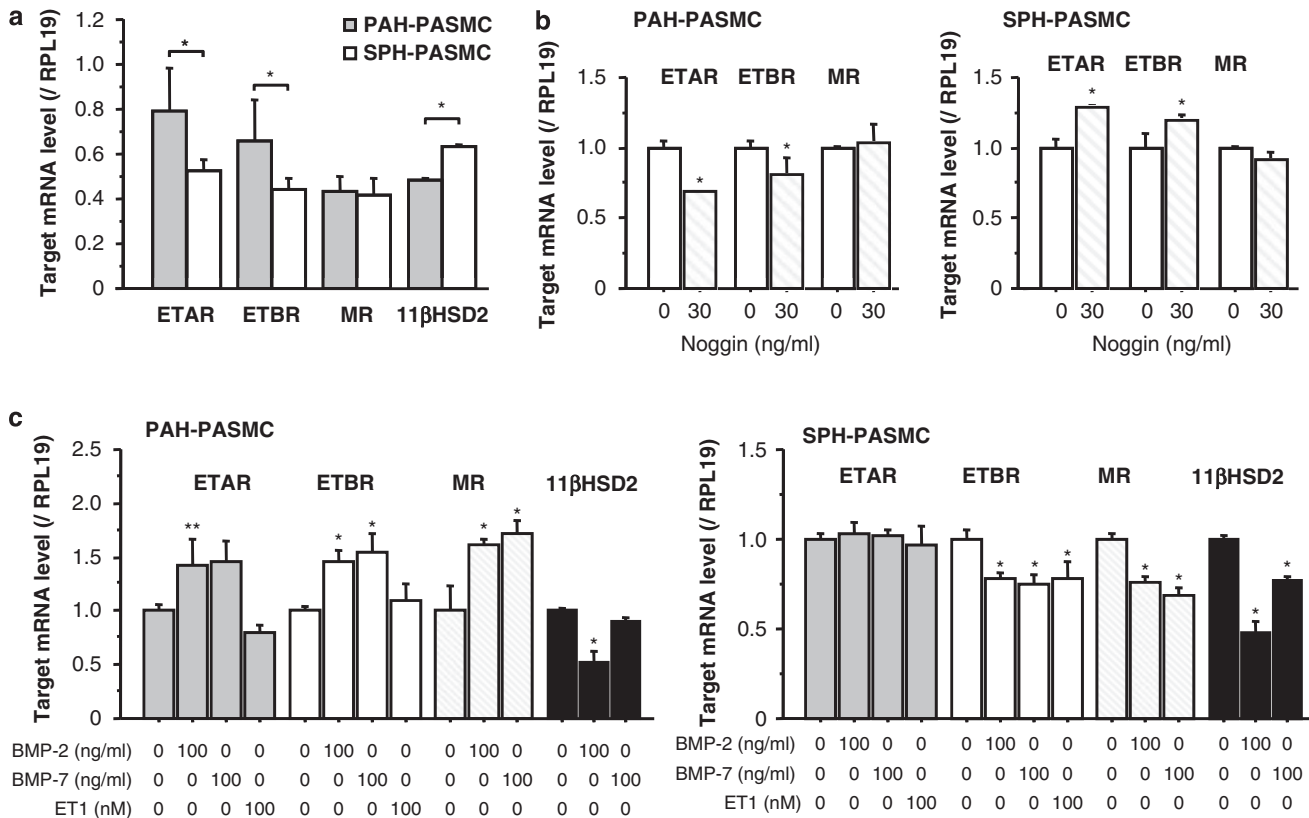
In aldosterone target cells, 11 $\beta$ HSD2 has crucial roles in conferring aldosterone sensitivity on MR by inactivating intracellular glucocorticoids before they occupy MR. In the present study, basal expression of 11 $\beta$ HSD2 in PASCs was relatively low in PAH lungs compared with SPH. The inhibitory effects of BMP-2 on 11 $\beta$ HSD2 expression and the MR upregulation by BMP-2 and BMP-7 may further enhance MR actions on PAH-PASC mitosis in response to endogenous mineralocorticoids and glucocorticoids *in vivo*. In addition to the genomic actions of aldosterone mediated through regulation of nuclear gene transcription and protein synthesis, aldosterone also elicits rapid and potentially nongenomic responses, including intracellular calcium, PKC, MAPK, Rho-kinase and PI3K, in a variety of cells.<sup>42</sup> The interaction between MR and its nongenomic responses is still controversial. Nevertheless, our data showed that eplerenone possibly inactivates aldosterone-induced PASC mitosis in PAH lungs, suggesting the possibility of clinical application of MR antagonists for controlling PAH pathogenesis.

Unbalanced Smad signaling induced by BMP and TGF- $\beta$  is functionally involved in the pathogenesis of PAH. Among the BMPRII mutants reported in PAH patients, Nishihara *et al.*<sup>43</sup> showed that missense mutations within the extracellular and kinase domains of

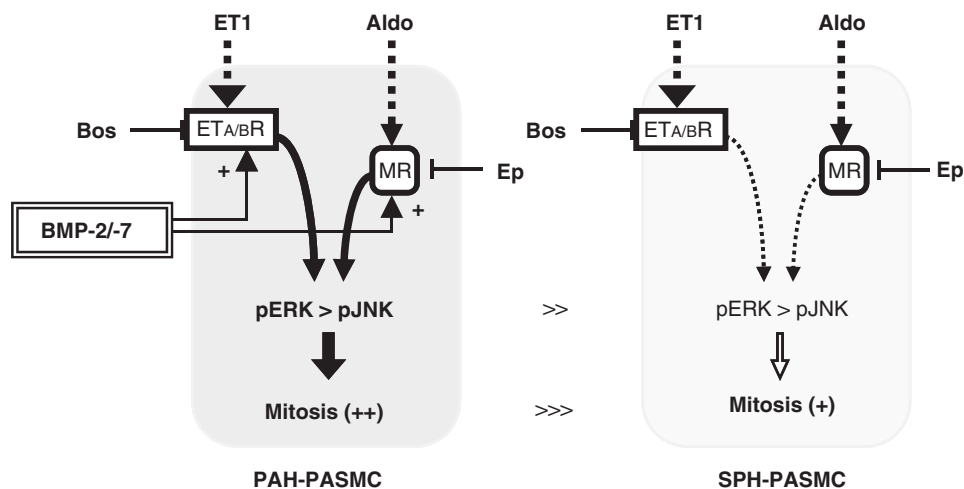
BMPRII abrogate signal transduction, which seems to be caused in part by the altered localization of these receptor domains. In contrast, BMPRII mutants with truncated cytoplasmic tails retained the ability to transduce normal BMP signaling, suggesting that BMPRII cannot be a single factor for PAH pathogenesis.<sup>43</sup> These findings have raised the possibility of the involvement of additional genetic and/or somatic factors to trigger the development of PAH lesions and further imply that causal factors for PAH development are heterogeneous.

Our present study revealed that mitotic effects of ET1, aldosterone and PDGF are, at least in part, MAPK (ERK1/2 and SAPK/JNK)-dependent. There is increasing evidence that other signaling pathways may also be involved in mediating BMP actions. Examples include TGF- $\beta$ -activated kinase-1, a member of the MAPK kinase family,<sup>44</sup> which is activated by BMP-4 or TGF- $\beta$ ,<sup>44,45</sup> and the Ras/Rac families of small GTP-binding proteins, which are also implicated in TGF- $\beta$  signaling. Moreover, the ERK1/ERK2 and SAPK/JNK pathways are linked to TGF- $\beta$ /BMP signal transduction in some cell types.<sup>46–48</sup> The data provided here show that the MAPK pathway is functionally involved in PAH-PASC mitosis, which is facilitated under the presence of combined stimuli of either ET1 or aldosterone and the BMP system.





**Figure 6** Effects of BMP on the expression of endothelin and mineralocorticoid receptors in PASCs. (a) Total cellular RNA was extracted from PAH- and SPH-PASCs, and the basal expression levels of ETAR, ETBR, MR and 11βHSD2 mRNA levels were examined by quantitative real-time PCR. (b, c) PASCs were treated with noggin (30 ng ml<sup>-1</sup>), BMP-2 and BMP-7 (100 ng ml<sup>-1</sup>) in combination with endothelin-1 (ET1; 100 nM) for 24 h. The expression levels of target genes were normalized to the RPL19 level in each sample. Results show the mean ± s.e.m. of data from three separate experiments, each performed in triplicate; \**P* < 0.05 vs. control groups and between the indicated groups.



**Figure 7** Possible interaction between endothelin, aldosterone and BMP receptor signaling in PASC proliferation. PAH-PASCs had higher mitotic activity and capacity of MAPK phosphorylation in response to endothelin-1 (ET1) compared with SPH-PASCs. The mitotic activity induced by ET1 and aldosterone (Aldo) is inhibited by bosentan (Bos) and eplerenone (Ep), respectively. PAH-PASCs, but not SPH-PASCs, also had mitotic sensitivity in response to Aldo. ET1 and Aldo elicit additive effects on PAH-PASC mitosis and MAPK activation, preferentially in the presence of BMP-2 and BMP-7. The expression of ET type-A/B receptors (ET<sub>A/B</sub>R) and mineralocorticoid receptor (MR) in PAH-PASCs was also upregulated in the presence of BMP-2 and BMP-7.

Collectively, the present data suggest that the BMP system may upregulate mitogenic sensitivity to exogenous ET1 and aldosterone in PAH-PASCs. This could be a feature distinct from SPH-PASCs,

which still have an autoregulatory system in response to exogenous ET1 and/or aldosterone. The relatively low penetrance of PAH within families supports a ‘two-hit’ hypothesis in which the vascular abnorm-

alities characteristic of idiopathic PAH are triggered by accumulation of genetic and/or environmental factors, including upregulation of vasoconstrictive hormones. The combination of increased endothelial injury associated with ET1 production and impaired suppression of PASMC proliferation, partly controlled by BMPs, could be critical for the cellular pathogenesis of PAH. However, there is still a case limitation in our present study, and the accumulation of basic experimental data derived from human samples would be absolutely necessary to elucidate the cellular mechanism leading to the development of idiopathic PAH and the characterization of the molecular basis of differential mechanisms of idiopathic and secondary PAH. The present findings may also permit further understanding of the molecular pathogenesis of PAH and novel therapeutic prospects to control ET1- and aldosterone-induced activation of MAPK for the prevention of excessive mitosis of PASMCs.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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- Rubin LJ. Primary pulmonary hypertension. *N Engl J Med* 1997; **336**: 111–117.
- Palevsky HI, Schloo B, Pietra GG, Weber KT, Janicki JS, Rubin E, Fishman AP. Primary pulmonary hypertension: vascular structure, morphology, and responsiveness to vasodilator agents. *Circulation* 1989; **80**: 1207–1221.
- Wilkins MR, Gibbs JS, Shovlin CL. A gene for primary pulmonary hypertension. *Lancet* 2000; **356**: 1207–1208.
- Nichols WC, Koller DL, Slovis B, Foroud T, Terry VH, Arnold ND, Siemieniak DR, Wheeler L, Phillips III JA, Newman JH, Conneally PM, Ginsburg D, Loyd JE. Localization of the gene for familial primary pulmonary hypertension to chromosome 2q31-32. *Nat Genet* 1997; **15**: 277–280.
- Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, Kalachikov S, Cayanis E, Fischer SG, Barst RJ, Hodge SE, Knowles JA. Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. *Am J Hum Genet* 2000; **67**: 737–744.
- Lane KB, Machado RD, Pauciuolo MW, Thomson JR, Phillips III JA, Loyd JE, Nichols WC, Trembath RC. Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. The International PPH Consortium. *Nat Genet* 2000; **26**: 81–84.
- Thomson JR, Machado RD, Pauciuolo 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. Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding BMPR-II, a receptor member of the TGF-beta family. *J Med Genet* 2000; **37**: 741–745.
- Morrell NW, Yang X, Upton PD, Jourdan KB, Morgan N, Sheares KK, Trembath RC. Altered growth responses of pulmonary artery smooth muscle cells from patients with primary pulmonary hypertension to transforming growth factor-beta(1) and bone morphogenetic proteins. *Circulation* 2001; **104**: 790–795.
- Rudarakanchana N, Flanagan JA, Chen H, Upton PD, Machado R, Patel D, Trembath RC, Morrell NW. Functional analysis of bone morphogenetic protein type II receptor mutations underlying primary pulmonary hypertension. *Hum Mol Genet* 2002; **11**: 1517–1525.
- Runo JR, Loyd JE. Primary pulmonary hypertension. *Lancet* 2003; **361**: 1533–1544.
- Gaiaid A, Yanagisawa M, Langleben D, Michel RP, Levy R, Shennib H, Kimura S, Masaki T, Duguid WP, Stewart DJ. Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N Engl J Med* 1993; **328**: 1732–1739.
- Jeffery TK, Morrell NW. Molecular and cellular basis of pulmonary vascular remodeling in pulmonary hypertension. *Prog Cardiovasc Dis* 2002; **45**: 173–202.
- Christman BW, McPherson CD, Newman JH, King GA, Bernard GR, Groves BM, Loyd JE. An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. *N Engl J Med* 1992; **327**: 70–75.
- Gaiaid A. Nitric oxide and endothelin-1 in pulmonary hypertension. *Chest* 1998; **114**: 208S–212S.
- Ambalavanan N, Bulger A, Murphy-Ullrich J, Oparil S, Chen YF. Endothelin-A receptor blockade prevents and partially reverses neonatal hypoxic pulmonary vascular remodeling. *Pediatr Res* 2005; **57**: 631–636.
- Rosenberg AA, Kennaugh J, Koppenhafer SL, Loomis M, Chatfield BA, Abman SH. Elevated immunoreactive endothelin-1 levels in newborn infants with persistent pulmonary hypertension. *J Pediatr* 1993; **123**: 109–114.
- Kunichika N, Landsberg JW, Yu Y, Kunichika H, Thistlethwaite PA, Rubin LJ, Yuan JX. Bosentan inhibits transient receptor potential channel expression in pulmonary vascular myocytes. *Am J Respir Crit Care Med* 2004; **170**: 1101–1107.
- Davie N, Haleen SJ, Upton PD, Polak JM, Yacoub MH, Morrell NW, Wharton J. ET(A) and ET(B) receptors modulate the proliferation of human pulmonary artery smooth muscle cells. *Am J Respir Crit Care Med* 2002; **165**: 398–405.
- Abraham WT, Reynolds MV, Badesch DB, Wynne KM, Groves BM, Roden RL, Robertson AD, Lowes BD, Zisman LS, Voelkel NF, Bristow MR, Perryman MB. Angiotensin-converting enzyme DD genotype in patients with primary pulmonary hypertension: increased frequency and association with preserved haemodynamics. *J Renin Angiotensin Aldosterone Syst* 2003; **4**: 27–30.
- Orte C, Polak JM, Haworth SG, Yacoub MH, Morrell NW. Expression of pulmonary vascular angiotensin-converting enzyme in primary and secondary plexiform pulmonary hypertension. *J Pathol* 2000; **192**: 379–384.
- Okada K, Bernstein ML, Zhang W, Schuster DP, Botney MD. Angiotensin-converting enzyme inhibition delays pulmonary vascular neointimal formation. *Am J Respir Crit Care Med* 1998; **158**: 939–950.
- Chung WK, Deng L, Carroll JS, Mallory N, Diamond B, Rosenzweig EB, Barst RJ, Morse JH. Polymorphism in the angiotensin II type 1 receptor (AGTR1) is associated with age at diagnosis in pulmonary arterial hypertension. *J Heart Lung Transplant* 2009; **28**: 373–379.
- Kouchi H, Nakamura K, Fushimi K, Sakaguchi M, Miyazaki M, Ohe T, Namba M. Manumycin A, inhibitor of ras farnesyltransferase, inhibits proliferation and migration of rat vascular smooth muscle cells. *Biochem Biophys Res Commun* 1999; **264**: 915–920.
- Ogawa A, Nakamura K, Matsubara H, Fujio H, Ikeda T, Kobayashi K, Miyazaki I, Asanuma M, Miyaji K, Miura D, Kusano KF, Date H, Ohe T. Prednisolone inhibits proliferation of cultured pulmonary artery smooth muscle cells of patients with idiopathic pulmonary arterial hypertension. *Circulation* 2005; **112**: 1806–1812.
- Takeda M, Otsuka F, Suzuki J, Kishida M, Ogura T, Tamiya T, Makino H. Involvement of activin/BMP system in development of human pituitary gonadotropinomas and non-functioning adenomas. *Biochem Biophys Res Commun* 2003; **306**: 812–818.
- Takeda M, Otsuka F, Nakamura K, Inagaki K, Suzuki J, Miura D, Fujio H, Matsubara H, Date H, Ohe T, Makino H. 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. *Endocrinology* 2004; **145**: 4344–4354.
- Von Bubnoff A, Cho KKY. Intracellular BMP signaling regulation in vertebrates: pathway or network? *Dev Biol* 2001; **239**: 1–14.
- Kourembanas S, Marsden PA, McQuillan LP, Faller DV. Hypoxia induces endothelin gene expression and secretion in cultured human endothelium. *J Clin Invest* 1991; **88**: 1054–1057.
- Wort SJ, Woods M, Warner TD, Evans TW, Mitchell JA. Endogenously released endothelin-1 from human pulmonary artery smooth muscle promotes cellular proliferation: relevance to pathogenesis of pulmonary hypertension and vascular remodeling. *Am J Respir Cell Mol Biol* 2001; **25**: 104–110.
- Channick RN, Simonneau G, Sitbon O, Robbins IM, Frost A, Tapson VF, Badesch DB, Roux S, Rainisio M, Bodin F, Rubin LJ. Effects of the dual endothelin-receptor antagonist bosentan in patients with pulmonary hypertension: a randomised placebo-controlled study. *Lancet* 2001; **358**: 1119–1123.
- Shichiri M, Kato H, Marumo F, Hirata Y. Endothelin-1 as an autocrine/paracrine apoptosis survival factor for endothelial cells. *Hypertension* 1997; **30**: 1198–1203.
- Wu-Wong JR, Chiou WJ, Dickinson R, Oppenorth TJ. Endothelin attenuates apoptosis in human smooth muscle cells. *Biochem J* 1997; **328**(Pt 3): 733–737.
- Remuzzi G, Perico N, Benigni A. New therapeutics that antagonize endothelin: promises and frustrations. *Nat Rev Drug Discov* 2002; **1**: 986–1001.
- Massague J. TGF-beta signal transduction. *Annu Rev Biochem* 1998; **67**: 753–791.
- Miyazono K, Kusanagi K, Inoue H. Divergence and convergence of TGF-beta/BMP signaling. *J Cell Physiol* 2001; **187**: 265–276.
- Aoki H, Fujii M, Imamura T, Yagi K, Takehara K, Kato M, Miyazono K. Synergistic effects of different bone morphogenetic protein type I receptors on alkaline phosphatase induction. *J Cell Sci* 2001; **114**: 1483–1489.
- Barst RJ, Langleben D, Badesch D, Frost A, Lawrence EC, Shapiro S, Naeije R, Galie N. Treatment of pulmonary arterial hypertension with the selective endothelin-A receptor antagonist sitaxsentan. *J Am Coll Cardiol* 2006; **47**: 2049–2056.
- Rubin LJ, Badesch DB, Barst RJ, Galie N, Black CM, Keogh A, Pulido T, Frost A, Roux S, Leone I, Landzberg M, Simonneau G. Bosentan therapy for pulmonary arterial hypertension. *N Engl J Med* 2002; **346**: 896–903.
- Star GP, Giovannozzo M, Langleben D. Effects of bone morphogenetic proteins and transforming growth factor-beta on *in-vitro* production of endothelin-1 by human pulmonary microvascular endothelial cells. *Vascu Pharmacol* 2009; **50**: 45–50.
- Funder JW, Pearce PT, Smith R, Smith AI. Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. *Science* 1988; **242**: 583–585.
- Edwards CR, Stewart PM, Burt D, Brett L, McIntyre MA, Sutanto WS, de Kloet ER, Monder C. Localisation of 11 beta-hydroxysteroid dehydrogenase—tissue specific protector of the mineralocorticoid receptor. *Lancet* 1988; **2**: 986–989.
- Funder JW. The nongenomic actions of aldosterone. *Endocr Rev* 2005; **26**: 313–321.

- 43 Nishihara A, Watabe T, Imamura T, Miyazono K. Functional heterogeneity of bone morphogenetic protein receptor-II mutants found in patients with primary pulmonary hypertension. *Mol Biol Cell* 2002; **13**: 3055–3063.
- 44 Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E, Matsumoto K. Identification of a member of the MAPKKK family as a potential mediator of TGF- $\beta$  signal transduction. *Science* 1995; **270**: 2008–2011.
- 45 Yamaguchi K, Nagai S, Ninomiya-Tsuji J, Nishida M, Tamai K, Irie K, Ueno N, Nishida E, Shibuya H, Matsumoto K. XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway. *EMBO J* 1999; **18**: 179–187.
- 46 Nakamura K, Shirai T, Morishita S, Uchida S, Saeki-Miura K, Makishima F. p38 Mitogen-activated protein kinase functionally contributes to chondrogenesis induced by growth/differentiation factor-5 in ATDC5 cells. *Exp Cell Res* 1999; **250**: 351–363.
- 47 Piek E, Heldin CH, Ten Dijke P. Specificity, diversity, and regulation in TGF- $\beta$  superfamily signaling. *FASEB J* 1999; **13**: 2105–2124.
- 48 Moore RK, Otsuka F, Shimasaki S. Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. *J Biol Chem* 2003; **278**: 304–310.