

# Mutations in Bone Morphogenetic Protein Type II Receptor Cause Dysregulation of Id Gene Expression in Pulmonary Artery Smooth Muscle Cells

## Implications for Familial Pulmonary Arterial Hypertension

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**Abstract**—Heterozygous germ line mutations in the gene encoding the bone morphogenetic protein (BMP) type II receptor occur in more than 80% of patients with familial pulmonary arterial hypertension. Because inhibitors of DNA binding (Id) genes are major targets of BMP/Smad signaling, we studied the regulation of these transcription factors in pulmonary artery smooth muscle cells harboring mutations in BMP type II receptor and control cells. Mutant cells demonstrated a marked deficiency in BMP4-stimulated Id1 and Id2 gene and protein expression compared with control cells. Mutant cells were deficient in Smad1/5 signaling in response to BMPs but also in extracellular signal-regulated kinase (ERK)1/2 activation. We provide evidence for an important interaction between Smad1/5 and ERK1/2 signaling in the regulation of Id gene expression. Thus, BMP4-induced Id1 expression was negatively regulated by ERK1/2 activation. The mechanism involves ERK1/2-dependent phosphorylation of the Smad1 linker region (serine 206), which limits C-terminal serine 463/465 phosphorylation and inhibits Smad nuclear accumulation. Furthermore, activation of ERK1/2 by platelet-derived growth factor BB also caused Smad1 linker region phosphorylation and inhibited BMP4-induced Id1 gene expression. In contrast, Id2 expression was positively regulated by ERK1/2. Moreover, we show that both BMP type II receptor mutation and Id1 knockdown leads to loss of growth suppression by BMPs. Taken together, these findings indicate an important interaction between ERK1/2 and Smad1/5 in the regulation of Id genes. Platelet-derived growth factor, via ERK1/2, further impairs the deficiency in Smad signaling found in BMP type II receptor mutant cells. The integration of these signals at the level of Id gene expression may contribute to the pathogenesis of familial pulmonary arterial hypertension. (*Circ Res.* 2008;102:1212-1221.)

**Key Words:** bone morphogenetic proteins ■ Id genes ■ pulmonary hypertension

Pulmonary arterial hypertension (PAH) is a severe clinical condition characterized by luminal obliteration of small pulmonary arteries by neointima formation.<sup>1-3</sup> Proliferation of myofibroblasts and smooth muscle cells in the arterial wall increases pulmonary vascular resistance and elevates pulmonary arterial pressure, ultimately leading to right ventricular failure. Endothelial dysfunction and proliferation also contribute to the process of vascular remodeling.<sup>4</sup> Although new treatments have improved symptoms of breathlessness and probably survival,<sup>5,6</sup> mortality remains high in these patients. The development of new approaches to therapy will depend partly on a greater understanding of the molecular mechanisms involved in the pulmonary vascular remodeling.

Recent studies have identified a major role for the bone morphogenetic protein (BMP) signaling pathway in the pathogenesis of familial PAH. The familial condition segre-

gates in an autosomal dominant manner with reduced penetrance.<sup>7,8</sup> Heterozygous germ line mutations in the gene encoding the BMP type II receptor (BMPR-II), a receptor member of the transforming growth factor  $\beta$  superfamily, have been identified in more than 80% of patients with familial PAH.<sup>9,10</sup> In addition, 15% to 26% of patients with idiopathic PAH have mutations in BMPR-II.<sup>11</sup> Furthermore, loss of BMPR-II expression is a feature of idiopathic PAH even in the absence of mutations in the *BMPR2* gene.<sup>3</sup> BMPR-II is a constitutively active serine-threonine kinase, which, in the presence of BMP ligand, heterodimerizes with and phosphorylates a type I receptor (BMPR1A or BMPR1B, also known as ALK-3 and ALK-6, respectively).<sup>12</sup> The activated type I receptor phosphorylates the downstream signaling Smad proteins at the C terminus (Smad1, -5, and -8), which translocate to the nucleus in the presence of a

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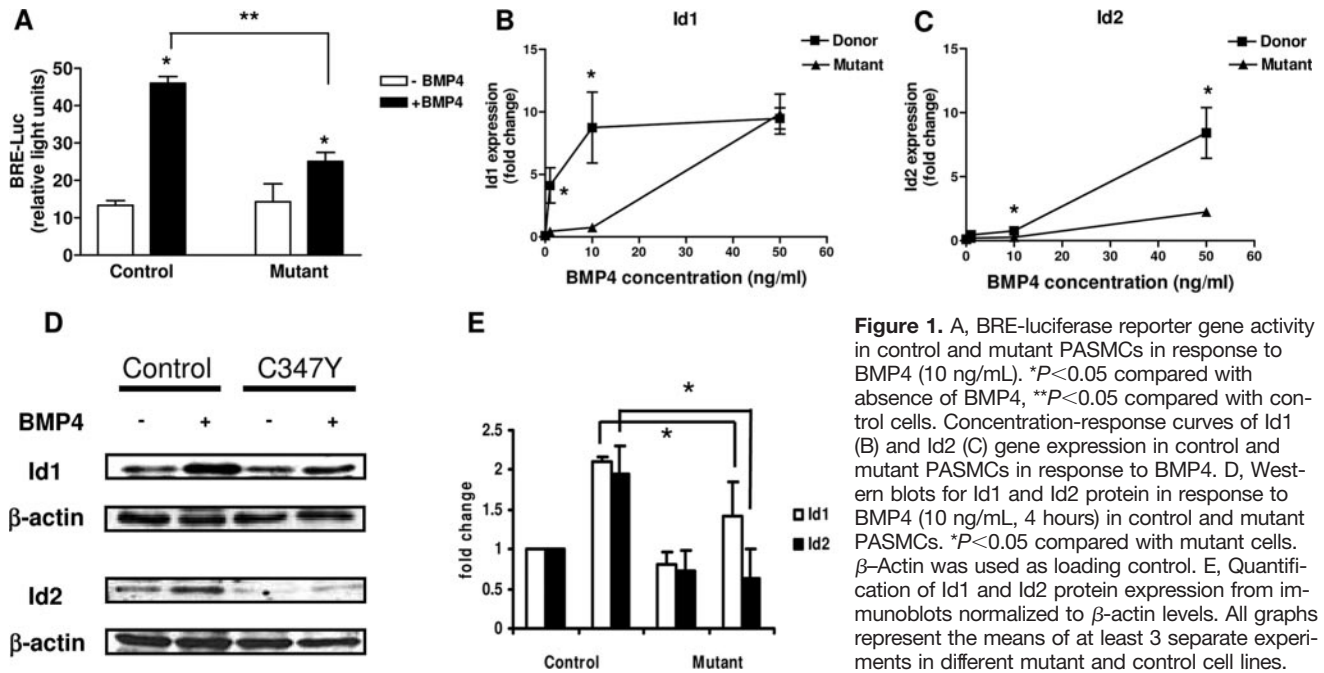
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**Figure 1.** A, BRE-luciferase reporter gene activity in control and mutant PASCs in response to BMP4 (10 ng/mL). \* $P < 0.05$  compared with absence of BMP4, \*\* $P < 0.05$  compared with control cells. Concentration-response curves of Id1 (B) and Id2 (C) gene expression in control and mutant PASCs in response to BMP4. D, Western blots for Id1 and Id2 protein in response to BMP4 (10 ng/mL, 4 hours) in control and mutant PASCs. \* $P < 0.05$  compared with mutant cells.  $\beta$ -Actin was used as loading control. E, Quantification of Id1 and Id2 protein expression from immunoblots normalized to  $\beta$ -actin levels. All graphs represent the means of at least 3 separate experiments in different mutant and control cell lines.

common partner Smad, Smad4, to regulate gene transcription. We have previously shown that BMPR-II mutation is associated with reduced ability to phosphorylate Smad1/5. However, BMPs can also signal via mitogen-activated protein kinase (MAPK) pathways in a Smad-independent manner.<sup>13,14</sup> Activation of the extracellular signal-regulated kinase (ERK)1/2 has also been shown to directly inhibit Smad1/5 signaling in epithelial cell lines,<sup>15</sup> but the role MAPK activation in vascular cells and the functional impact of this interaction on the regulation of BMP-responsive genes is unclear.

A major transcriptional target of BMP signaling is the inhibitor of DNA binding (Id) family of proteins.<sup>12,16</sup> Id proteins (Id1 to -4) are basic helix-loop-helix transcription factors that lack a DNA binding domain. These proteins bind with high affinity to the ubiquitously expressed E protein family of basic helix-loop-helix transcription factors and inhibit their binding to target DNA.<sup>17</sup> This important function of Id proteins confers a central role in the regulation of gene expression and hence cellular differentiation and proliferation.<sup>17-19</sup>

Here, we demonstrate dysregulation of Id gene expression in human pulmonary artery smooth muscle cells (PASCs) harboring mutations in BMPR-II. Transcriptional regulation of Id1 and Id2 by BMPs was markedly impaired in mutant cells. Failure of Id1 gene induction was associated with a failure to respond to the growth suppressive effects of BMPs. These studies further demonstrate an important interaction between ERK1/2 and Smad1/5 pathways and suggest a mechanism by which growth factors such as platelet-derived growth factor (PDGF) can impair BMP signaling via ERK1/2 activation. The impairment of Id gene expression in mutant cells may have important consequences for cell proliferation and differentiation in familial PAH.

## Materials and Methods

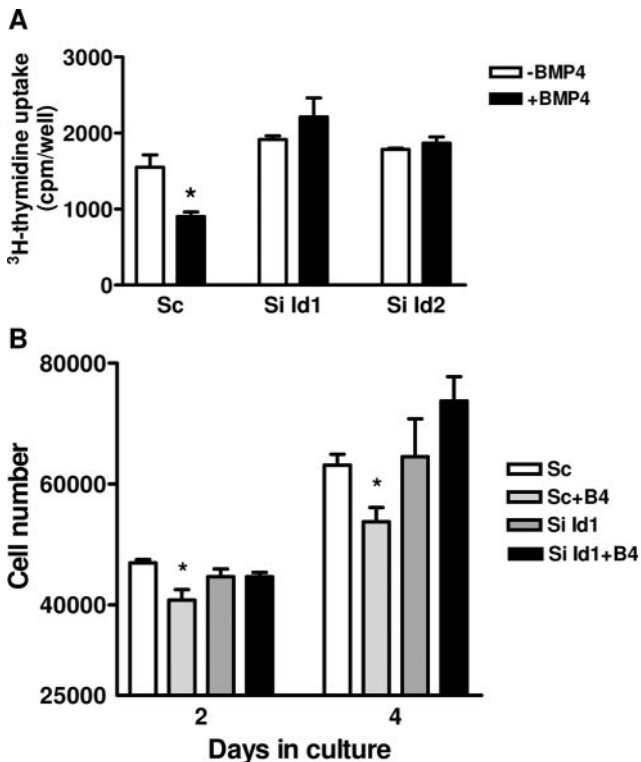
### Human Pulmonary Artery Smooth Muscle Cell Culture

Pulmonary artery smooth muscle cells (PASCs) were derived from peripheral pulmonary arteries (<2 mm external diameter), as previously described,<sup>20</sup> or lobar arteries for growth studies. Cells were derived from the lungs of patients undergoing heart lung transplantation for PAH (n=4), known to harbor a mutation in the *BMPR2* gene. These included 2 patients with a mutation in the kinase domain of *BMPR2* in which arginine or tyrosine is substituted for cysteine at position 347 (C347R and C347Y)<sup>10</sup>; a missense mutation in the cytoplasmic tail of *BMPR2*, leading to a serine in place of asparagine at position 903 (N903S)<sup>20</sup>; an exon 1 nonsense mutation at amino acid 9, W9X, predicted to lead to haploinsufficiency.<sup>10</sup> Control PASCs were obtained from patients undergoing lung resection for suspected malignancy (n=5). The Papworth Hospital ethical review committee approved the study, and subjects or relatives gave informed written consent. For details regarding cell culture, see the expanded Materials and Methods section in the online data supplement at <http://circres.ahajournals.org>.

### Immunoblotting

PASCs were seeded at  $5 \times 10^5$  in 6-cm dishes and grown to  $\approx 95\%$  confluence. After 48 hours of quiescence, cells were treated with or without BMP4 or BMP6 (0.1 to 100 ng/mL) in 0.1% FBS/DMEM for up to 8 hours. At specified time points, cells were lysed in buffer as described previously.<sup>13</sup> Samples were electrophoresed by 10% SDS-PAGE and then transferred to nitrocellulose membrane. For studies of Smad1 and ERK1/2 activation, blots were incubated with polyclonal rabbit anti-phospho-Smad1/5 antibody (serine 463/465 1:1000) or anti-phospho-ERK1/2 (1:1000) (Cell Signaling Technology Inc), as previously described.<sup>13</sup> To confirm equal protein loading, blots were stripped and reprobed using anti-Smad1 (1:500) or anti-ERK1/2 (1:1000). In additional experiments, cells were preincubated with a selective inhibitor of ERK1/2, U0126, or platelet-derived growth factor (PDGF)-BB. Ras activity was determined using a Ras Activation Assay Kit (Upstate), according to the instructions of the manufacturer.

To assess Smad1 linker region s206 phosphorylation on immunoblots, we used a polyclonal rabbit anti-phospho (s206) Smad1



**Figure 2.** A, [<sup>3</sup>H]-thymidine incorporation studies demonstrate that siRNA knockdown of Id1 (Si Id1) or Id2 (Si Id2) abolished the inhibition of DNA synthesis by BMP4 (10 ng/mL) in control PSMCs over 24 hours. B, In proliferation studies, knockdown of Id1 with siRNA abolishes the growth inhibitory effect of BMP4 in control PSMCs. Cells were grown in DMEM/10% FBS with or without BMP4 and counted after 2 and 4 days of treatment. The scramble (Sc) nontarget siRNA was transfected as a negative control. \**P*<0.05 compared with Id1 siRNA-treated cells. Results represent means of 3 separate experiments.

antibody (1:100).<sup>21</sup> Horseradish peroxidase-conjugated anti-rabbit IgG (Pierce, 1:5000) was used as a secondary antibody.

In additional experiments, nuclear and cytosolic fractionations were performed to determine phospho-Smad1/5 localization (Pierce). Separation of the nuclear fraction was demonstrated with an antibody against Histone 1B (Upstate Biotechnology). The purity of cytosolic fractions was confirmed using an anti- $\alpha$ -tubulin antibody (Sigma).

Anti-Id1 (sc-488) and anti-Id2 (sc-489) (Santa Cruz Biotechnology) were used at 1:500, followed by anti-rabbit secondary antibody (1: 1000; Dako).

### Reporter Gene Assays and Constructs

A BMP-response element linked to a luciferase reporter (BRE-luc), derived from the mouse Id1 promoter,<sup>22</sup> was used to quantify BMP/Smad-dependent gene transcription. In addition, to directly determine the effect of ERK1/2 activation on Id promoter activity, we used a 2.2-kb luciferase construct in the pGL-3 vector encoding the 5' regulatory region of the human Id1 promoter<sup>23</sup> and a 2.7-kb luciferase human Id2 promoter in the pGL3 basic vector.<sup>24</sup> Cotransfection with *Renilla* luciferase was used as a transfection control (pRL-TK, Promega). Constitutively active MEK1 with an enhanced green fluorescent protein tag (pcDNA3-MEK1E) (Simon Cook, Babraham Institute, Cambridge, UK) was used to directly assess the role of ERK1/2 activation on Id promoter activity. Transient transfection of PSMCs was achieved by electroporation using the Amaxa Nucleofector system, according to the instructions of the manufacturer.

### Localization of Phospho-Smad1/5 by Confocal Immunofluorescence

The proportion of cells displaying nuclear localization of phospho-Smad1 was determined by confocal microscopy. For details, see the online data supplement.

### Real-Time RT-PCR

Quiescent PSMCs were incubated with BMP4 (0.1 to 50 ng/mL) or DMEM alone for 1 or 4 hours. In some experiments, cells were preincubated with U0126 or cotreated with PDGF-BB (10 ng/mL). See the online data supplement for RT-PCR methods.

### Small Interfering RNA Knockdown of Id Genes and BMPR-II

Control human PSMCs were treated with small interfering (si)RNA for Id1 or Id2 siRNA (see online data supplement for details). Cells were grown in 10% FBS/DMEM in the presence or absence of BMP4 (10 ng/mL) and counted on days 2 and 4 using a hemocytometer. Cell viability was assessed with Trypan blue. For [<sup>3</sup>H]-thymidine incorporation studies, after 48 hours of siRNA treatment, cells were incubated with BMP4 10 ng/mL for a further 12 hours, with 0.5  $\mu$ Ci per well methyl-[<sup>3</sup>H]-thymidine added for the final 12 hours. In parallel experiments, cells were plated in 6-well plates and treated similarly. Total RNA was extracted from these cells to assess the efficiency of Id1 and Id2 knockdown by real-time RT-PCR.

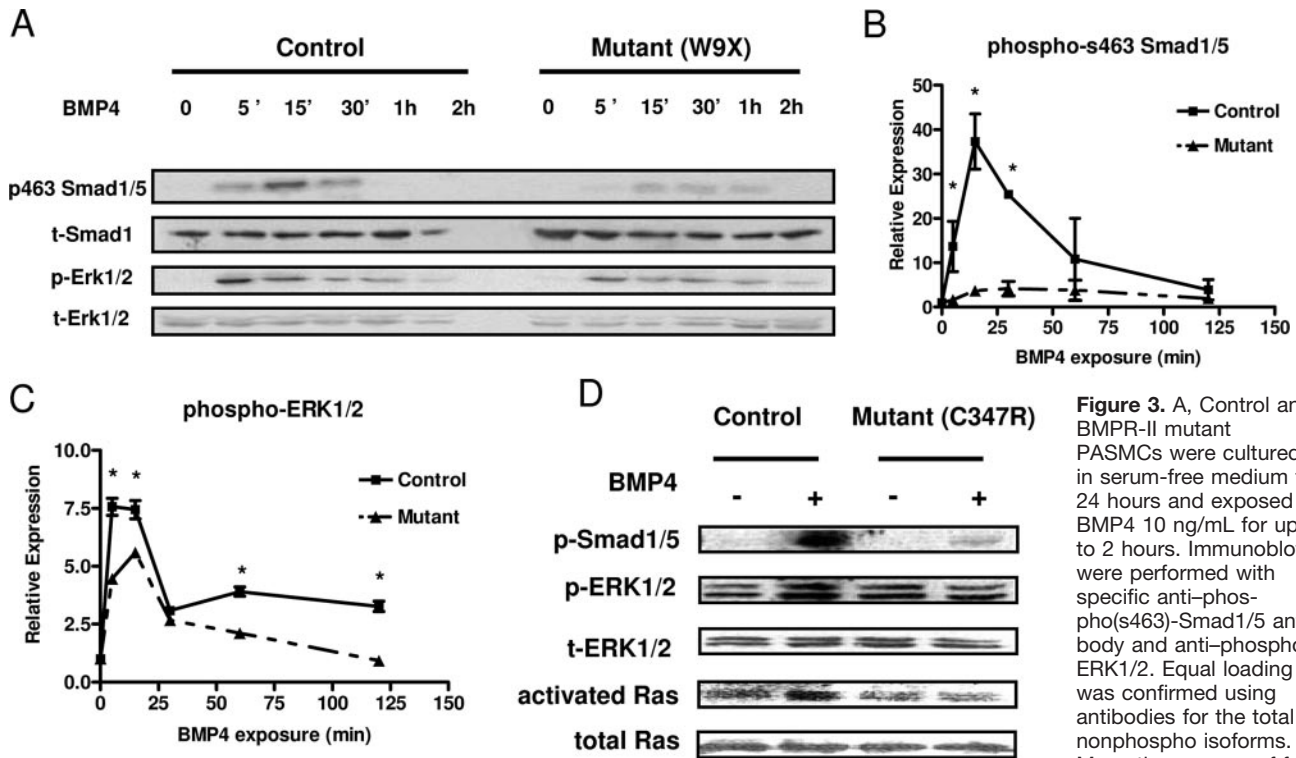
### Statistics

Data are presented as means  $\pm$  SE. Data between groups were compared using a 2-tailed *t* test or a 1-way ANOVA, followed by Tukey's honestly significant difference test, whichever was appropriate. *P*<0.05 indicated a statistically significant result.

## Results

### Differential Regulation of Id Gene Expression in BMPR-II Mutant Cells

We first confirmed that activation of the BRE-luc reporter gene by BMP4 was reduced in all mutant cell isolates compared with control cells (Figure 1A). Because the BRE-luc is derived from the mouse Id1 promoter, we determined the expression of Id1 and Id2 transcripts in control and mutant cells. Basal levels of Id1 and Id2 gene expression were similar in control and mutant cells by quantitative RT-PCR. Exposure of control PSMCs to BMP4 (50 ng/mL) for 4 hours resulted in a 9.5-fold increase in Id1, although the response was maximal at a concentration of 10 ng/mL BMP4 (Figure 1B). The induction of Id1 gene expression was markedly reduced in mutant cells at lower concentrations of BMP4 (0.1 to 10 ng/mL) (Figure 1B). The findings with Id2 were similar though mutant cells were markedly resistant to the induction of Id2 mRNA over the range of concentrations of BMP4 used (Figure 1C). Western blotting of whole cell lysate after 4 hours of stimulation with BMP4 (10 ng/mL) confirmed the lack of induction of Id1 and Id2 protein in mutant cells (Figure 1D and 1E). To confirm that BMP4 was mediating effects via BMPR-II, we performed BMPR-II knockdown with siRNA. Knockdown of BMPR-II (>80% confirmed by real-time RT-PCR) markedly inhibited BMP4-stimulated Id1 and Id2 gene expression (Figure I in the online data supplement).



**Figure 3.** A, Control and BMPR-II mutant PASCs were cultured in serum-free medium for 24 hours and exposed to BMP4 10 ng/mL for up to 2 hours. Immunoblots were performed with specific anti-phospho(s463)-Smad1/5 antibody and anti-phospho-ERK1/2. Equal loading was confirmed using antibodies for the total nonphospho isoforms. Mean time course of for BMP4 stimulated phospho-Smad1 (B) and phospho-ERK1/2 (C) activity from 3 control and 3 mutant cell lines. \**P*<0.05 compared with corresponding time point. D, Demonstration of reduced Ras-ERK1/2 and Smad1/5 signaling in mutant PASCs compared with control. Blots are representative of at least 3 separate experiments.

**Id1 Contributes to the Growth Inhibitory Effects of BMP4**

To assess the functional contribution of Id1 and Id2 to BMP-mediated growth suppression in PASCs, we transfected cells with Id1- or Id2-specific siRNA and performed [<sup>3</sup>H]-thymidine incorporation and proliferation assays in response to BMP4. The efficiency of Id1 and Id2 knockdown in these experiments was >80% and >90%, respectively (supplemental Figure II). BMP4 reduced [<sup>3</sup>H]-thymidine incorporation in PASCs transfected with a scrambled nontargeting siRNA over 24 hours, as expected. In contrast, transfection with Id1 or Id2 siRNA caused resistance to the growth suppressive effects of BMP4 (Figure 2A). In proliferation assays, we confirmed that siRNA knockdown led to a sustained reduction in Id1 mRNA by quantitative RT-PCR (>80% at 2 days and >70% at 4 days, compared with control levels). After 4 days, the growth of control human PASCs treated with transfection reagent alone or with scrambled nontarget control siRNA was significantly inhibited by BMP4 (Figure 2B). Cells in which Id1 had been reduced by siRNA knockdown exhibited loss of the antiproliferative effect of BMP4 (Figure 2B).

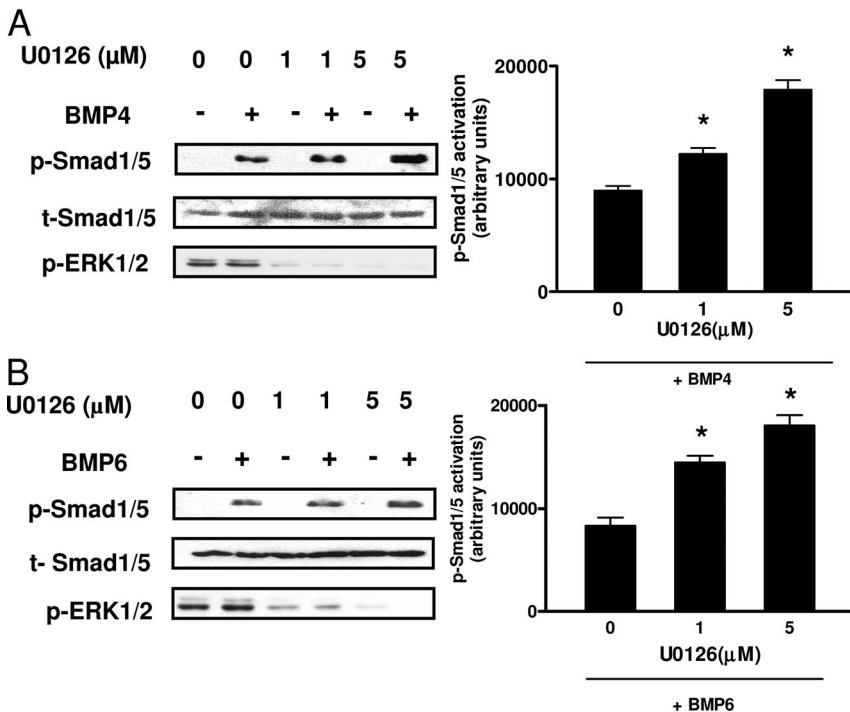
**Reduced Activation of Smad1/5 and ERK1/2 Pathways by BMP4 in BMPR-II Mutant PASCs**

To begin to determine the signaling pathways regulating the expression of Id genes in control and BMPR-II mutant cells, we characterized the phosphorylation of ERK1/2 and C-terminal Smad1/5 in control cells and cells harboring mutations in BMPR-II. In control cells, BMP4 (10 ng/mL)

caused a time-dependent activation of Smad1/5 and ERK1/2 (Figure 3A). As expected, we observed a reduction in Smad1/5 C-terminal phosphorylation in all BMPR-II mutant cells (Figure 3A, 3B, and 3D). Activation of ERK1/2 was also reduced in mutant PASCs compared with control cells (Figure 3A, 3C, and 3D). To further determine the activation status of the ERK1/2 pathway in control and mutant cells, we determined Ras activity following BMP4 stimulation. Ras activation and ERK1/2 activity was reduced in all mutant cells, suggesting a deficiency of the Ras-ERK1/2 pathway in these cells (Figure 3D).

**ERK1/2 Activation Antagonizes Smad1/5 Phosphorylation in PASCs**

Activation of MAPK has been shown to functionally cooperate with Smad signaling in some cells but to oppose Smad signaling in others.<sup>12,25</sup> In particular, it has been shown in epithelial cells<sup>26</sup> that ERK1/2 activation by epidermal growth factor causes phosphorylation of PXSP motifs in the linker region of Smad1 and inhibits nuclear accumulation of Smad1. We therefore determined whether functional antagonism occurs downstream of the BMP receptors in PASCs. In control cells, preincubation with the ERK1/2 inhibitor U0126 inhibited the phosphorylation of ERK1/2 in response to BMP4 and increased the C-terminal phosphorylation of Smad1/5 in a concentration-dependent manner (Figure 4A). There were no changes in the level of total Smad1 in these experiments. Similar experiments with BMP6 stimulation also showed that C-terminal phosphorylation of Smad1/5 was increased by preincubation with U0126 (Figure 4B).



**Figure 4.** A, Preincubation with the ERK1/2 inhibitor U0126 increased BMP4-induced (10 ng/mL) Smad1/5 phosphorylation and inhibited ERK1/2 activation in a concentration-dependent manner, with unchanged total Smad1/5 levels. B, Preincubation with U0126 also increased BMP6-induced (10 ng/mL) Smad1/5 phosphorylation. Blots are representative of at least 3 separate experiments. Densitometric analyses of phospho-Smad1/5 activity in response to BMP4 in the presence and absence of U0126 are presented in the accompanying graphs. \* $P < 0.05$  compared with cells not exposed to U0126.

To determine whether ERK1/2 activation antagonized nuclear accumulation of Smads, we examined the localization of phospho-Smad1/5 by confocal immunofluorescence microscopy. Stimulation of control PASCs with BMP4 (10 ng/mL) for 1 hour caused phospho-Smad1 nuclear accumulation in  $44.6 \pm 3.5\%$  of cells. Preincubation with 1 and 5  $\mu\text{mol/L}$  U0126 increased nuclear accumulation to  $58.4 \pm 6.8\%$  and  $63.5 \pm 3.4\%$  of cells, respectively (Figure 5A and 5C). In mutant cells, we observed reduced BMP4-stimulated phospho-smad1/5 nuclear localization at 1 hour ( $7.8 \pm 1.2\%$ ) compared with control cells. Preincubation with U0126 did not significantly increase Smad1/5 nuclear localization in response to BMP4 in mutant cells (Figure 5B and 5D).

To further confirm the effect of activated ERK1/2 on C-terminal phosphorylated Smad1, we separated nuclear and cytosolic fractions and probed with antiphospho-Smad1/5 antibody. BMP4-stimulated (10 ng/mL) nuclear accumulation of phospho-Smad1/5 at 1 hour, which was accentuated by preincubation with U0126 (Figure 5E). Conversely, direct activation of ERK1/2 by costimulation with PDGF-BB antagonized the BMP-stimulated nuclear accumulation of phospho-Smad1/5 (Figure 5E).

To determine the functional significance of the interaction between PDGF-BB and BMP signaling, we determined the effect of BMP4 and PDGF-BB on the proliferation of control and BMPR-II mutant PASCs. In control cells, PDGF-BB opposed the antiproliferative effect of BMP4. In contrast, in mutant cells the BMP4 failed to suppress PDGF-BB-induced proliferation (Figure 6).

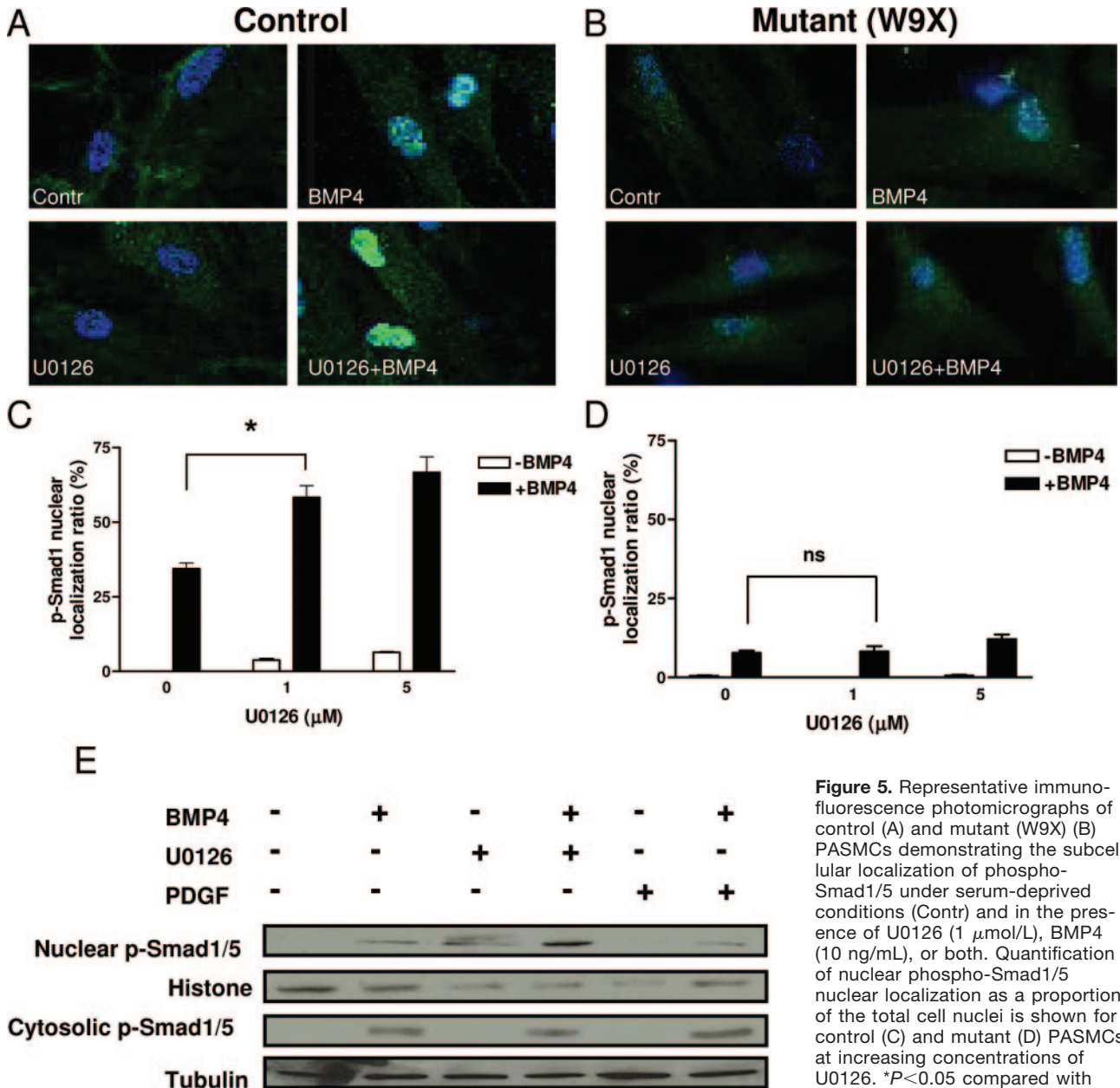
### Smad1 Linker Region Phosphorylation Regulates Nuclear Localization of C-Terminal Phospho-Smad1 in PASCs

To directly assess the effect of ERK1/2 activation on Smad1 linker region phosphorylation, we used an antibody that

specifically recognizes phosphorylation of the Ser206 residue in the linker region of Smad1. BMP4 caused phosphorylation of both the C-terminal Ser463 and linker region Ser206, whereas activation of ERK1/2 with PDGF-BB caused only Ser206 phosphorylation (Figure 7A). Coincubation of BMP4 with PDGF-BB caused increased activation of Ser206 compared with either agent alone and reduced C-terminal phosphorylation of Ser463 (Figure 7A and 7B). We further confirmed that U0126 inhibited the s206 phosphorylation in response to BMP4 or PDGF-BB (supplemental Figure III). To determine the effect of BMPR-II mutation on nuclear/cytoplasmic partitioning of C-terminal phospho-Smad1/5, we compared control and mutant cells in response to BMP4. Mutant cells demonstrated reduced nuclear and cytoplasmic accumulation of phospho-Smad1/5 at 1 hour (supplemental Figure IV). In addition, we determined the effect of mutation on Smad1 linker region phosphorylation. In response to BMP4, reduced Ser206 and Ser463 phosphorylation was observed in mutant cells, compared with control cells (Figure 7C and 7D). Reduced Ser206 phosphorylation in mutant cells was consistent with the defect in ERK1/2 activation described above.

### Contribution of ERK1/2 Signaling to BMP4-Stimulated Id Gene Expression

We determined whether ERK1/2 inhibition affects BMP-mediated transcriptional responses using the Smad1/5-responsive BRE-luc reporter gene. BMP4 consistently activated BRE-luc in control PASCs. ERK1/2 inhibition increased the BMP4-dependent activation of BRE-luc (Figure 8A). Next, we used real time RT-PCR to determine the involvement of ERK1/2 signaling in BMP4-stimulated Id gene expression in PASC. Preincubation of PASCs with U0126 led to an increase in BMP4-stimulated Id1 gene expression, consistent with the hypothesis that ERK1/2 was



**Figure 5.** Representative immunofluorescence photomicrographs of control (A) and mutant (W9X) (B) PSMCs demonstrating the subcellular localization of phospho-Smad1/5 under serum-deprived conditions (Contr) and in the presence of U0126 (1 μmol/L), BMP4 (10 ng/mL), or both. Quantification of nuclear phospho-Smad1/5 nuclear localization as a proportion of the total cell nuclei is shown for control (C) and mutant (D) PSMCs at increasing concentrations of U0126. \**P*<0.05 compared with BMP4 alone. Results are representative of 3 separate experiments with different mutants and controls. E, PSMCs were incubated with BMP4 (10 ng/mL) for 30 minutes in the presence or absence of U0126 (1 μmol/L) or PDGF-BB (10 ng/mL). Nuclear and cytoplasmic fractions were collected. Histone and tubulin blots show the loading of nuclear and cytoplasmic fractions, respectively.

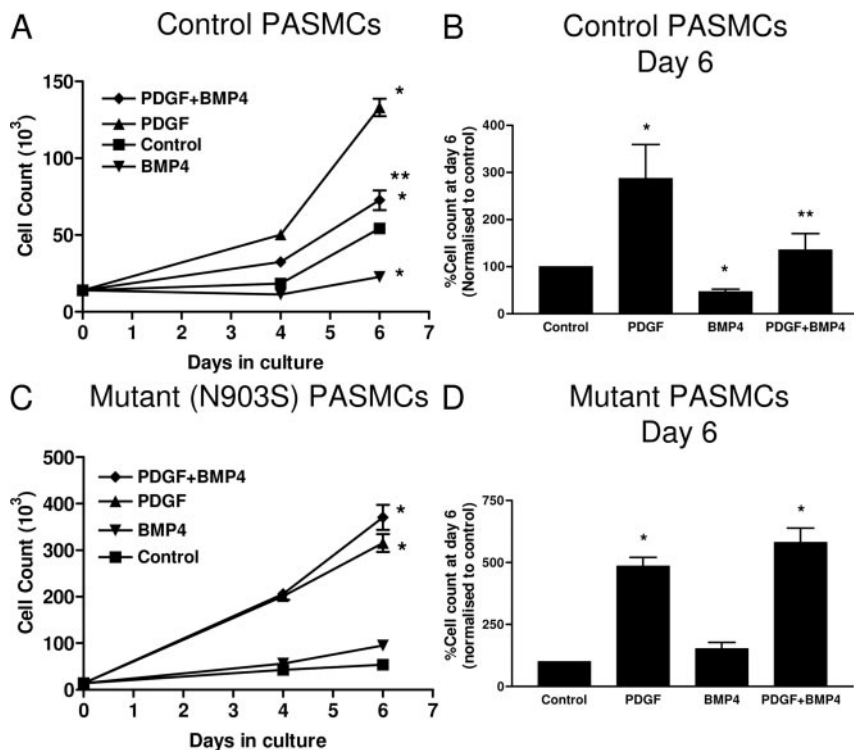
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involved in functional antagonism of Id1 gene expression (Figure 8B). Moreover, coincubation of cells with PDGF-BB markedly inhibited BMP4-stimulated Id1 gene expression (Figure 8B). In contrast, ERK1/2 inhibition led to a reduction in BMP4-stimulated Id2 gene expression in control cells, suggesting that ERK1/2 positively regulates expression of Id2 in PSMCs (Figure 8C). However, PDGF-BB alone had no significant effect on Id2 gene expression and coincubation of PDGF-BB, and BMP4 did not further increase Id2 expression compared with BMP4 stimulation alone (Figure 8C). To further confirm the differential regulation of Id1 and Id2 gene expression by ERK1/2, we transiently cotransfected PSMCs with Id1 or Id2 promoters linked to luciferase reporter genes with constitutively active MEK1 or pcDNA3 control vectors.

Overexpression of MEK1 suppressed BMP4 activation of the Id1 promoter (Figure 8D) but enhanced BMP4 stimulation of the Id2 promoter (Figure 8E).

### Discussion

The present study demonstrates several novel findings regarding BMP signaling in PSMCs and the functional consequences of BMPR-II mutation. This is the first study to demonstrate a defect in the transcriptional regulation of Id genes in cells harboring mutations in BMPR-II. The differential regulation of these important BMP target genes in PSMCs from patients with BMPR-II mutation is likely to have major consequences for cell function. Classically, Id proteins bind with basic helix-loop-helix transcription fac-



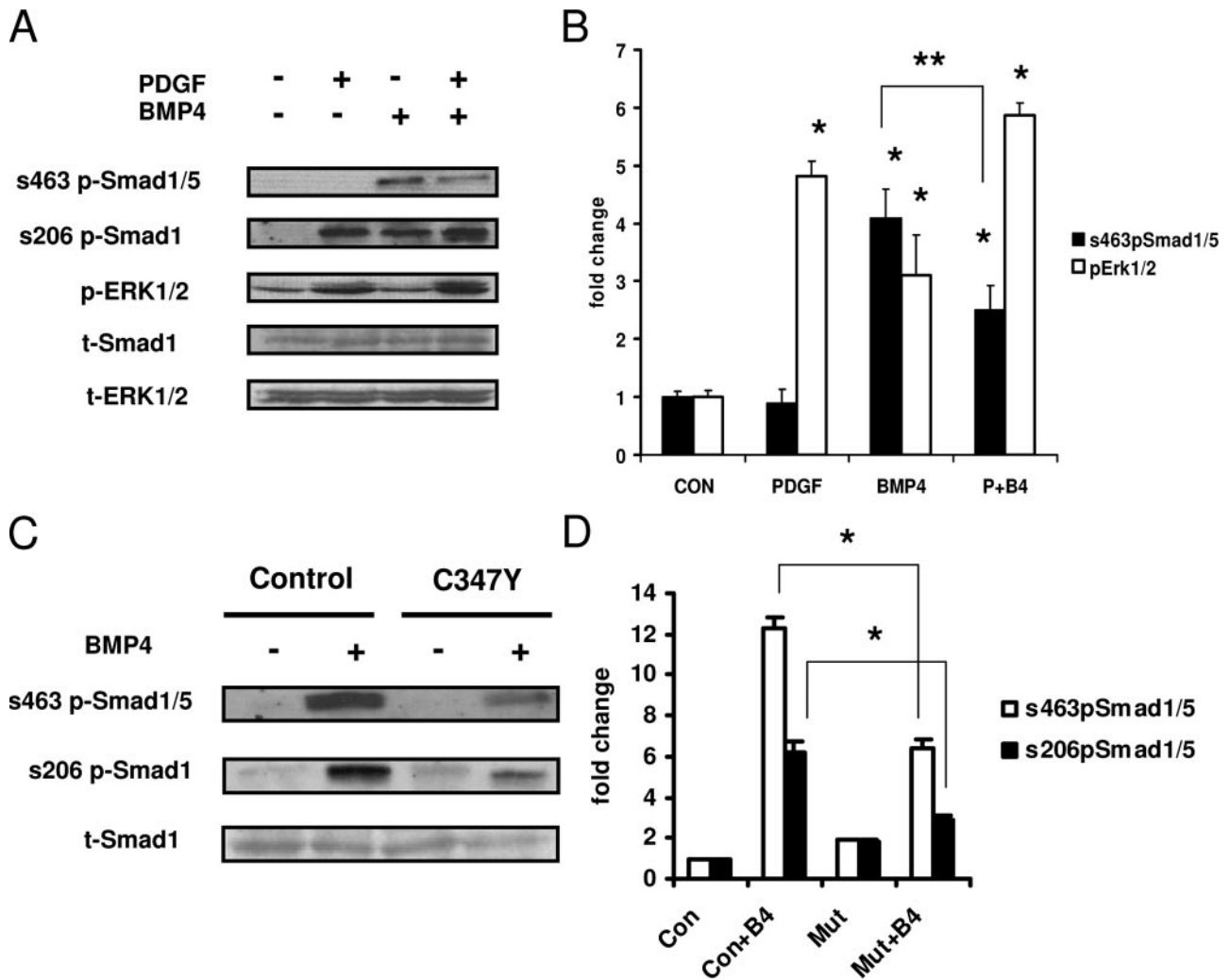
**Figure 6.** Representative growth curves of control (A) and BMPR-II mutant (C) PSMCs grown over 6 days in 5% FBS (control) in the presence or absence of PDGF-BB (10 ng/mL), BMP4 (10 ng/mL), or both. \* $P < 0.05$  compared with corresponding control value. Mean cell counts at day 6 are shown for 3 control cell lines (B) and 3 mutant lines (D). Graphs illustrate the ability of PDGF-BB to oppose BMP4 growth inhibition in control cells and the failure of BMP4 growth inhibition in mutant cells. \* $P < 0.05$  compared with control.

tors and limit their binding to target DNA.<sup>16</sup> The Id genes are thus critical regulators of cell transcriptional activity,<sup>16,19</sup> playing important roles in cell growth and differentiation.<sup>27,28</sup> Our data show that BMP4 stimulated Id1 and Id2 gene expression is impaired both in mutant cells and in control cells following siRNA knockdown of BMPR-II. In addition, we showed that knockdown of Id1 partly prevented the antiproliferative effects of BMP4 in control PSMCs. We have previously reported that PSMCs harboring disease-causing mutations in BMPR-II are also resistant to the growth suppressive effects of BMPs.<sup>20</sup> It was previously shown that hypoxia impairs the regulation of Id1 gene expression in PSMCs as a potential mechanism for hypoxia-induced pulmonary vascular remodeling,<sup>29</sup> suggesting a plausible common mechanism for vascular remodeling within the pulmonary circulation. Taken together, our data suggest that BMPR-II mutation causes loss of BMP mediated growth inhibition via a failure of Id gene transcription. Further work is needed to determine the specific roles of individual Id genes in PSMC function and differentiation. The identity of the partner transcription factors for Id1 and Id2 in pulmonary vascular cells also remain to be determined.

We sought to define the likely signal transduction pathways downstream of BMPR-II—regulating Id gene expression and the impact of mutation in BMPR-II. We show that mutation in BMPR-II reduces BMP-stimulated Smad1/5 and ERK1/2 activation in human PSMCs. We have previously shown reduced Smad1/5 signaling in mutant PSMCs,<sup>20</sup> but the effect of mutation on MAPK signaling is controversial.<sup>13,30</sup> Reduced BMP-stimulated ERK1/2 signaling in mutant cells is supported by 3 separate lines of evidence in our study: (1) from controlled time course comparisons of phospho-ERK1/2 activity in control and mutant cells, (2) by

demonstration of the reduced activation of Ras in mutant cells, and (3) by the reduced phosphorylation of the ERK1/2 phosphorylation site (Ser206) on the linker region of Smad1. Previous studies have suggested that MAPK pathways are constitutively activated in the presence of a mutation in BMPR-II, in the absence of any BMP stimulation.<sup>13,30</sup> In one of these, from our own laboratory, the constitutive activation of p38 MAPK was observed in a mouse epithelial cell line transiently transfected with mutant BMPR-II.<sup>13</sup> This difference may represent cell type-specific BMP signaling, because we have not observed this phenomenon in human PSMCs.<sup>20</sup> A further report did show evidence for activation of ERK1/2 in mutant cells, but this was in the presence of serum (5%) rather than the serum-free conditions used in the present study. Thus, our data show that PSMCs harboring mutations in BMPR-II, when studied under serum-free conditions, demonstrate a reduced capacity to phosphorylate ERK1/2 and the C terminus of Smad1/5 in response to BMP stimulation, compared with control cells.

We further report the presence of an important functional interaction between Smad1/5 and ERK1/2 pathways downstream of the BMP receptors in normal PSMCs. Following preincubation with a selective inhibitor of ERK1/2, subsequent BMP4 or BMP6 stimulation led to increased levels of phospho-Smad1/5 by immunoblotting and increased translocation of Smad1/5 to the nucleus. It was previously shown in epithelial cell lines that ERK1/2 activated by growth factors such as epidermal growth factor inhibit nuclear translocation of Smad1 via linker region phosphorylation.<sup>15,26</sup> Similarly, in the present study, we demonstrate that PDGF, an activator of ERK1/2 signaling, can antagonize BMP induced C-terminal Smad phosphorylation and increase linker region phosphorylation. We also demonstrated that this potentially important



**Figure 7.** A, Representative immunoblots of s463- and s206-Smad1 phosphorylation in response to BMP4 (10 ng/mL), PDGF (10 ng/mL), or a combination of both in normal PSMCs. B, Mean data from 3 separate experiments in which the phosphorylated signal is presented as a ratio of the total Smad or total ERK1/2 protein. \* $P < 0.05$  compared with control unstimulated signal, \*\* $P < 0.05$  compared with BMP4 alone. C, Representative immunoblots of s463- and s206-Smad1 phosphorylation in control (Con) and BMPR-II mutant (mut) (C347Y) PSMCs in response to BMP4 (B4). D, Mean data from 3 independent comparisons in control and mutant cells quantified by image analysis and normalized to total Smad1 levels. \* $P < 0.05$ .

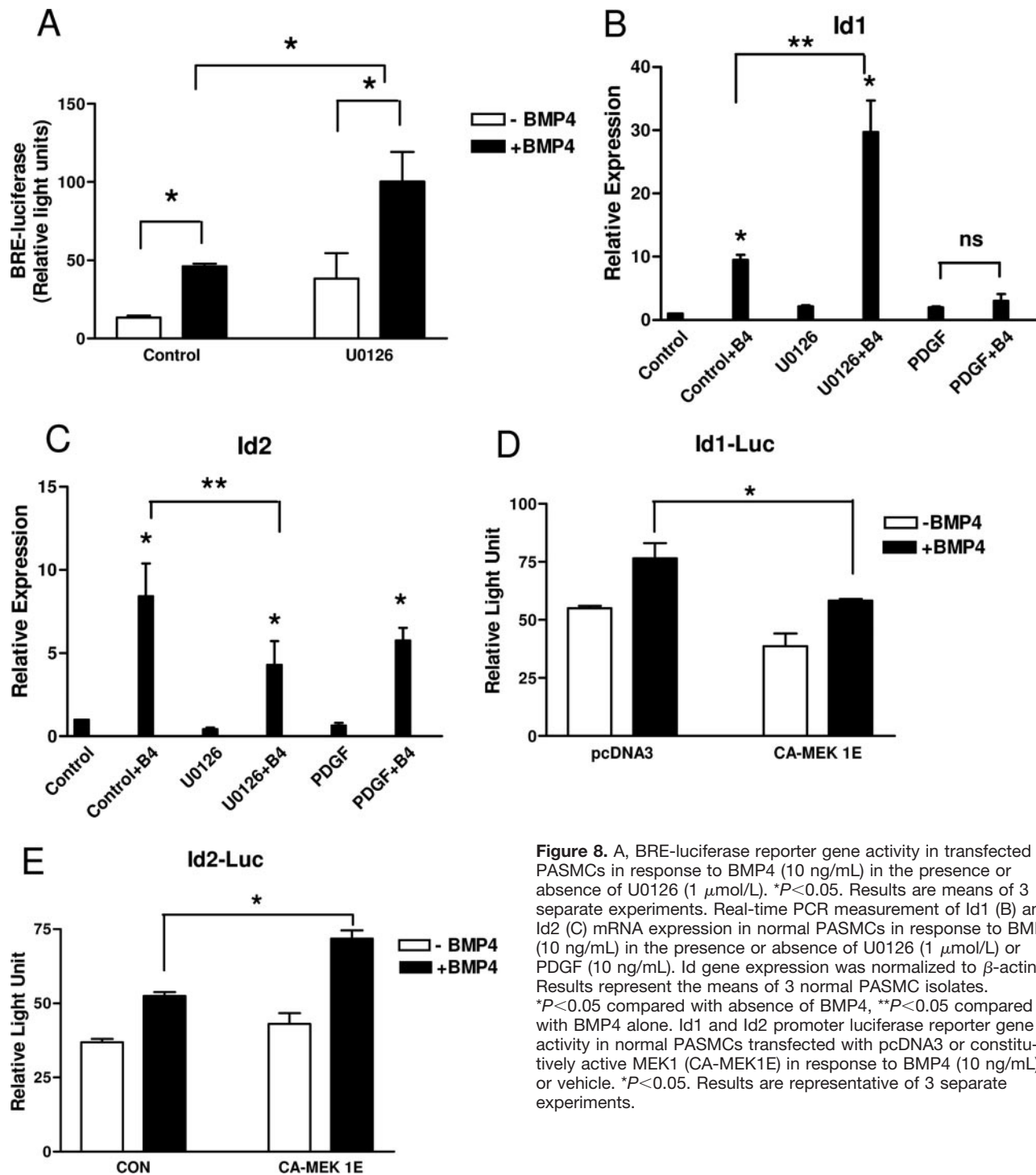
interaction operates directly downstream of the BMP receptor complex to modify Smad signaling and BMP transcriptional responses. The mechanism by which ERK1/2 inhibition increases whole cell C-terminal phospho-Smad1 levels in our study is likely explained by the recent observation that Smad1 linker region phosphorylation primes Smad1 for recognition and polyubiquitination by Smurf1 and subsequent degradation.<sup>21</sup>

In BMPR-II mutant cells, we observed a reduced level of BMP-induced nuclear translocation of Smad1/5, as expected. The reduction of phospho-Smad1/5 in mutant cells was seen both in cytoplasmic and nuclear compartments, indicating that the primary defect is a failure of cytoplasmic Smad1/5 activation in these cells. Interestingly, we observed no significant effect of ERK1/2 inhibition on BMP-stimulated Smad1/5 phosphorylation in mutant cells. This is consistent with the suggestion that BMP-stimulated ERK1/2 pathways are also defective in mutant cells and are contributing less to the inhibition of Smad signaling.

We determined that ERK1/2 exerts differential effects on Id1 and Id2 gene expression. Thus ERK1/2 negatively regulates Id1 gene expression but positively regulates Id2 gene expression in PSMCs. Preincubation of cells with the ERK1/2 inhibitor U0126 increased BMP4-dependent Id1 expression but inhibited Id2 gene expression. We further confirmed the differential requirement for ERK1/2 in the regulation of Id1 and Id2 promoters by the transient overexpression of constitutively active MEK1. Furthermore, PDGF activation of ERK1/2 inhibited BMP-induced Id1 gene expression, although it had no significant effect on Id2 gene expression. These findings suggest important differences in the coupling of ERK1/2 to Id1 and Id2 gene expression in these cells.

We chose to use PDGF as an exogenous activator of ERK1/2 in our studies to examine the effect of parallel activation of MAPK on BMP-induced C-terminal Smad phosphorylation and downstream gene expression. We found that PDGF-induced activation of ERK1/2 powerfully antag-





**Figure 8.** A, BRE-luciferase reporter gene activity in transfected PASCs in response to BMP4 (10 ng/mL) in the presence or absence of U0126 (1  $\mu$ mol/L). \* $P$ <0.05. Results are means of 3 separate experiments. Real-time PCR measurement of Id1 (B) and Id2 (C) mRNA expression in normal PASCs in response to BMP4 (10 ng/mL) in the presence or absence of U0126 (1  $\mu$ mol/L) or PDGF (10 ng/mL). Id gene expression was normalized to  $\beta$ -actin. Results represent the means of 3 normal PASC isolates. \* $P$ <0.05 compared with absence of BMP4, \*\* $P$ <0.05 compared with BMP4 alone. Id1 and Id2 promoter luciferase reporter gene activity in normal PASCs transfected with pcDNA3 or constitutively active MEK1 (CA-MEK1E) in response to BMP4 (10 ng/mL) or vehicle. \* $P$ <0.05. Results are representative of 3 separate experiments.

onized Smad1/5 C-terminal activation, inhibited nuclear accumulation of phospho-Smad1/5 and inhibited Id1 gene expression in PASCs. Furthermore, we show that the ability of PDGF to inhibit C-terminal activation is associated with increased s206 linker region phosphorylation of Smad1. PDGF is widely implicated in the process of vascular remodeling in the systemic and pulmonary circulations, and novel therapies that target the PDGF receptor kinase in PAH are under investigation.<sup>31</sup> Thus, improved Smad signaling and BMP dependent gene transcription could be 1 of the benefi-

cial effects of PDGF inhibition in PAH. Moreover, these observations provide a mechanism by which key growth regulatory pathways, acting via MAPKs, can lead to a critical reduction in BMP/Smad signaling. Genetic studies have confirmed that disease gene penetrance is less than 50% in familial PAH, and it is recognized that additional environmental or genetic factors are necessary to bring about clinical manifestation of disease. Our observations demonstrate that growth factors, such as PDGF, can further impair BMP signaling and may represent a “second hit” necessary for

disease manifestation. In support of this, we confirmed that the cells harboring mutations in BMPR-II lose the capacity to antagonize the proliferative effects of PDGF-BB.

In summary, we have demonstrated that BMP-dependent gene transcription in PASMCs depends on the integration of Smad and ERK1/2 activity. ERK1/2 activation antagonizes Smad1/5-dependent gene transcription by Smad linker region phosphorylation in the case of Id1 or may positively regulate gene transcription in the case of Id2. Mutation in BMPR-II causes a deficiency of Smad1/5 and ERK1/2 activation in PASMCs, leading to reduced transcription of Id1 and Id2. Reduced Id1 expression, caused by either deficiency of BMPR-II or inhibition of Id1 gene transcription by ERK1/2, contributes to the failure to respond to the growth suppressive effects of BMPs in PASMCs. The failure of activation of Id gene expression in mutant PASMCs may contribute to abnormal pulmonary vascular cell proliferation in familial PAH.

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### Disclosures

None.

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## Mutations in Bone Morphogenetic Protein Type II Receptor Cause Dysregulation of Id Gene Expression in Pulmonary Artery Smooth Muscle Cells: Implications for Familial Pulmonary Arterial Hypertension

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## **Supplemental Methods**

### ***Cell culture***

Cells were maintained in 10% FBS/Dulbecco's modified Eagle Medium (DMEM) and used between passage 4 and 6. The smooth muscle phenotype of isolated cells was confirmed by positive immunofluorescence with antibodies to  $\alpha$  smooth muscle actin, smooth muscle specific myosin, fibronectin, and vimentin. For proliferation studies proximal PSMCs were plated at a density of 20,000 cells/well in 24 well plates. Cells were serum starved for 24 hours and then incubated in 5%FBS/DMEM alone, in the presence of 10ng/ml PDGF-BB, BMP4 10ng/ml or co-incubated with both PDGF-BB and BMP4. Medium and ligands were replenished every 48 hours. Cells were trypsinised and counted using a trypan blue exclusion method on days 0, 4 and 6.

### ***Localization of phospho-Smad1/5 by confocal immunofluorescence***

Cells were cultured in 8-well slide chambers and after 24 hours quiescence, were incubated with BMP4 (10ng/ml) or serum free media for 1 hour. In some experiments cells were preincubated with U0126 for 30mins. Cells were then washed with Tris buffered saline (TBS) and fixed in methanol at -20°C for 10min. After further washing with TBS slides were placed in blocking buffer (10% goat serum, 1% BSA, 0.02% NaN<sub>3</sub> in 1x PBS) at room temperature for 60 min. Cells were then incubated overnight with polyclonal rabbit anti-phospho-Smad1/5(serine 463/465 1:100) at 4°C, then with fluorescent (Alexa Fluor 488) goat anti-rabbit IgG (Invitrogen) for 60 min at room temperature. Cells were counterstained with propidium iodide (Vector Laboratories, CA) mounted and phospho-Smad1/5 localization was visualized by

confocal microscopy (TCSSB Leica). The proportion of cells displaying nuclear localization of phospho-Smad1 was determined.

### ***siRNA knockdown of Id genes and BMPR-II***

Control PSMCs were plated in 24 well plates at a density of 15,000 cells per well and incubated overnight in 10%FBS/DMEM. Following 24 hours quiescence in 0.1%FBS/DMEM, cells were washed twice in Optimum I, and then incubated at 37°C in 500µL Optimum I for 3 hours. Cells were treated with either the transfection reagent alone, non-targeting scrambled siRNA with transfection reagent or siRNA for Id1, Id2 or BMPR-II with transfection reagent. The final concentration of siRNA was 10nM. Cells were incubated with the transfection complex for 4 hours at 37°C, following which the medium was added with DMEM containing 10%FBS with or without BMP4 (10ng/mL). The sequence used for Id1 siRNA was 5' AAGAGGAATTACGTGCTCTGT 3' (Biospring MBH). For BMPR-II and Id2 the siRNAs were obtained from Dharmacon (On-TargetPlus SMARTpool).

### ***Real time RT-PCR***

Total RNA was extracted from PSMCs using TRIzol reagent, then reverse transcribed using Superscript III First-strand Synthesis Super Mix (Invitrogen). Quantitative-PCR was performed using SYBR1 GreenER qPCR SuperMix and samples were run on BioRad iCycler iQ. Primers were used to allow amplification of Id1 and Id2, and normalised to  $\beta$ -actin, which was included in each sample run. Primer sequences were as follows: Id1: forward, 5'ctgctctacgacatgaacggc; reverse, 5'tgacgtgctggagaatctcca; Id2: forward 5'gacccgatgagcctgtatac; reverse,

5' ggtgctgcaggattccatct;  $\beta$ -actin: forward, 5' gcaccacaccttctacaatga; reverse, 5' gtcattctctcgcggttggc.

### **Supplemental data**

**Online Figure I.** Real time PCR of Id1 (A) and Id2 (B) gene expression following siRNA knockdown (24h) of BMPR-II in PSMCs followed by 1h treatment with BMP4 (10ng/ml). \*P<0.05 compared with siCP. DH1, Dharmafect transfection reagent alone; siB2, siRNA for BMPR-II; siCP, siControl Pool. (C) Real time PCR showing the efficiency of BMPR-II mRNA knockdown in these experiments. \*\*P<0.05 compared with siCP. All results represent the means of 3 repeat experiments.

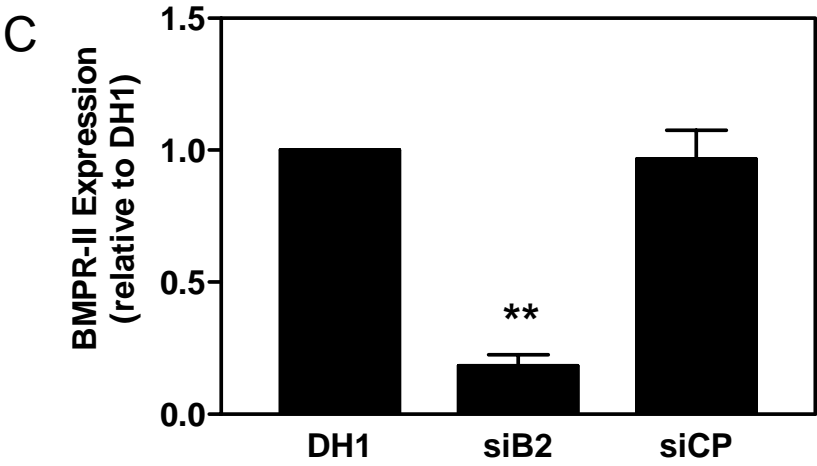
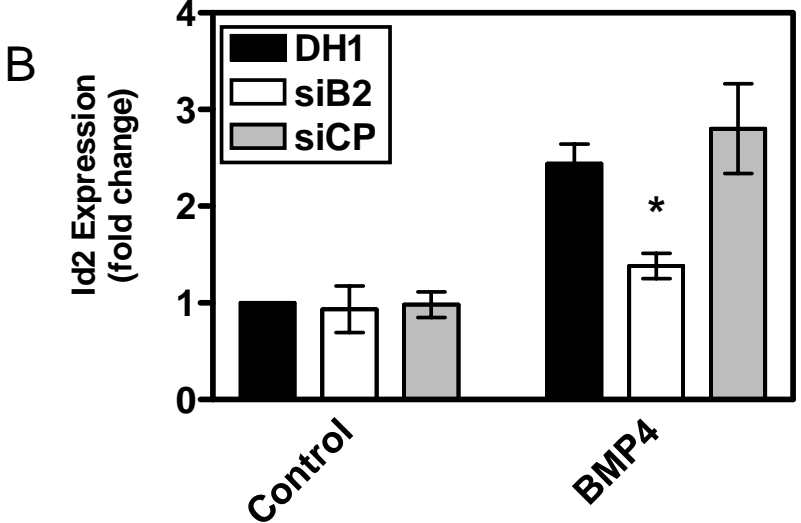
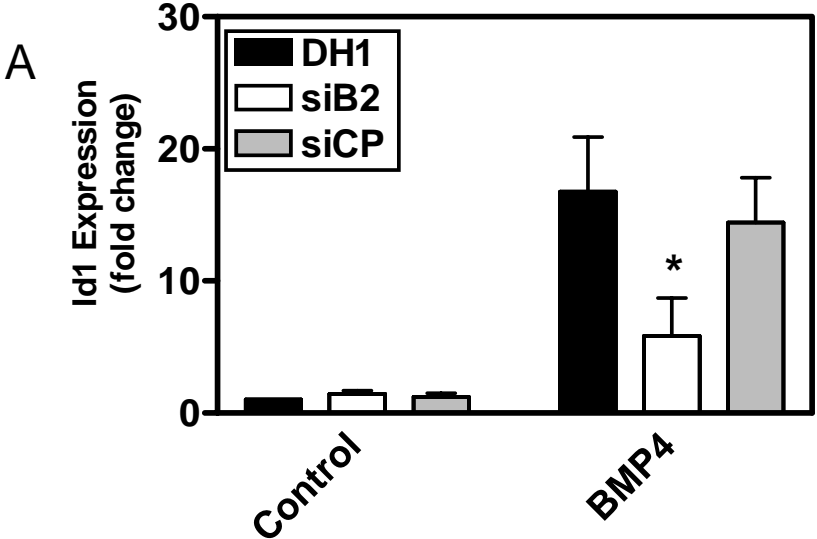
**Online Figure II.** Real time PCR for Id1 (A) and Id2 (B) mRNA expression following 48 hour incubation with siRNA for Id1 (siId1) or Id2 (siId2). Immunoblotting for Id1 (C) and Id2 (D) protein 48 hours after treatment with the corresponding siRNA. Sc, scrambled non-targeting siRNA. Blots shown are representative of n=3 separate experiment. Graphs represent mean data from separate experiments; \*P<0.05 compared with Sc.

**Online Figure III.** (A) Representative immunoblots showing s206- and s463-Smad1 phosphorylation in response to 1h incubation with BMP4 (10ng/ml) or PDGF (10ng/ml) in the presence or absence of U0126.  $\beta$ -actin was used as loading control. Quantification of s206- (B) and s463-Smad1 (C) phosphorylation and the effects of

U0126 is shown in the bar charts. Mean results are from 3 separate experiments on different cell lines. \*P<0.05 compared with paired value.

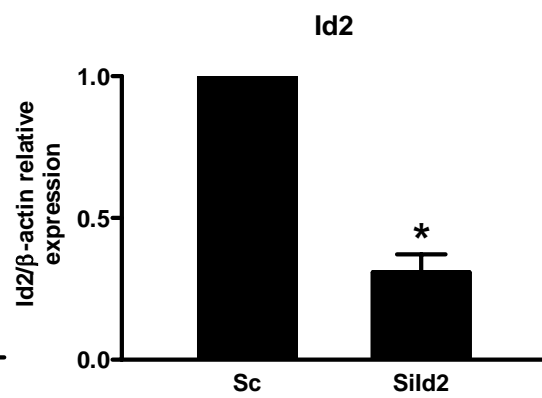
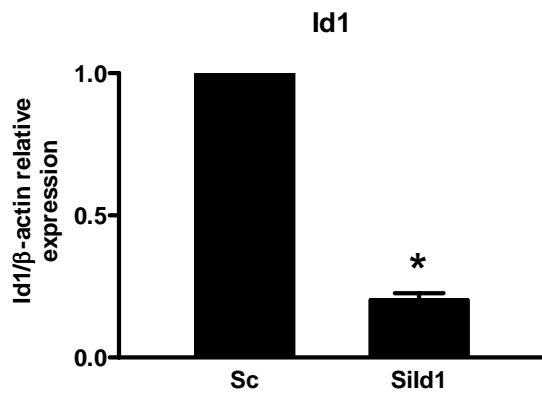
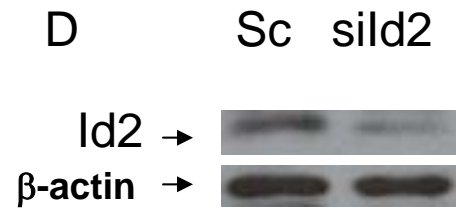
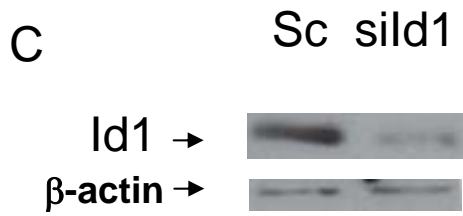
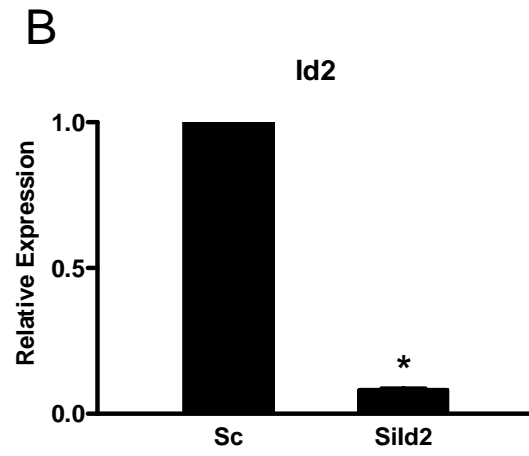
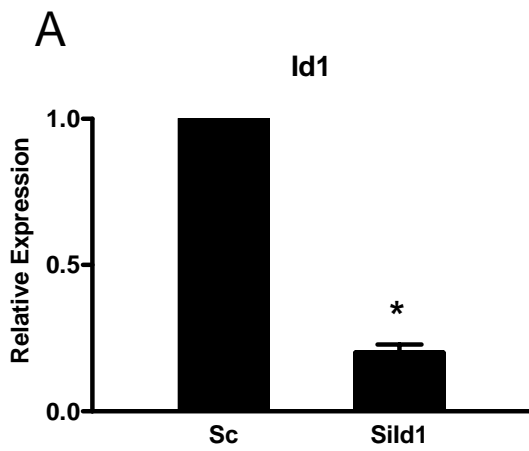
**Online Figure IV.** (A) Representative immunoblots of s463-Smad1/5 phosphorylation following BMP4 (10ng/ml) stimulation in control and mutant (C347R) PASMCs for 1h. Nuclear and cytoplasmic protein was run separately. Tubulin was used as a marker of cytoplasmic protein and histone confirmed the nuclear protein fraction. (B) Quantification of nuclear/cytoplasmic partitioning of s463-Smad1 phosphorylation in control (Con) and mutant (Mut) cells following BMP4 (B4) stimulation. Graph represents mean results from 3 different control and mutant isolates. \*P<0.05 compared with untreated conditions.

Online Figure I



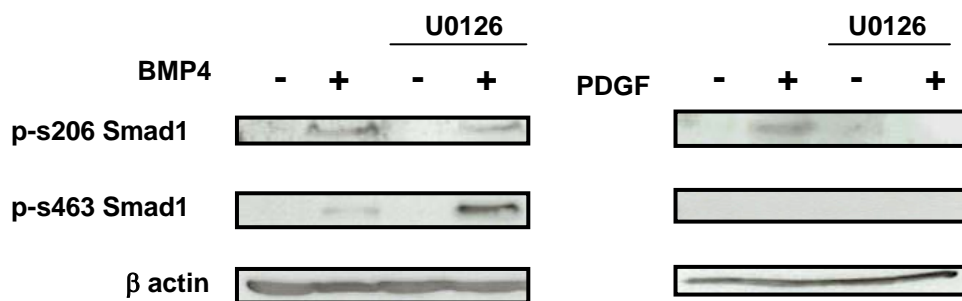


# Online Figure II

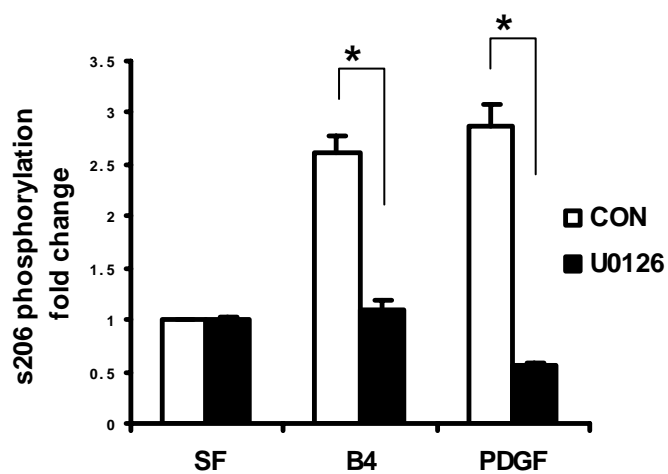


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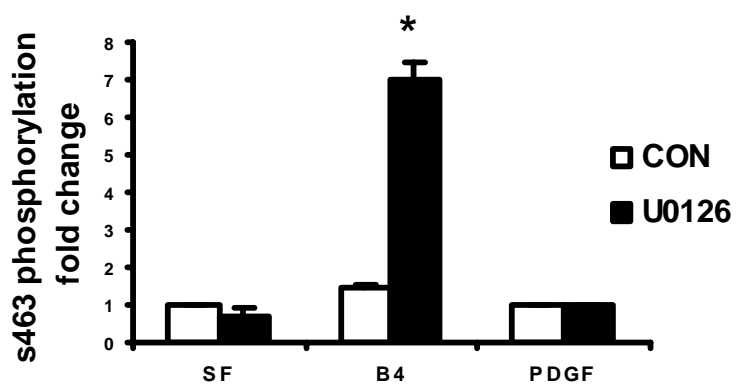
## A



## B

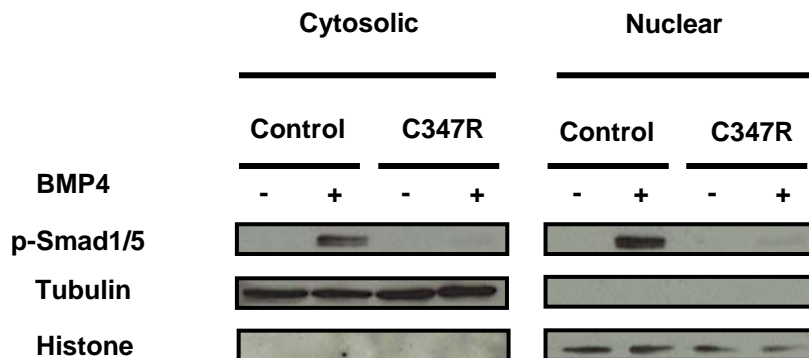


## C



# Online Figure IV

A



B

