Id proteins are critical downstream effectors of BMP signaling in human pulmonary arterial smooth muscle cells

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PULMONARY ARTERIAL HYPERTENSION (PAH) is a severe clinical condition associated with a poor prognosis and high mortality. Occlusive remodeling of the distal pulmonary vasculature is the major pathological finding in PAH. Proliferation of myofibroblasts and smooth muscle cells in the pulmonary arterial wall increases pulmonary vascular resistance and elevates pulmonary arterial pressure, ultimately leading to right ventricular failure and eventually death (20, 26, 29). A greater understanding of the molecular mechanisms involved in pulmonary vascular remodeling will facilitate new approaches for therapy.

Bone morphogenetic protein type II receptor (BMPR-II) mutations are responsible for the majority (>70%) of cases of heritable PAH and have been reported in 15–40% of apparently sporadic idiopathic cases (6, 15, 34). Loss of BMPR-II or dysfunction of BMP signaling are now recognized in several preclinical models of PAH, including those induced by hypoxia, monocrotaline, and high flow (10, 16). BMPs are pleiotropic cytokines involved in a wide range of vascular cell function including proliferation, migration, differentiation, and apoptosis, but how BMPR-II mutations cause these abnormalities remains uncertain.

The inhibitor of DNA binding family of proteins (Id proteins) are major downstream mediators of BMP signaling (19). Id proteins are basic helix-loop-helix transcription factors that lack a DNA binding domain. These proteins bind to the ubiquitously expressed E protein family members with high affinity and inhibit their binding to target DNA (5, 11). This special function of Id proteins confers a central role in the regulation of gene expression and hence cell differentiation and proliferation (24, 31). Until now, four members of the Id family, Id1–4, have been identified in mammalian cells. They are encoded by separate genes and demonstrate individual expression patterns and protein structure, which may contribute to their different functions. For example, Id2 is largely expressed in immune cells (8, 40), and only Id1 lacks the consensus CDK2 phosphorylation site at its NH2 terminus (5). Our recent study revealed that Id1 and Id2 are induced by BMPs in pulmonary artery smooth muscle cells (PASMCs) through a canonical Smad-dependent pathway (37) and that BMPR-II mutation reduced the BMP-stimulated induction of Id1 and Id2 in these cells. We further showed that Id1 and Id2 are involved in the inhibition of PASMC proliferation by BMP4. We have also shown that agents enhancing BMP/Smad/Id signaling in PASMCs can restore the growth-suppressive effects of BMPs in BMPR-II mutant cells (38, 39).

In the present study, we undertook a systematic analysis of the regulation of Id1–4 in PASMCs via a range of growth factors, cytokines, and BMPs. Having identified Id1 and Id3 as major targets of BMP signaling in these cells, we show that the induction of both Id1 and Id3 is dependent on intact BMPR-II and that Id3 promotes PASMC growth suppression. These studies provide further evidence for a central role of canonical BMP signaling via Id genes in the regulation of PASMC...
proliferation, loss of which likely contributes to the abnormal growth of vascular cells in PAH.

MATERIALS AND METHODS

Human PASMC culture. Human PASMCs were derived from peripheral pulmonary arteries (<2 mm external diameter), as previously described (36). BMPR2 mutant cells were derived from the lungs of patients undergoing heart lung transplantation for PAH (n = 3), known to harbor a mutation in the BMPR2 gene. These included mutant sites at position 347 (C347Y), position 899 (R899X), and amino acid 9 (W9X). Normal PASMCs were obtained from patients undergoing lung resection for suspected tumors and taken from regions of lung remote from the pathology (n = 5). The Papworth Hospital ethical review committee approved the study, and subjects or relatives gave informed written consent.

Immunoblotting. PASMCs were grown to 95% confluence. After 24-h quiescence with incubation in 0.1% FBS/Dulbecco modified Eagles medium (DMEM), cells were treated with BMPs for up to 48 h. At specified time points, cells were lysed in buffer as described (37). Samples were electrophoresed by 12% SDS-PAGE and then transferred to PVDF membrane (GE Healthcare). For studies of BMPR2, Smad1/5, Id1, and Id3, blots were incubated with polyclonal mouse anti-BMPR2 (1:1,000, BD Biosciences), polyclonal rabbit antiphospho-Smad1/5 (1:1,000, Cell Signaling Technology), polyclonal rabbit anti-Id1 (1:1,000, Biocheck), polyclonal rabbit anti-Id3 (1:1,000, CalBioreagents), as previously described (39). To confirm equal protein loading, blots were stripped and reprobed using anti-β-actin (1:4,000, Sigma).

Real-time Q-PCR. Quiescent PASMCs were incubated with BMPs (10 ng/ml) or DMEM alone for 4 or 24 h. Total RNA was extracted using TRIzol reagent, then reverse transcribed using High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems. Quantitative PCR was performed using SYBR Green JumpStart Taq ReadyMix (Sigma), and samples were run on Applied Biosystems StepPlusOne Realtime PCR System. Primers were used to allow amplification and normalized to β-actin, which was included in each sample run. Human Id3 was conducted with Quanti Tect Primer Assay (QT01673336), and Id4 was conducted with Quanti Tect Primer Assay (QT00234920), which were purchased from Qiagen. Other primers purchased from Sigma are listed in Table 1.

siRNA knockdown of BMPR2. Human PASMCs were plated at 20,000/well in 24-well plates in normal culture medium. Before the knockdown experiment, medium was changed to DME1 containing 10% FBS without antibiotics. Cells were then transfected with small interfering (si)RNA for BMPR2 (Dharmacon) or control siRNA (siCP nontargeting pool, Dharmacon) using Dharmafect transfection reagent, following the manufacturer’s instructions. Four hours later, transfection media was changed to normal culture medium; 24 h later, medium was changed to 0.1 FBS/DME1 medium and left overnight. Cells were treated with BMPs for 24 h and then lysed for immunoblotting.

Lentiviral transfection of Id3. Pseudotyped vectors were generated by transfection of plasmid DNA into 293T cells using a calcium phosphate method, as previously described (38). Transfections were performed in six-well plates using optimized ratios of constructs. The efficiency of transfection of PASMCs was assessed by green fluorescent protein reporter expression 72 h after transduction. Levels of Id3 were assayed by immunoblotting.

Proliferation assays. Cell proliferation was quantified by cell number using a hemocytometer as previously described (22). In knockdown and overexpression experiments, cells were grown in 10% FBS/DME1 and counted on day 3. Cell viability was assessed with Trypan blue exclusion. Each isolate was studied at least three times under each condition, and the mean values were taken from all studies.

**Fig. 1.** Induction of Id genes by a panel of factors in pulmonary artery smooth muscle cells (PASMCs). A: relative abundance of mRNA transcripts for Ids in human PASMCs. Cell lysates were collected from PASMCs after 24-h quiescence in 0.1% FBS Dulbecco modified Eagles medium (DMEM). The calculation of transcript abundance relies on ΔΔCt between the gene of interest and 2 housekeeping genes (β-actin and β-2-microglobulin). We applied the assumption of equal PCR threshold values for different Id genes and used the ΔΔCt method to calculate the relative expression of each gene to β-actin after normalizing to β-2-microglobulin. B: real-time PCR with human Id1 primers shows the mRNA expression in human PASMCs after 24-h treatment with bone morphogenetic protein-4 (BMP4) (10 ng/ml), BMP6 (10 ng/ml), BMP9 (10 ng/ml), IL-1β (10 ng/ml), Angiotensin II (AngII) (200 mmol/l), serotonin (5-HT) (1 μmol/l), β-estradiol (50 mmol/l), platelet-derived growth factor (PDGF)-bb (5 ng/ml), and thrombin (1 U/ml). We used β-actin as the housekeeping gene to calculate results using the 2−ΔΔCt method (*P < 0.05 compared with control).
conducted with any single cell line. In parallel experiments, to allow determination of gene or protein expression, cells were plated in six-well plates and treated similarly. Immunoblotting was used to demonstrate the efficiency of Id3 overexpression.

**BrdU incorporation in PASMCs in vitro.** PASMCs grown to 70% confluence in six-well tissue culture plates were infected with empty lentivirus or Id3 lentivirus. Cells were synchronized by serum deprivation overnight followed by culture for 3 days in DMEM supplemented with 10% FBS. BrdU, at a final concentration of 10 μM, was added for the last 24 h. The tissue culture plates were washed, and a BrdU cell proliferation assay was performed according to the manufacturer’s instructions (BD Biosciences).

BrdU incorporation assays were combined with DNA dyes (7AAD), allowing two-color flow cytometric characterization of cells that are actively synthesizing DNA (BrdU incorporation) relative to their phase in the cell cycle (i.e., G0/G1, S, or G2/M) and the quantification of apoptotic cells.

**Immunohistochemistry.** Five-micron-thick paraffin sections of lung sections from wild-type (n = 3) and R899X BMPRII mutants (n = 6) were coimmunostained using a previously described protocol (2). In brief, sections were exposed to high-pressure antigen retrieval using a pressure cooker containing 0.1 M sodium citrate buffer pH 6.0 for 2 min. Following nonspecific antibody blocking in 10% fetal calf serum in PBS, sections were incubated overnight at 4°C with 1:50 dilution of rabbit anti-Id3 (catalog no. M094, CalBioreagents; and catalog no. 41834, Abcam) and 1:100 dilution of mouse anti-smooth muscle actin (catalog no. LM557, Life Technologies) for 1 h at room temperature. Sections were then mounted in Vecta Mounting Medium (Vector Laboratories) and examined using a Leica SP1 confocal laser-scanning microscope.

**Statistical analysis.** Statistical analysis was carried out on the DAB-stained slides using a Lumenera software with an infinity USB 2.0 camera (Lumenera) attached to a Leica microscope. The number of smooth muscle cell nuclei positive for Id3 in normal and hypertensive arteries was counted in at least 10 arteries in each study group. In addition, the mean signal intensity of the Id3-positive nuclei in the lung parenchyma was determined using ImageJ 1.47k software by converting the DAB-stained images to 32 bit; we then set the threshold to do binary conversion and then measured the intensity of positive staining. The readings of the mean signal intensity were exported to GraphPad Prism 5 for statistical comparison between the study groups using Student’s t-test, with a statistical difference seen between the two study groups; P < 0.05.

**RESULTS**

**Id gene expression in human PASMCs.** First we investigated the expression pattern of Id1–4. All four Ids were expressed...
under basal conditions (0.1% FBS/DMEM) in PASMCs. Id4 was expressed at the lowest level. Although Id2 is known to be mainly expressed in immunocytes, we found abundant expression in PASMCs with a similar level to Id1 and Id3 (Fig. 1A). Because Id genes have been reported to be regulated by a number of factors (12, 25, 30), we next examined the effects of a range of growth factors and cytokines with relevance to PAH pathobiology on Id gene expression. This included BMPs, angiotensin II (Ang II), platelet-derived growth factor (PDGF), interleukin-1β (IL-1β), β-estradiol, and thrombin. Results showed that BMP4 and 6 were the major regulators of Id1. BMP9 had a much smaller impact on Id1 gene expression. Of the other factors studied, the changes in Id1 gene expression were smaller and inconsistent. Thus BMPs demonstrate the greatest impact on Id gene expression, in particular BMP4 and 6 (Fig. 1B).

The induction of Id genes by BMPs is dependent on BMPR-II. We next investigated the importance of BMPR-II in the induction of Id1–4 gene expression by BMP4 and 6. Specific siRNA was used to knockdown BMPR-II in PASMCs. We first confirmed the efficiency of knockdown of BMPR-II mRNA and protein is greater than 90% and 80%, respectively (Fig. 2, A and B), 48 h after knockdown; BMP4 (10 ng/ml) and BMP6 (10 ng/ml) were incubated with PASMCs for 24 h. Measurement of Id1–4 gene expression showed similar levels of induction of Id genes by BMP4 and 6 (Fig. 2, C–F). However, the induction of Id1 (14-fold) and Id3 (22-fold) was generally greater than the fold induction of Id2 (7-fold) and Id4 (4-fold) in control cells treated with BMP4. Of note, the induction of all four Id genes was markedly reduced following BMPR-II knockdown, with Id1 and Id3 being the most consistently affected. Id1 induction by BMP4 and BMP6 was decreased by 57.4% and 74.0%, respectively. Id3 induction by BMP4 and BMP6 was decreased by 59.1% and 76.8%, respectively. Id4 induction by BMP4 and BMP6 was decreased by 56.3% and 59.2%, respectively. Id2 induction by BMP4 was decreased by 67.3%, but its induction by BMP6 was not affected by BMPR-II knockdown (Fig. 2). We further employed Western blotting using antibodies against Id1 and Id3 to confirm the impact of BMPR-II knockdown on the induction of Id1 and 3 protein levels (Fig. 3).

Effect of BMPR-II mutation on Id1/3 gene and protein induction by BMPs. To further investigate the potential impact of BMPR-II mutation on the induction of Id genes expression, we carried out experiments in PASMCs from healthy control and patients with PAH harboring BMPR-II mutations. The latter included three different BMPR-II mutations at the following sites: C347Y, W8X, and R899X (Fig. 4, A and B) (28). Because BMP signaling is dynamic and dependent on the cellular context (15), we determined the time course of induction of Id1 and Id3 protein levels following stimulation with BMP4 or BMP6 (Fig. 5, A and B). We observed that both BMP4 and BMP6 induced an early expression of Id1 and Id3 in control PASMCs, with the expression peaking at 2 h, then a plateau of sustained expression for up to 8 h, before tailing off toward 24 h (Fig. 5, C and D). In contrast, in BMPR-II mutant PASMCs, there was a marked diminution of the Id induction in response to BMPs. Figure 4 shows a comparison of the Id3
protein induction in three control and three mutant cells lines at the 1-h time point, confirming the major impact of BMPR-II mutation on the induction of Id3. These data for Id3 are similar to Id1 protein expression, which was published in our previous report (37).

Reduced expression of Id3 in pulmonary arteries from BMPR-II mutant mice and in heritable PAH. The Id3 antibody used in these studies is suitable for use in immunohistochemistry (14). We took advantage of this to examine the expression of Id3 in the lungs of mice harboring a heterozygous knockin of the human BMPR-II mutation, R899X, and wild-type littermates (3). In wild-type mouse lung, Id3 immunostaining was observed in cells of the lung parenchyma but also in PASMCs surrounding small peripheral pulmonary arterioles. In the lungs of BMPR-II R899X mice, the immunostaining of Id3 appeared less intense in PASMCs (Fig. 6, C). Further studies on human tissues demonstrated positive staining for Id3 colocalized with α-smooth muscle actin staining in the walls of small pulmonary arterioles (Fig. 7). Similar to the murine studies, the expression of Id3 in remodeled pulmonary arterioles from patients with heritable PAH was conspicuously low or absent (Fig. 7).

Id3 is involved in PASMC cell cycle regulation and proliferation. To determine the impact of Id3 on cell proliferation, PASMCs were transduced with lentivirus to overexpress Id3 or empty virus as a control. Thereafter, proliferation was stimulated with 10% FBS. PASMCs were counted at day 3. The overexpression of Id3 reduced the proliferative capacity of PASMCs in both control and mutant cells (P < 0.05) (Fig. 8A). Overexpression of Id3 was confirmed by immunoblotting of transfected cell lysates (Fig. 8B). To examine the direct role of Id3 on the cell cycle, we used a BrdU incorporation assay to examine the S phase of serum-stimulated PASMCs. After overnight starvation to synchronize the cell cycle, PASMCs were incubated with BrdU for 16 h (10 μg/ml), and then cells were collected for flow cytometry analysis. In control cells, 6.2% of cells contained newly synthesized DNA, whereas, following Id3 overexpression, only 3.0% of cells were in S phase (Fig. 8C). Id3 overexpression did not significantly increase the number of apoptotic cells (Id3, 3.88% vs. control, 3.40%) (Fig. 8C). Following prolonged BrdU incubation (40 h), the inhibitory effect of Id3 overexpression on proliferation was enhanced with 2.9% cells in S phase compared with 12.0% of control transfected cells. At this time point, there was again no significant difference comparing apoptotic cell numbers following Id3 overexpression (3.99%) vs. control (3.64%).

LPS exacerbates the defect in BMP signaling in BMPR-II mutant PASMCs. To examine the possible role of inflammatory stimuli as a second hit in PAH pathogenesis, we examined the impact of LPS on BMP signaling in PASMCs (Fig. 9). In control cells, LPS showed a nonsignificant trend toward reducing BMP-stimulated Id3 expression at higher concentrations of LPS (10 μg/ml). In BMPR-II mutant cells, LPS alone had no effect on Id3 expression but significantly inhibited BMP4 stimulated Id3 expression in a concentration-dependent manner from 0.1 μg/ml to 10 μg/ml (P < 0.05).

DISCUSSION

In this study, we systematically characterized the induction of Id genes in response to a range of BMPs and other cytokines and growth factors, relevant to PAH. Id1 and Id3 consistently demonstrated higher levels of expression and the strongest induction by BMPs. We also revealed that LPS further reduced Id3 gene expression in BMPR-II mutant PASMCs. In addition, our study demonstrates the role of Id proteins on cell cycle regulation in PASMCs and provides further evidence that Id proteins are potential therapeutic targets to inhibit PASMC proliferation and vascular remodeling in PAH.

Id proteins function as dominant-negative transcriptional regulators, as suggested by their name: inhibitors of DNA binding. The expression levels and function of the four members the Id gene family vary in different organs. For example, Id2 is mainly expressed in immune cells, and Id4 is usually expressed at lower levels than other Ids (24, 30). Id2 expression is also high in developing lung epithelial cells, whereas Id1 and 3 are expressed in the developing lung mesenchyme (27). Much of the published research on Id genes is focused on cancer biology, although, in recent years, their function in cardiovascular diseases has attracted attention. Studies have suggested an important role of Id proteins in maintaining vessel homeostasis, and Id proteins have been implicated in angiogenesis and atherosclerosis (4, 18, 23). Our findings provide a further understanding of the role of Id proteins in the vasculature, particularly in the pathophysiology of PAH.
Fig. 5. Time courses of regulation of Id1 and Id3 by BMP4 and BMP6 in BMPR-II wild-type and BMPR-II mutant PASMCs. Western blots for Id1 and Id3 protein in response to BMP4 (A) and BMP6 (B) (10 ng/ml, 1 to 24 h) in control and mutant PASMCs. β-Actin was used as loading control. C and D: quantification of Id1 and Id3 protein expression from immunoblots normalized to β-actin levels. Time-dependent expression of Id1 and Id3 in control and mutant PASMCs in response to BMP4 (C) and BMP6 (D). All graphs represent the means of at least 3 separate experiments in different mutant and control cell lines.

Fig. 6. Id3 protein expression in lung tissue from BMPR2+/+ mice and BMPR+/− mice. A: light microscopy images of small pulmonary arteries in mouse tissue from a BMPR2+/+ and a BMPR+/− mouse stained with antibodies for Id3. Smooth muscle cells in a small pulmonary artery stained positive with α-smooth muscle actin (SMA) antibody (inset), solid arrows represent Id3-positive staining, arrow heads represent Id3-negative staining. B: quantification of Id3-positive cells in the vascular media from BMPR+/− mice is significantly less than BMPR+/+ mice (*P < 0.05). C: quantification of the Id3 signal intensity in the lung parenchyma. N = 4–6 animals per group, (*P < 0.05).
In 2000, the link between BMPR-II mutation and PAH was established (10, 16). Loss of BMPR-II and dysfunction of BMP signaling are now recognized to be involved in aberrant smooth muscle cell and endothelial cell proliferation and apoptosis in PAH. However, the observation that disease penetrance is low (~20–30%) in families carrying BMPR2 mutations and the fact that heterozygous BMPR-II knockout mice develop mild or no PAH suggest that a ‘second hit’ or further dysfunction of BMP signaling may be necessary to initiate and accelerate the disease (12). Our previous reports have demonstrated that BMPRII mutation in PASMCs is associated with loss of downstream Smad1/5 phosphorylation (37) and loss of Id1 gene and protein expression. Here we extend those findings to show that Id1 and Id3 are the most abundant Id transcripts in PASMCs and are most strongly regulated by BMP4 and BMP6. Knockdown studies of BMPR-II accompanied by experiments in PASMCs harboring mutations in BMPR-II show that Id1 and Id3 induction is dependent on BMPR-II. Inflammation is emerging as a potential second hit in the setting of BMPR-II mutation. For example, BMPR-II heterozygous mice develop pulmonary hypertension when adenovirus-expressing 5-lipoxygenase is introduced into the lung (33). In addition, patients with idiopathic and familial PAH demonstrate increased circulating levels of inflammatory cytokines (32). In the present study, we provide further evidence for a role for inflammation as a second hit in PAH associated with BMPR-II mutations. We chose to use LPS as a well-characterized pro-inflammatory stimulus. LPS exposure further reduced the induction of Id genes in mutant PASMCs. The mechanism underlying this effect is the subject of ongoing work in our laboratory.

BMP9 has recently been reported to upregulate Id gene expression in endothelial cells (9). BMP9 signals predominantly via BMPR-II complexed with ALK1, which is expressed at a high level on ECs but not on PASMCs (35), which likely explains the low level of Id gene induction in response to BMP9 in PASMCs. We tested the effect of a range of cytokines and growth factors on the induction of Id genes in the present study. The rationale is that several of these factors have been shown to regulate Id gene expression in other cell types (7, 17, 30) and are implicated in the pathobiology of PAH. For example, TGF-β and PDGF are implicated in pulmonary vascular remodeling and have been shown to antagonize BMP/Id signaling (7, 37). IL-1β is produced by activated macrophages, a major immune cell effector of lung inflammation. This cytokine is an important mediator of the inflamma-
Id3 is involved in the regulation of proliferation in PASMCs. A: cell counting at day 3 after lentiviral infection in 10% DMEM shows a significant reduction in cell number in Id3-overexpressed PASMCs compared with empty virus-infected cells (P < 0.05). B: immunoblot confirms the overexpression of Id3 by viral transduction compared with empty virus (Φ) in PASMCs (lines: 75M and 77M) after 5-day infection, with β-actin staining as loading control. *P < 0.05 compared with untransduced control cells, **P < 0.05 compared with untransduced mutant cells. C: flow cytometric analysis of PASMCs incubated with 1 μM BrdU for 16 h and 40 h after quiescence overnight. Cells were treated with DNase and incubated with BrdU-FITC for 20 min at room temperature. Representative BrdU FITC vs. 7-AAD dot plots shows regions (R) from control or Id3 overexpressed PASMCs. R3: G0/G1, R4: S phase, R5: G2+M, R6: apoptotic. At both 16 and 40 h, Id3 overexpression reduced the proportion of cells in the S phase. There were no consistent differences in the number of apoptotic cells between the control and Id3 overexpressed groups at 16 h and 40 h.

Fig. 8. Id3 is involved in the regulation of proliferation in PASMCs. A: cell counting at day 3 after lentiviral infection in 10% DMEM shows a significant reduction in cell number in Id3-overexpressed PASMCs compared with empty virus-infected cells (P < 0.05). B: immunoblot confirms the overexpression of Id3 by viral transduction compared with empty virus (Φ) in PASMCs (lines: 75M and 77M) after 5-day infection, with β-actin staining as loading control. *P < 0.05 compared with untransduced control cells, **P < 0.05 compared with untransduced mutant cells. C: flow cytometric analysis of PASMCs incubated with 1 μM BrdU for 16 h and 40 h after quiescence overnight. Cells were treated with DNase and incubated with BrdU-FITC for 20 min at room temperature. Representative BrdU FITC vs. 7-AAD dot plots shows regions (R) from control or Id3 overexpressed PASMCs. R3: G0/G1, R4: S phase, R5: G2+M, R6: apoptotic. At both 16 and 40 h, Id3 overexpression reduced the proportion of cells in the S phase. There were no consistent differences in the number of apoptotic cells between the control and Id3 overexpressed groups at 16 h and 40 h.

Id3 is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis, and has been implicated as a mediator of PAH (32). Angiotensin II increases blood pressure and promotes vascular remodeling acting through the angiotensin type 1 receptor in hypoxic pulmonary hypertension (21). Serotonin (5-HT) is widely implicated in PAH and antagonizes Smad signaling in the hypoxic mouse model of pulmonary hypertension (12). In PAH, there is a well-recognized female predominance. β-Estradiol is the predominant estrogen produced during the reproductive years both in terms of absolute serum levels as well as in terms of estrogenic activity. Thus we examined whether these important mediators directly regulate Id gene expression but found that only BMP4 and 6 robustly regulate expression of Id genes in PASMCs.

Our previous study showed that PASMCs with BMPR-II mutations were resistant to the growth inhibitory effects of BMP4 (13, 22). Here we further provide evidence to support the critical role of BMP signaling in regulating PASMC proliferation. We measured absolute cell number as well as new DNA synthesis (proportion of cells in S phase) and demonstrated that the reduced cell number after Id3 overexpression is due to a reduction in the number of cells in S phase. There was no consistent effect of Id3 overexpression on apoptosis of PASMCs. We sought to demonstrate whether the dysregulation of Id genes, and particularly our new findings of reduced induction of Id3 in mutant cells, could also be observed in the pulmonary circulation in situ. Immunohistochemistry demonstrated decreased Id3 expression in small pulmonary arteries and the lung parenchyma in BMPR-II-deficient mice. In addition, the reduced expression of Id3 was observed in the remodeled pulmonary arteries of patients with human PAH. Taken together, these findings provide further evidence for a critical role for Id1 and 3 in the proliferation of pulmonary vascular smooth muscle cells and the process of pulmonary vascular remodeling. In our experiments, we observed no effect of Id3 overexpression on rates of apoptosis in PASMCs although it was reported that Id1 regulates prostate cancer cell apoptosis (42), and BMP4 has been shown to induce apoptosis in PASMCs (41).

The potential role of Id1 and 3 has been explored previously in the chronically hypoxic mouse model of pulmonary hypertension (14). These investigators reported that Id1 and 3 expression are increased by hypoxia in a BMP-dependent
Fig. 9. LPS reduced Id3 expression following BMP4 stimulation in mutant PASMCs. A: immunoblots of Id3 protein levels in control and mutant PASMCs after treatment with 0.1% FBS contained DMEM, LPS (10 μg/ml), BMP4 (10 ng/ml), BMP4 + LPS (0.1 μg/ml), BMP4 + LPS (1 μg/ml), or BMP4 + LPS (10 μg/ml) for 24 h. β-Actin was used as a loading control. B: quantification of Id3 protein expression level in mutant PASMCs. C: quantification of Id3 protein expression level in mutant PASMCs. #P < 0.05 compared with 0.1% FBS DMEM, ns: no significant difference compared with 0.1% FBS DMEM, NS: no significant difference compared with BMP4 treat alone. **P < 0.05 compared with BMP4 (10 ng/ml)-treated mutant cells. Results represent the means of 3 independent experiments.

fashion in PASMCs. Id1 knockout mice did not develop more severe pulmonary hypertension during chronic hypoxic exposure than wild-type mice, which was thought to be due to the compensation by Id3 (14). Indeed, potential compensation of Id3 for Id1 is well recognized from studies in angiogenesis (30). In the present study in the PASMCs from BMPRII-deficient patients with human PAH, the expression of both Id1 and 3 was reduced, which may be critical for disease development. Because the double knockout of Id1 and 3 is embryonic lethal, it would be most useful to explore the pulmonary vascular phenotype of a conditional Id1/3 double knockout mouse to demonstrate definitely the role of this pathway in vivo (30).

Together, our study presents the indispensable role of BMPR-II on the regulation of Id gene expression. We provide evidence that Id3 regulates PASMC cell cycle and proliferation and show that inflammation, such as that produced by LPS, could be a potential second hit for the development of PAH in BMPR-II mutant carriers. These studies further strengthen the potential for targeting the Id1/3 pathway for therapeutic intervention in vascular diseases and particularly in PAH.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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AUTHOR CONTRIBUTIONS

Author contributions: J.Y. and N.W.M. conception and design of research; J.Y., X.L., Y.L., M.S., L.Y., L.L., and R.A.-L. performed experiments; J.Y. and X.L. analyzed data; J.Y., L.Y., R.A.-L., and N.W.M. interpreted results of experiments; J.Y. and N.W.M. approved final version of manuscript.

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