Hypoxia Regulates Bone Morphogenetic Protein Signaling Through C-Terminal–Binding Protein 1

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Abstract—Bone morphogenetic protein receptor 2 (BMPR2) mutations have been linked to familial pulmonary arterial hypertension (PAH), but the molecular pathways leading to this severe pathology remain poorly characterized. We report that hypoxia, a paramount stimulus for the development of pulmonary hypertension, suppresses the expression of inhibitor of differentiation 1 (Id1), a downstream target of the BMPR2 pathway, in human pulmonary artery smooth muscle cells (HPASMC). This attenuation of BMP signaling by hypoxia is conveyed through a repression of the transcriptional activity of the BMP responsive element (BRE) through mechanisms involving the transcriptional corepressor C-terminal–binding protein 1 (CtBP-1) and histone deacetylases (HDACs). Concordantly, overexpression of CtBP-1 suppressed BMP signaling, whereas small interfering RNA against CtBP-1 efficiently enhanced BMP stimulation of Id1 gene expression. Scavengers of reactive oxygen species had no effect on the hypoxic regulation of Id1, but, significantly, enhancement of the intracellular NADH/NAD\(^+\) ratio mimicked the effects of hypoxia. These results indicate that attenuation of BMP signaling can occur through modulation of CtBP-1 activity by hypoxia-induced changes in the NADH/NAD\(^+\) ratio. Our findings, taken in context with the observed prevalence of pulmonary arterial hypertension associated with BMPR2 mutations, define converging molecular pathways that lead to the development of pulmonary hypertension, through either genetic or epigenetic loss of function of components of the BMP signaling pathway. (Circ Res. 2006;99:240-247.)

Key Words: hypoxia • bone morphogenetic protein • C-terminal–binding protein 1 • inhibitor of differentiation 1 • histone deacetylases • pulmonary hypertension

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor (TGF)-\(\beta\) superfamily and are multifunctional proteins that regulate proliferation, differentiation, and apoptosis in various cell types.1–4 Two BMP type I receptors (BMPR1A and BMPR1B) and a single type 2 receptor (BMPR2) have been identified in mammals, and all 3 harbor serine/threonine kinase activity.5–8 In addition, activin type 2 receptor B (ActRIIB) can also act as BMPR2. BMPR2 binds to BMP and then phosphorylates and activates BMPR1,9,10 which subsequently phosphorylates and activates the so-called receptor-activated Smad (R-Smad) proteins including Smad1, Smad5, and Smad8. The activated R-Smad proteins oligomerize with Smad4 and translocate to the nucleus where they bind to the BMP responsive elements (BRE) in the transcriptional control regions of target genes, inducing their expression. The BMP signaling pathway is negatively regulated by the inhibitory Smad proteins, Smad6 and Smad7.11–14 In addition, a number of Smad-interacting proteins have been characterized that can regulate BMP signaling.15–17

C-terminal–binding protein 1 (CtBP-1) is a transcriptional corepressor that interacts with a variety of proteins, including histone deacetylase (HDAC), Smad6, and the transcriptional repressor ZEB.14,18–28 Binding of CtBP-1 to ZEB is regulated by the nicotinamide adenine dinucleotides (NAD\(^+\) and NADH), with NADH being 2 to 3 orders of magnitude more effective.25,28 An increase in cellular NADH results in stronger interaction between CtBP-1 and ZEB and increased repressor activity of CtBP-1.25,28 By detecting changes in nuclear NAD\(^+\)/NADH ratio, CtBP-1 can function as a redox sensor for transcription that links transcription to cellular metabolism and respiration.28

Recent genetic studies have revealed that mutations in BMPR2 are associated with familial pulmonary arterial hypertension (PAH)29–35; however, the mutation may be a predisposing factor and often requires a second inciting stimulus to result in clinical disease. Hypoxia accompanies various lung diseases and is recognized as an important stimulus for nonfamilial pulmonary hypertension. Exposure to chronic hypoxia leads to pulmonary hypertension in several animal models, and these tools have been invaluable in the investigation of the pathogenesis of this disease.36–40 Nevertheless, despite significant progress in this area, the molecular mechanisms and signaling pathways that underlie the development of pulmonary hypertension remain poorly understood. In this study, we investigated the effects of...
hypoxia on BMP signaling in human pulmonary artery smooth muscle cells (HPASMC) to explore potential crosstalk between these pathways that are linked with pulmonary hypertension. We found that hypoxia suppressed BMP signaling pathways that regulate the expression of the inhibitor of differentiation 1 (Id1) gene, one of the most important target genes of BMP.41–46 Our examination of the molecular mechanisms underlying hypoxic regulation of BMP signaling defines molecular pathways that converge in inducing pulmonary hypertension through genetic (mutations) or epigenetic (hypoxia) loss of function of components of the BMP signaling pathway.

**Materials and Methods**

**Cell Culture, Hypoxia, and Chemical Treatments**
HPASMC were purchased from Cambrex (Walkersville, Md) and grown in SmGM-2 media (Cambrex) containing 5% FBS until 80% confluence under normoxia (21% O₂, 5% CO₂). Hypoxia treatment was performed in an Invivo2 400 Hypoxia Workstation (Biotrace International BioProducts, Bothell, Wash) at 0.5% O₂, 5% CO₂, 94.5% N₂. Starvation was performed in SmBG-2 media containing 0.05% FBS and 0.1% BSA. For the various treatments, HPASMC were incubated with 2 μg/mL trichostatin A (TSA) overnight to inhibit HDAC activity, 250 μmol/L cobalt chloride (hypoxia mimic), or 1 mmol/L TEMPO (reactive oxygen species [ROS] scavenger) for 1 hour in starvation medium under normoxic or hypoxic conditions. HPASMC were stimulated with recombinant human BMP-2 (R&D Systems, Minneapolis, Minn) in SmBG-2 containing 0.05% FBS and 0.1% BSA.

**Electrophoretic Mobility Shift Assay**
Preparation of nuclear extract and electrophoretic mobility shift assay (EMSA) were performed essentially as by Katagiri et al.47 See the online data supplement, available at http://circres.ahajournals.org.

**Phosphorylation of Smad1/5/8 and Smad5 Proteins**
HPASMC were up to 80% confluence and subjected to starvation overnight in normoxia or hypoxia for 24 hours. The cells were then stimulated with BMP-2 (20 ng/mL) or BMP-4 (50 ng/mL) for 0, 15, 30, and 90 minutes as indicated. After washing with PBS, the cells were lysed in RIPA buffer. Phospho-Smad1/5/8 levels were determined by Western blot analysis using an antibody recognizing phospho-Smad1/5/8.

**NADH/NAD⁺ Ratio Estimation**
Free cellular NADH/NAD⁺ ratio was estimated by enzymatically measuring the lactate and pyruvate concentrations as described previously.25,56

**Results**

**Hypoxia Suppresses BMP Signaling in HPASMC**
To investigate the effects of hypoxia on BMP signaling in HPASMC, we examined the expression of Id1, an important target gene of BMP stimulation. Western blot analysis showed that, under normoxic conditions, expression of the Id1 gene was induced 4-fold within 1 hour of treatment with BMP-2 (Figure 1a and 1b). Id1 protein was transiently induced by BMP-2, reached the highest levels within 1 to 2 hours of stimulation, and then declined to basal levels 24 hours after the treatment (Figure 1c and 1d). However, Id1 induction was significantly reduced when cells had been exposed to hypoxia (0.5% O₂, 94.5% N₂, 5% CO₂) overnight before stimulation with the same amount of BMP-2 (Figure 1a through 1d). Hypoxia did not accelerate the degradation of Id1 protein; in fact, it may have moderately increased the half-life of Id1 protein (supplemental Figure I). Consistent with protein levels, Id1 mRNA levels were increased ∼3-fold.
within 1 hour of BMP-2 stimulation as quantified by Northern blot analysis (Figure 1e and 1f). In contrast, induction of mRNA in hypoxic cells was significantly suppressed compared with normoxic cells (Figure 1e and 1f). Expression of Id1 mRNA was transiently induced by BMP-2, reaching its highest levels 1 to 2 hours after addition of BMP-2, and was diminished shortly afterward (Figure 1e and 1f). However, the half-life of Id1 mRNA was not reduced by hypoxia as demonstrated by transcription inhibition studies with actinomycin D (data not shown). These results suggest that hypoxia suppresses BMP-2–stimulated Id1 gene expression mainly at the transcriptional level.

A BMP responsive element (BRE) has been identified in the promoter region (−984 to −866) of the human Id1 gene. The BRE consists of multiple Smad-binding elements (SBE) and a GC-rich region but no hypoxia-inducible factor-1 (HIF-1) binding consensus sequence 5′-RCGGT-3′. Both SBE and the GC-rich region are required for BMP-stimulated Id1 gene expression. We investigated the effect of hypoxia on BRE activation by BMP-2 using luciferase as a reporter gene. A human Id1 promoter fragment (−984 to −866) containing the putative BRE was synthesized (Figure 2a). Adenovirus (Ad.BRE-Pr-Luc) containing BRE, SV40 basic promoter, and the luciferase reporter gene (Luc) was produced (Figure 2b), and cells were infected with these viruses. BMP-2 increased luciferase activity 4.5-fold in normoxic HPASMC infected with Ad.BRE-Pr-Luc but not in cells infected with the control virus (Ad.Pr-Luc) that contains only SV40 promoter and the Luc gene without BRE (Figure 2b). Mutation of SBE also abolished BMP-2–stimulated luciferase expression (data not shown). These results confirm that the BMP-2–induced luciferase activity observed in Ad.BRE-Pr-Luc–infected HPASMC was mediated by the BRE. In hypoxic HPASMC, BMP-2–stimulated luciferase activity was reduced by ~50% compared with normoxic cells, indicating that hypoxia suppresses BMP-induced BRE activation in these cells.

To further confirm hypoxic effect on BRE-dependent BRE activation, we performed EMSA to examine the interaction between Smad proteins and BRE. We used BRS1 (BMP-responsive sequence) containing 3 SBE, and BRS2 (the GC-rich sequence) of BRE (Figure 2a), respectively, as probes as they have been shown to interact with Smad1 protein in a BMP-dependent manner in the nuclear extract from C2C12 cells. Two major DNA/protein complexes (A and B) were formed from the incubation of BRS2 with HPASMC nuclear extract, and the formation of complex A was dramatically induced by BMP-2 in normoxic cells (Figure 2d). This induction was significantly reduced by hypoxia (Figure 2d). Cold BRS2 oligo, but not excessive nuclear factor (NF)-κB oligo, competed with complex A (Figure 2e). Furthermore, the formation of complex A was prevented by antibody against Smad1, but not by antibodies against Smad2 and TGF-β type 2 receptor (TGFβRII) (Figure 2f). Thus, for the first time we showed BMP-dependent Smad1–BRE interaction in primary HPASMC. Similar effects of hypoxia on the BMP-dependent BRS1–Smad1 interaction were also observed in HPASMC (data not shown).

**Figure 2.** Hypoxia represses BRE activation in HPASMC. a, Sequence of BRE in the human Id1 promoter. b, Schematic diagram for adenoviral vectors used for BRE activity assay. c, Fold induction of luciferase activity in normoxic and hypoxic HPASMC infected with Ad.Pr-Luc or Ad.BRE-Pr-Luc and stimulated with BMP-2 for 8 hours. Luciferase activity was measured from 4 to 5 independent experiments, normalized to β-gal activity, and presented as mean ± SD. *P<0.05 vs normoxic cells. d, Effect of hypoxia on BMP-2–mediated BRS2/protein complex formation. EMSA was performed with nuclear extract prepared from HPASMC that had been starved in normoxia and hypoxia overnight and treated with BMP-2 (60 ng/mL) for 1 hour. e, Competition analysis of BRS-2/protein complexes with cold BRS2 or NF-κB oligo. f, Formation of BRS-2/protein complexes was inhibited by antibody against Smad1, but not by antibodies against Smad2 or TGFβRII.

**Hypoxia Does Not Inhibit BMP-Dependent Smad1/5/8 Phosphorylation**

To investigate the molecular basis underlying hypoxic suppression of Id1 expression, we investigated the abundance of BMP receptors and Smad1 and phosphorylation of Smad1/5/8 in normoxic and hypoxic HPASMC. Hypoxic and normoxic HPASMC have similar levels of BMPR1, BMPR2, and Smad1 as determined by Western blot analysis (supplemental Figure II). The levels of phospho-Smad1/5/8 increased to a similar degree in response to BMP-2 and BMP-4 under both normoxic and hypoxic conditions (Figure 3a through 3c). These results indicate that hypoxia does not regulate activation of Smad1/5/8 in HPASMC. Similar levels of phospho-Smad1/5/8 were detected in nuclear extracts from BMP-2–treated normoxic and hypoxic HPASMC (supplemental Figure III).
CtBP-1 Function Is Required for Hypoxic Regulation of BMP Signaling

Recently, Smad6 has been shown to recruit the transcriptional corepressor CtBP-1 to Smad1/Smad4 complexes and to inhibit BMP-2–stimulated gene expression. Furthermore, a number of additional Smad-associated corepressors have also been identified and shown to block BMP-dependent signaling through recruiting CtBP-1 to the R-Smad/Smad4 complexes. Thus, we investigated the role of CtBP-1 in the hypoxic regulation of Id1 expression. Hypoxia did not alter the expression of CtBP-1 (Figure 3a). CtBP-1 levels were significantly downregulated by infecting HPASMC with virus expressing CtBP-1 small interfering RNA (siRNA) (Figure 4a, lanes 1 and 2) but not with control siRNA (H1 siRNA, lanes 3 and 4). Downregulation of CtBP-1 significantly attenuated the hypoxic effect on BMP-2–stimulated luciferase activity (Figure 4b), compared with cells expressing control siRNA whose CtBP-1 levels were unaffected (Figure 4a, lanes 3 and 4). In contrast, overexpression of human CtBP-1 under normoxia resulted in decreased induction of luciferase by BMP-2, compared with control cells overexpressing GFP (Figure 4c; Figure 4a, lanes 5 and 6). These results indicate that CtBP-1 regulates Id1 gene expression and is required for hypoxic suppression of BMP signaling.

HDAC Activity Is Required for Hypoxic Repression of BMP Signaling

HDAC can be recruited by CtBP-1, and a number of inhibitory regulators of BMP pathway recruit CtBP-1 and/or HDAC to the R-Smad/Smad4 complexes. To investigate whether HDAC is involved in the hypoxic regulation of these pathways, HPASMC were treated with TSA, a known HDAC-specific inhibitor. TSA did not significantly affect BMP-2–induced Id1 expression in normoxic cells (Figure 5a, lane 5). However, TSA prevented suppression of Id1 expression by hypoxia (Figure 5a, lane 6). In agreement, BRE activity was induced to similar levels in TSA-treated hypoxic and normoxic HPASMC in response to BMP-2 (Figure 5b). These results indicate that HDAC activity is required for hypoxic regulation of BMP-mediated Id1 gene expression. Furthermore, chromatin immunoprecipitation (ChIP) assay with antibody against HDAC1 indicated that hypoxia enhanced the recruitment of HDAC1 to a sequence (−1140 to −824) of Id1 promoter that contains the BRE (Figure 5c), whereas such enhancement was not seen in ChIP with negative control antibody against angiopoietin-1 (data not shown).

Cobalt Chloride Inhibits BMP Signaling in HPASMC

The repressor activity of CtBP-1 has been shown to be regulated by the cellular redox state (NADH/NAD + ra-
tio),25,26 with increased NADH/NAD\(^+\) ratio increasing repressor activity. Hypoxia is known to increase NADH/NAD\(^+\) ratio in a variety of cell types, including PASMC,25,53–55 and, similarly, the heavy metal cobalt has also been shown to increase cellular NADH/NAD\(^+\) ratio.25,56 Thus, we examined the effect of cobalt chloride on BMP signaling. As in hypoxic cells (Figure 6a, lane 4), in HPASMC pretreated with 250 μmol/L CoCl\(_2\) for 1 hour, BMP-2–stimulated Id1 induction was significantly reduced (Figure 6a, lane 5) compared with control cells (Figure 6a, lane 2). Similarly, BMP-2–stimulated luciferase induction was significantly repressed by cobalt treatment (Figure 6b). NADH/NAD\(^+\) ratios were increased by 4.4- and 3.5-fold in hypoxic and cobalt-treated cells, respectively (Figure 6c). These data suggest that cellular redox state regulates BMP pathways, and hypoxia may regulate these pathways through increasing cellular NADH/NAD\(^+\) ratio.

Effect of ROS on BMP Signaling

It has been suggested that mitochondria may function as oxygen sensors by increasing ROS generation during hypoxia.46 Previous reports have shown that mitochondrial ROS are required for hypoxic induction of gene expression.58 To determine whether hypoxia-generated ROS are involved in the regulation of BMP-2–mediated Id1 gene expression, we treated hypoxic HPASMC with the membrane permeable ROS scavenger TEMPOL (1 mmol/L) for 1 hour before stimulation with BMP-2. TEMPOL did not prevent the suppression of Id1 expression by hypoxia (Figure 7a). Additional ROS scavengers such as diphenyleneiodonium chloride (DPI) and Tiron had similar effects (data not shown). To directly examine the effect of ROS on BMP signaling, we pretreated HPASMC with 200 μmol/L H\(_2\)O\(_2\) for 1 hour to increase cellular ROS. Interestingly, opposite to hypoxia, BMP-2–stimulated luciferase induction was significantly enhanced by H\(_2\)O\(_2\) (Figure 7b).

Effect of Id1 on HPASMC Migration

To explore the biological function of Id1, we performed migration assays. Overexpression of Id1 in HPASMC caused...
Figure 8. Effect of Id1 overexpression on HPASMC migration. HPASMC were infected with adenoviruses expressing β-gal or Id1 for 48 hours. Migration was induced by incubation with PDGF (25 ng/mL) for 4 hours. Mean±SD of percent migration of three independent experiments is shown (*P<0.01 vs cells overexpressing β-gal).

a significant decrease in the migration of these cells, compared with cells overexpressing β-galactosidase (β-gal) in response to platelet-derived growth factor (PDGF) (Figure 8).

Discussion
Recent genetic studies have found an association between PAH and mutations in the BMPR2 gene. Expression of a negative dominant BMPR2 in smooth muscle cells led to the development of PAH phenotype in mice. Thus, disruption of BMP signaling in vascular cells plays an important role in the pathogenesis of PAH. Hypoxia is an important stimulus linked to pulmonary hypertension in humans and has been used in animal models to mimic the human disease.

Although hypoxia and disruption of BMPR2 signaling are important factors linked to the development of pulmonary hypertension, it is not known whether hypoxia can modulate BMP pathways. In this study, we showed that hypoxia suppresses BMP signaling and BMP-2–mediated Id1 gene expression in HPASMC. Hypoxic suppression of Id1 gene expression did not involve hypoxia-inducible factor-1 (HIF-1), a key transcription factor known to regulate the expression of several genes. Instead, the effects of hypoxia were prevented by downregulation of CtBP-1 and inhibition of HDAC activity by TSA but not by scavengers of ROS. Moreover, BMP pathways were suppressed by overexpression of CtBP-1 or treatment with cobalt and enhanced by H2O2. Thus, hypoxic regulation of the BMP signaling cascade requires CtBP-1 and HDAC activity and is independent of HIF-1 or ROS.

Our results from Smad1/5/8 phosphorylation assays indicate that activation of Smad pathways is unaffected by hypoxia, whereas CtBP-1 is critical for the hypoxic regulation of Id1 gene expression. This is evidenced by the fact that overexpression of CtBP-1 attenuated BMP signaling, whereas knockdown of CtBP-1 by siRNA prevented the hypoxic suppression of BMP signaling. It has been reported that the repressor activity of CtBP-1 is regulated by NADH/NAD+ ratios and that hypoxia increases cellular NADH/NAD+ ratios, leading to increased interaction between CtBP-1 and the repressor ZEB. Thus, we propose that hypoxia may suppress BMP signaling through increasing cellular NADH/NAD+ ratios. Indeed, we found significantly elevated NADH/NAD+ ratio on exposure of cells to hypoxia or treatment with cobalt chloride. Both hypoxia and cobalt have been shown to regulate E-cadherin promoter activity and p53-dependent transcription through increasing NADH/NAD+ ratios and CtBP-1 activity. CtBP-1 interacts with a wide variety of transcriptional repressors, including HDAC, and HDAC activity is required for the suppressor activity of CtBP-1. In concordance, we found that hypoxia enhanced recruitment of HDAC1 to BRE and inhibition of HDAC activity prevented hypoxic suppression of BMP pathways. Additional repressor(s) may be required for hypoxic suppression of BMP signaling. A number of additional Smad-associated corepressors have been identified that block BMP-dependent signaling through recruiting CtBP-1 and/or HDAC to the R-Smad/Smad4 complexes. The transcriptional repressor ZEB2 has been shown to form complexes with CtBP-1/HDAC and inhibit BMP signaling, whereas ZEB1 recruits p300/CPB and promotes BMP signaling. In addition, activation of p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) 1/2, and Akt in PASMC is transiently induced by hypoxia. The role of ZEB2 and Smad6 and these kinases in the hypoxic regulation of BMP signaling and the effect of hypoxia on ZEB1/p300 recruitment to BMP-chromatin remain to be investigated. Other possible epigenetic regulation mechanisms including potential methylation of CpG motifs immediately upstream of the Id1 SBE in response to hypoxia also remain to be explored.

Furthermore, a recent study reported that chronic hypoxia increased Id1 levels in immortalized mouse pulmonary microvascular endothelial cells and whole lung lysate of mice through inducing BMP-4 production. These differences may be cell type specific or attributable to the difference in species and culture conditions. Alternatively, the effect of BMP4 on the pulmonary vasculature could be mediated through BMPR2-independent signaling pathways. More work is needed to decipher the complex pathways mediated by the BMP family and its receptors.

HIF-1 is a well-characterized oxygen regulated transcription factor. There is a putative hypoxia responsive element (HRE) approximately 0.4 kb upstream of the BRE in the promoter of the Id1 gene, as noted by sequence analysis. However, this putative HRE is dispensable for hypoxic regulation of BMP signaling in HPASMC because the BRE in our reporter construct contains no HRE, and yet it is hypoxia responsive.

Hypoxia has been reported to produce ROS in cells, and ROS are thought to function as oxygen sensors that are required for hypoxia-induced gene expression. However, our findings suggest that they are not involved in hypoxic regulation of BMP signaling, because treatment with ROS scavengers did not block the effects of hypoxia on BMP pathways. More compelling evidence came from our findings.
that, in contrast to hypoxia, \( \text{H}_2\text{O}_2 \) enhanced the effect of BMP-2 on Id1 gene expression.

Id1 has been shown to inhibit differentiation and promote proliferation in certain cell types, and Id1 overexpression enhances tube formation and migration of endothelial cells.\(^{41–46} \) However, Id1 has been also shown to promote apoptosis in a variety of cell types both in vitro and in vivo, including cardiomyocytes.\(^{41–46} \) In addition to the inhibitory effect of Id1 on migration, we have recently found that Id1 overexpression induced apoptosis and inhibited proliferation of HPASMC (manuscript in preparation). These findings suggest that Id1 may play an important role in BMP-mediated apoptosis and inhibition of proliferation of vascular cells and that downregulation of Id1 may contribute to vascular remodeling and the development of pulmonary hypertension.

Based on our findings and the known interactions among CtBP-1, HDAC, and ZEB2, we propose a model of cross-talk between BMP signaling and oxygen sensing/hypoxia response. Hypoxia may suppress BMP-2–stimulated Id1 gene expression by increasing the repressor activity of CtBP-1, as a result of a hypoxia-induced increase in cellular NADH/NAD\(^+\) ratio, leading to the formation of inhibitory complexes, such as ZEB2/CtBP-1/HDAC. The latter bind to and inhibit the activity of R-Smad/Smad4 complexes, leading to suppression of BMP-2–mediated Id1 gene activation. This model proposes that genetic lesions of BMPR2 and hypoxia-induced attenuation of BMP signaling represent convergent pathways underlying PAH.

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**Disclosures**

None.

**References**


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Detailed Methods

Western Analysis: HPASMC were washed with PBS and lysed in RIPA buffer (50 mM Tris-Cl, pH7.4; 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1 mM EDTA) containing 1x MiniComplete (proteinase inhibitors from Roche, Minneapolis, MN). Protein content was determined using DC method (Bio-Rad, Hercules, CA). The antibodies were purchased from commercial sources: Id1, Smad1 (T-20), Smad2 (I-20), BMP1A, BMP2, angiopoietin 1, angiopoietin 2 and HDAC1 from (Santa Cruz Biotechnologies, Santa Cruz, CA), phospho-Smad 1, TGF-β type 2 receptor (TGFβRII), Smad5 and CtBP-1 from Upstate Cell Signaling Solutions Cell (Upstate, NY), Phospho-Smad1/5/8 from Cell Signaling (Beverly, MA), and β-actin from Sigma (St. Louis, MO).

Northern Analysis: Total RNA was isolated from HPASMC using RNeasy RNA isolation kit (Qiagen, Valencia, CA). Twenty µg RNA were subjected to electrophoresis in a denaturing agarose gel. RNA was transferred onto cellulose membrane, UV cross-linked and subjected to pre-hybridization in Quick-Hyb solution (Stratagene, La Jolla, CA) for 2 h. Id1 probe was prepared by reverse transcription (RT)-PCR amplification and labeled with 32P-dCTP using Prime II labeling kit (Amersham, Piscataway, NJ) and was added to the membrane followed by incubation for 4 h at 60 °C. The membrane was washed and exposed to X-film. The intensity of RNA bands was quantified using NIH image software and normalized against β-actin mRNA.

Electrophoretic mobility shift assay (EMSA): HPASMC were grown in SmGM-2 complete medium up to 80% confluence and subjected to serum starvation in normoxia or hypoxia over night. The cells were then treated with BMP-2 (60 ng/ml) for 60 min. Preparation of nucleic extract and EMSA were performed essentially as previously described by Katagiri et al,¹ except that sonicated salmon sperm DNA was added to the binding reaction instead of poly(dI:dC). BRS1 (BMP responsive sequence 1, 5’CAGGCCTGGCGTCTAACGGTCTGAGCCGCTGG
TTCAGACGCTGACACAGACCGG3′) and BRS2 (5′CATGGCGACCGCCCGCGCGCGCAGCCT3′) were used as probes in EMSA. Synthetic BRS1 and BRS2 oligos were annealed and labeled with 32P-γ-ATP and T4 polynucleotide kinase. NF-κB oligo (Promega, Madison, WI) was used as nonspecific competitor. Antibodies against Smad1, Smad2 and TGFRII were used in supershift assay. DNA-protein complexes were resolved by electrophoresis in 4% polyacrylamide gels in 0.5 x TBE (44.5 mM Tris base, 44.5 mM boric acid and 1 mM EDTA).

**Chromatin immunoprecipitation (CHIP) assay:** CHIP was performed according to the procedures provided by Upstate Cell Signaling Solutions Biotechnology. Briefly, HPASMC were exposed to hypoxia for 24 h and used for CHIP assay. CHIP was performed according to the procedures provided by Upstate Cell Signaling Solutions.

After reverse crosslinking of protein/DNA complexes with NaCl, DNA was purified by extraction with phenol/chloroform in the presence of 10 μg yeast tRNA (Sigma, St. Louis, MO), and precipitated with 2.5 vol of ethanol and 0.1 vol of 3 M sodium acetate (pH 5.2). The primers used for PCR amplification were as follows: 5′GTCTGGGTTGGGAGACTCGCAGGT3′ (sense) and 5′CGCGGCAGCTGCGAGATACGTT3′ (antisense). PCR reaction was performed in 20 μl containing 1x FailSafe PCR PreMix D (Epicentre Biotechnologies, Madison, WI), 0.25 μM each primer, 2 μl DNA sample and 1 U Taq polymerase (Qiagen, Valencia, CA). PCR reaction was analyzed by 1.6 % agarose gel electrophoresis. DNA fragment was visualized by ethidium bromide staining.

**Production of Recombinant Adenoviruses:** AdMax Adenovirus Creation System (Microbix, Ontario, Canada) was used to produce adenovirus expressing human CtBP-1 with a Flag tag (Ad.CtBP-1), β-gal (Ad.β-Gal) and GFP (Ad.GFP), respectively. The cDNA sequence encoding
Flag-CtBP-1 were cloned by RT-PCR amplification from HPASMC total RNA with specific primers: 5’GAATTCGCCACCATGGATTACAAGGATGACGACGATAAGATGGGCAGCTC GCACCTTGCTCAACAAAGGGC3’ (sense); 5’GTCGACTACAACTGGTCACTGGCGTGGTCT CTACC3’ (antisense). The cDNA clones were sequenced to ensure accuracy of the sequences. The GFP and β-Gal coding sequences were excised from the plasmids pBI-GFP (BD Biosciences, San Jose, CA) and pRSV-Gal (Promega, Madison, WI), respectively. The coding sequences were cloned into the shuttle plasmid pDC316 that harbors a MCMV promoter (Microbix). Co-transfection of QBI293A cells (Q.Biogene, Quebec, Canada) with the shuttle plasmid and viral genomic DNA produced the desired viruses through site-directed recombination.

To produce adenovirus expressing CtBP-1 siRNA (target sequence GAACTGTGTCAACAAGGAC), synthetic oligodeoxynucleotides were cloned into pSilencer3.1-H1neo (Ambion, Austin, TX) that contains the human H1 promoter and allows in vivo transcription by RNA pol III to generate siRNA. The DNA fragment containing the H1 promoter and siRNA sequence was cloned into plasmid pDC312 (Microbix). Adenovirus expressing siRNA (Ad.CtBP-1siRNA) was generated by co-infection as described above. In a similar way, adenovirus was generated expressing H1 control siRNA (Ad.H1siRNA) that does not share homology with the human genes (Ambion).

To construct the adenoviral vector (Ad.BRE-Pr-Luc) containing BRE and the luciferase reporter gene, synthetic BRE (-985 bp and -957 bp) of the human Id1 gene was cloned into plasmid pGL3promoter (Promega). The DNA fragment containing BRE and the luciferase gene was then excised and cloned into the plasmid pDC312. Recombinant adenovirus was generated by co-transfection as described above.

**Manipulation of Ctbp-1 Expression:** CtBP-1 was overexpressed in HPASMC by infection with adenovirus expressing Flag-CtBP-1 (Ad.CtBP-1). HPASMC were grown on 12-well dishes until
80% confluence and infected with Ad.CtBP-1 or Ad.GFP (MOI=5) for 24 h. To down-regulate CtBP-1, HPASMC were grown on 100 mm dishes up to 80-90% confluence and infected with Ad.CtBP-1siRNA, or Ad.H1siRNA (MOI=50) overnight. Cells were trypsinized and seeded onto 6-well dishes and incubated for 6 h. Cells were then subjected to starvation, infection with Ad.BRE-Pr-Luc and luciferase assay.

**Luciferase and β-gal Activity Assay:** HPASMC were grown on 12-well or 24-well plates up to 80% confluence and starved overnight. The cells were infected with Ad.BRE-Pr-Luc (MOI=10) and Ad.β-Gal (MOI=3) that was used as infection control and to normalize luciferase activity. Six hours later, cells either remained in normoxia or were subjected to hypoxia overnight, and then incubated with 0 or 20 ng/ml BMP-2 for an additional 8 h. Luciferase and β-gal activities were determined using the luciferase and β-gal assay kits, respectively, following the manufacturer’s instructions (Promega, Madison, WI).

**Migration assay:** Migration assays were performed using Transwell (Corning Costar Corp) 24-well tissue culture inserts with 8-µm pores. HPASMC infected with Ad.Id1 or Ad.β-Gal (MOI=10) for 48 h were trypsinized and resuspended in SmBG-2 containing 0.1% BSA at 4x10^5 cells/ml. Equal volume of cell suspension was added to the insert (0.3 ml/insert) and 0.3 ml of SmBG-2 containing 0.1% BSA and 0 or 25 ng/ml PDGF was added to the lower chamber. The cells were incubated for 4 h at 37 °C and 5% CO₂. The inserts were washed with PBS and the cells that did not transmigrate were wiped off with Q-tips. The inserts with transmigrated cells were then incubated with the substrate solution (10 mM P-nitrophenol phosphate, 10 mM sodium acetate, 0.1% Triton X-100, pH5.8) (0.45 ml/well) for 1.5 h at 37 °C. After addition of 0.05 ml 1 N NaOH to quench the reaction, OD₄₁₀ was measured using a microplate reader.
Reference List


Effect of hypoxia on Id1 protein degradation in HPASMC. HPASMC were stimulated with serum for 1 h. The cells were then replaced with pre-equilibrated normoxic (N) and hypoxic (H) starvation medium (SmBG-2 containing 0.1% BSA) and incubated in normoxia and hypoxia for additional 1, 2, 3, 4, 8 and 20 h. The cells were lysed and Id1 protein was determined by Western analysis.
Effect of hypoxia on BMP receptors (BMPR1A and BMPR2), Smad1 and angiopoetin 2 expression in HPASMC. HPASMC were incubated in normoxia (N) and hypoxia (H) for 6, 12 and 24 h. The cells were lysed and the protein levels of BMPR1A, BMPR2, Smad1 and angiopoein 2 were determined by Western analysis.
Effect of hypoxia on nuclear accumulation of phospho-Smad1/5/8 in HPASMC. HPASMC were starved overnight under normoxic or hypoxic conditions, and then stimulated with BMP-2 (20 ng/ml) for 1 h. Nuclear extract was prepared from the cells and subjected to Western analysis with antibody recognized phospho-Smad1/5/8.
HDAC inhibitor TSA prevents hypoxic suppression of BMP signaling. HPASMC infected with Ad.BRE-Pr-Luc were pretreated with TSA and then stimulated with BMP-2 for 8 h under normoxic or hypoxic conditions. Luciferase activity was measured from 3 independent experiments, normalized to β-gal activity and presented as mean±SD of RLU.