Bone Morphogenetic Protein 4 Promotes Pulmonary Vascular Remodeling in Hypoxic Pulmonary Hypertension

David B. Frank, Amir Abtahi, D.J. Yamaguchi, Suzanne Manning, Yu Shyr, Ambra Pozzi, H. Scott Baldwin, Joyce E. Johnson, Mark P. de Caestecker

Abstract—We show that 1 of the type II bone morphogenetic protein (BMP) receptor ligands, BMP4, is widely expressed in the adult mouse lung and is upregulated in hypoxia-induced pulmonary hypertension (PH). Furthermore, heterozygous null Bmp4<sup>ΔucZ<sub>+</sub></sup> mice are protected from the development of hypoxia-induced PH, vascular smooth muscle cell proliferation, and vascular remodeling. This is associated with a reduction in hypoxia-induced Smad1/5/8 phosphorylation and Id1 expression in the pulmonary vasculature. In addition, pulmonary microvascular endothelial cells secrete BMP4 in response to hypoxia and promote proliferation and migration of vascular smooth muscle cells in a BMP4-dependent fashion. These findings indicate that BMP4 plays a dominant role in regulating BMP signaling in the hypoxic pulmonary vasculature and suggest that endothelium-derived BMP4 plays a direct, paracrine role in promoting smooth muscle proliferation and remodeling in hypoxic PH. (Circ Res. 2005;97:496-504.)

Key Words: bone morphogenetic proteins  endothelial cells  hypoxic pulmonary hypertension  signaling pathways  Smad  vascular remodeling  vascular smooth muscle cell proliferation

Chronic hypoxia is the most common underlying cause of secondary pulmonary hypertension (PH) in humans. This is associated with the development of fixed defects in the pulmonary vasculature, including medial wall thickening and muscularization of the peripheral vasculature, both mimicked by a rodent model of hypoxic PH in rats and mice. Numerous studies using these experimental models provide evidence of an imbalance in the secretion of vasoactive agents and mitogens in the pulmonary vasculature, leading to structural changes in the pulmonary vasculature.

Genetic studies in patients with familial primary pulmonary hypertension (FPPH) have identified mutations in 1 of the 3 known type II bone morphogenetic protein receptors (BMP-RII), BMPR2. BMP-RII is a member of the transforming growth factor β family of receptors that acts downstream of the BMP family of ligands, and ligands interact with 2 classes of transmembrane receptors, termed type I receptors, (ALK2, 3, and 6), and the type II receptors (BMP-RII and Act-RIIA and -IIB). Ligand binding induces type I-receptor phosphorylation by type II receptors, leading to activation of downstream signaling including the classical Smad1/5/8 and the alternative p38 and extracellular signal–regulated kinase, mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and protein kinase C pathways. Evidence of a role for this signaling pathway in hypoxic PH was shown in 2 recent in vivo studies. By manipulating BMP-RII signaling through 2 different approaches, these studies demonstrated that BMP-RII can promote opposite effects on the pulmonary vasculature under different conditions.

To explore the mechanisms mediating these effects, we looked at the regulation BMP ligands and downstream signaling in a mouse model of hypoxia-induced PH. One of these ligands, BMP4, is widely expressed in the adult mouse lung and is upregulated by hypoxia in vivo. These changes are of functional significance as heterozygous null Bmp4 mutant mice show a reduction in hypoxic pulmonary vascular smooth muscle cell (VSMC) proliferation and remodeling, leading to a significant delay in the development of PH following exposure to hypoxia. These findings suggest that BMP4 plays an important role in promoting hypoxic pulmonary vascular remodeling, suggesting a more complex role for BMP signaling in the development of PH.

Materials and Methods

Quantification and Localization of BMP-Signaling Components

To assess Bmp4 expression in the Bmp4<sup>ΔucZ<sub>+</sub></sup> mouse, a modified β-galactosidase assay on trachea-perfused lungs was performed, as described. Immunohistochemistry was performed using citrate antigen retrieval, biotinylated secondary antibodies with Vectastain Elite ABC amplification (Vector), and developed with diaminobenzidine (Sigma) or 3-amino-9-ethylcarbazole reagents (Zymed). Primary antibodies used for both Western blotting and immunohistochemistry included rabbit polyclonal antibodies for phospho-Smad1/
5/8 (Upstate, catalogue No. 06702; Cell Signaling, catalogue No. 9511) and β1 (Santa Cruz, catalogue No. SC488), monoclonal anti-BMP4 antibody (Santa Cruz, catalogue No. SC12721), and a β-actin monoclonal antibody (Sigma, catalogue No. A2228) for normalization of all Westerns. Quantitative RT-PCR (Q-RT-PCR) analysis of lung RNA was performed using a Light Cycler (Roche) and primers crossing exons for Bmp2, 4, 5, 6, and 7 and β-actin (Table 1 in the online data supplement available at http://circres.ahajournals.org). Values are expressed as fold-change in the ratio of Bmp2, 4, 5, 6, or 7/β-actin mRNA copies.

For analysis of protein expression in endothelial cells (ECs), primary mouse pulmonary microvascular ECs (mPMVECs) were recovered from wild-type and Bmp4−/− mutant mice, as described,14 and cultured up to P4 in medium (EGM-2; Clonetics). Temperature-sensitive, conditionally immortalized mPMVECs were isolated in the same manner from H-2Kb-tsA58 SV40 large T Ag transgenic mice16 and were characterized by Matrigel-based capillary formation and expression of endothelial-specific markers15 (online supplemental data section No. 1 and online Figure I). Following expansion, cells were maintained at 37°C in the absence of interferon-γ for 5 days, before serum starving and incubating in 1% oxygen. BMP4 protein was detected by Western blot in cell lysates and conditioned media after pull-down using heparin Sepharose beads. Additionally, other proteins were detected with rabbit polyclonal antibodies to phospho-Smad1/5/8, Id1, and hypoxia-inducible factor (HIF)-1α (Novus Biologicals, catalogue No. NB100449) and normalized to β-actin.

Experimental PH

The Bmp4 mutant mice (Bmp4+/−), bred on an ICR background, were a gift from Bridig Hogan (Duke University, Durham, NC). Eight to ten-week old heterozygous and wild-type littermates were exposed to 10% normobaric oxygen for 4 days, 3, and 5 weeks. Right ventricular systolic pressure (RVSP), heart rates, RV weights, and lung-tissue collection were performed as described in online supplemental data section No. 2. Experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Analysis of medial wall thickening was performed as described in online supplemental data section No. 3, using a blind-coded analysis. For peripheral muscularization, sections were stained by double immunofluorescence using mouse anti–α-SM actin (Sigma, catalogue No. A5228) and rabbit anti–von Willebrand Factor (Dako, catalogue No. A0082). Peripheral vessels were defined as those small vessels distal to the terminal, muscularized bronchioles, and classified as fully muscularized, partially muscularized, or nonmuscularized.

Analysis of Cell Proliferation and Migration

Isolation of mouse pulmonary artery smooth muscle cells (mPASMCs) was performed as described previously.19 More than 95% of these cells expressed α-SM actin and γ-SM actin by immunofluorescence and were used between P1 to P9. mPASMC proliferation was analyzed by [3H]thymidine incorporation performed in quadruplicate. After 48-hour serum starvation, media were treated with either BMP ligands, a 1:2 dilution of 24-hour 1% oxygen, or normoxic conditionally immortalized mPMVEC-conditioned media with or without recombinant Noggin (Alpha Diagnostics) or neutralizing anti-BMP4 monoclonal antibody (R&D systems, catalogue No. MAB757), and cells were cultured for an additional 24 hours at 1% oxygen while labeling with [3H]thymidine. Cell-migration assays were performed using 8 μmol/L polycarbonate Transwell filter inserts (Costar) precoated with growth-factor reduced Matrigel (BD Bioscience). mPASMCs were washed and plated in serum-free media into the upper chamber, and the lower chamber was filled with serum-free media treated with the respective BMP ligands or conditioned immortalized mPMVEC-conditioned media, as described above. Plates were placed in 1% oxygen for 5 hours, fixed, and stained overnight with 2% crystal violet. After washing in PBS, cells in the upper chamber were removed using cotton buds and remaining cells counted in 5 randomly selected fields at ×200 magnification for each Transwell, repeated in triplicate.

In vivo cell proliferation was determined by double immunofluorescence using rabbit polyclonal anti–proliferating cell nuclear antigen (PCNA) (Santa Cruz, catalogue No. SC7907), and monoclonal anti–α-SM actin and nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Proliferation index was determined by counting the proportion of PCNA and actin-positive cells in ~40 small/medium-sized arterioles accompanying muscularized airways.

Statistical Analysis

For universal data analysis, the Student t test was applied for testing the unadjusted mean difference between the study groups. For multivariate data analysis, the general linear model (2-way ANOVA) with adjusted least-squares means was used for testing the adjusted mean difference between study groups. All tests of significance were 2-sided, and differences were considered statistically significant when the probability value was P<0.05. All data were expressed as means±SEM.

Results

BMP4 Is Selectively Upregulated in the Lungs of Hypoxic Mice

To evaluate the regulation of BMP ligands during hypoxic PH, we obtained lung-tissue samples from wild-type mice after 3 weeks of exposure to hypoxia. Bmp2, 4, 5, 6 and 7 mRNA levels were quantified using real-time RT-PCR. We saw a 2- to 3-fold increase in Bmp2 and Bmp4 mRNAs in the hypoxic mice compared with normoxic controls but no significant changes in mRNA levels for any of the other Bmp ligands (Figure 1A).

As BMP4 plays a vital role in lung branching and morphogenesis,19,20 we focused on the analysis of Bmp4 in hypoxic PH. Bmp4 mRNA levels rose steadily at 4 days, peaked at 3 weeks, and returned to normoxic levels after 5 weeks of exposure to 10% oxygen (Figure 1B). Analysis of BMP4 protein expression confirmed that these changes in Bmp4 mRNA were associated with a significant increase in both mature secreted and preprotein forms of BMP4 in whole-lung lysates following exposure to hypoxia for 4 days (Figure 1C, lanes 1 through 12, and 1D). We used the Bmp4lacZ−/− reporter knock-in mice17 to localize Bmp4-dependent LacZ expression in the adult mouse lung. Using this mouse, we showed that LacZ is widely expressed in EC, VSMC, and epithelial compartments of the mouse lung (Figure 1E), with prominent expression in ECs seen both in muscularized and nonmuscularized vessels throughout the lung (Figure 1F and 1G).

Impaired BMP4 Signaling in Hypoxic Bmp4lacZ−/− Mutant Mice

We used heterozygous null Bmp4lacZ−/− mice to examine the functional role of BMP4 in hypoxic PH. Heterozygous null Bmp4lacZ−/− mutant mice exhibit minor renal, craniofacial, and skeletal phenotypes on different backgrounds,21 but we observed no evidence of structural defects in the lungs or vasculature on the ICR background used for these studies. Q-RT-PCR in lung samples from Bmp4lacZ−/− mutant mice showed a 50% decrease in the basal expression of Bmp4 mRNA compared with wild-type mice, with no increase in Bmp4 expression following exposure to hypoxia at any of the time points tested (Figure 1B). Analysis of BMP4 protein confirmed that there was a loss of hypoxia-induced BMP4
expression in Bmp4lacZ/+ mutant mice, although basal expression was unchanged compared with wild-type mice (Figure 1C, lanes 13 through 24, and 1D).

We next examined the effects of hypoxia on the activation of two downstream components of the BMP-signaling pathway, the BMP-receptor–activated Smad proteins, Smad1, 5, and 8, and Id1, an inhibitory transcriptional cofactor that mediates a range of Smad-dependent responses in different cell types.22 Activation of Smad1/5/8 was detected using phospho-Smad1/5/8 antibodies from another commercial source (Cell Signaling; data not shown). Id1 was also induced in the pulmonary vasculature of hypoxic wild-type but not Bmp4lacZ/+ mutant mice (Figure 2H through 2K). Id1 was prominently expressed in the VSMCs of the hypoxic pulmonary vasculature. Specificity of this antibody was confirmed in an earlier study showing absent staining in tumor vasculature from Id1-null mutant mice.23 Loss of hypoxia-induced vascular phospho-Smad1/5/8 and expression occurred despite the fact that Bmp2 mRNA was more highly upregulated in the lungs of hypoxic Bmp4lacZ/+ mutant than wild-type mice (Figure 2L).

**Impaired Functional Responses to Chronic Hypoxia in Bmp4lacZ/+ Mice**

To explore the functional significance of these changes in BMP4 expression, we induced PH by placing wild-type and Bmp4lacZ/+ mutant mice in hypoxic chambers for 4 days, 3 weeks, and 5 weeks. Whereas we saw very little to no change in RV pressure and hypertrophy after 4 days of hypoxia, there was a marked increase in RVSP and RV hypertrophy in wild-type mice after 3 weeks of hypoxia, both of which were significantly attenuated in Bmp4lacZ/+ mice (Figure 3A and 3B). Multivariate statistical analysis indicated that neither sex nor weight of the mice had significant confounding effects on these differences (online Table II). In addition, although there was a significant increase in hematocrit in hypoxic mice...
After 5 weeks of hypoxia, there was a consistent increase in RVSP at 5 weeks in the Bmp4 mutant, this protection was not statistically significant. Whereas there was a significant increase in arterial wall thickness in wild-type mice after 3 weeks at 10% oxygen, these changes were absent in mice Bmp4 mutant mice (Figure 4A through 4G). In addition, there was a marked increase in the proportion of peripheral muscularized vessels in wild-type mice exposed to hypoxia, which was significantly reduced in the heterozygous Bmp4 mutants after 3 and 5 weeks of exposure to hypoxia, although the effect was less pronounced at 5 weeks (Figure 5A through 5E). Similar changes were observed by immunohistochemical analysis using horse radish peroxidase–conjugated antibodies to detect α-SM actin expression in the lung periphery (Figure 5F through 5I).

**Pulmonary Vascular Remodeling**

We analyzed the effects of the Bmp4 mutation on two parameters of vascular remodeling, medial wall thickening of muscular arteries, and muscularization of small, peripheral vessels.\(^2\)

Whereas there was a significant increase in arterial wall thickness in wild-type mice after 3 weeks at 10% oxygen, these changes were absent in mice Bmp4 mutant mice (Figure 4A through 4G). In addition, there was a marked increase in the proportion of peripheral muscularized vessels in wild-type mice exposed to hypoxia, which was significantly reduced in the heterozygous Bmp4 mutants after 3 and 5 weeks of exposure to hypoxia, although the effect was less pronounced at 5 weeks (Figure 5A through 5E). Similar changes were observed by immunohistochemical analysis using horse radish peroxidase–conjugated antibodies to detect α-SM actin expression in the lung periphery (Figure 5F through 5I).
ation. As previous studies have shown that proliferative effects of hypoxia occur early in the development of PH,\textsuperscript{2,3} we examined VSMC proliferation and apoptosis after 4 days of hypoxia. Although we were unable to detect any effect of hypoxia on cellular apoptosis in wild-type or heterozygous \textit{Bmp4} mutant mice by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (data not shown), we did observe an increase in the proportion of PCNA-positive pulmonary VSMCs in hypoxic wild-type mice, which was reduced in the \textit{Bmp4} \textit{lacZ}/+/H11001 mutants (Figure 6A through 6J).

**Hypoxia-Induced BMP4 Expression in Pulmonary Microvascular Endothelial Cells**

We used conditionally immortalized mouse PMVECs to evaluate the potential origin and mechanisms regulating BMP4 expression in the hypoxic lung. When grown under hypoxic conditions, these cells secrete BMP4 into the cell-culture supernatant (Figure 7A, conditioned media). Similar results were obtained using primary wild-type mPMVECs (Figure 7B, lanes 1 through 4). This response was lost in mPMVECs derived from \textit{Bmp4}^{+/+} mutant mice (Figure 7B, lanes 5 through 8), indicating that loss of hypoxia-induced BMP4 expression in \textit{Bmp4}^{+/+} mutant mice occurs cell autonomously. In addition, analysis of BMP-dependent Smad/Id1 signaling in hypoxic conditionally immortalized mPMVECs demonstrated parallel changes in phospho-Smad1/5/8, Id1, and HIF-1\alpha expression (Figure 7A, cell lysates).

**Paracrine Signaling by BMP4 Mediates EC-VSMC Interactions**

To determine whether hypoxia-induced BMP4 expression by ECs could induce paracrine effects on VSMCs, we isolated primary mPASMCs and cultured them in the presence of conditioned media from hypoxic conditionally immortalized mPMVECs. Two parameters of VSMC activation, cellular proliferation and migration, were analyzed under hypoxic conditions. In addition, to determine whether these effects were BMP dependent, experiments were performed both with and without the addition of saturating concentrations of the BMP antagonist Noggin, as previously described,\textsuperscript{23,24} or a commercially available BMP4-specific neutralizing antibody. Incubation of mPASMCs with conditioned media from conditionally immortalized mPMVECs exposed to 1% oxygen or normoxia for 24 hours demonstrated increased mPASMC proliferation and migration compared with baseline, and both responses were inhibited by addition of Noggin or the neutralizing BMP4 antibody inhibited these responses, indicating that they are BMP4 dependent (Figure 8A and 8B). Furthermore, BMP4 induced a dose-dependent increase in thymidine incorporation and migration that was only observed in early passage, cultured mPASMCs (Figure 8C through 8E). In contrast to BMP4, BMP2, which was also upregulated in the hypoxic lung, had opposite effects on cellular proliferation, but it promoted mPASMC migration (Figure 8D and 8E).

**Discussion**

These studies demonstrate that \textit{Bmp4}-dependent \textit{LacZ} expression is widely distributed in the mouse lung and that
BMP4 mRNA and protein are upregulated during the early phases of hypoxic PH. This response was lost in the Bmp4 heterozygous null mutant mice, indicating that these mice provide a useful model to study the functional effects of hypoxia-induced BMP4 expression. Furthermore, whereas we showed that the related BMP family member Bmp2 is also upregulated in the hypoxic lung, decreased BMP4 expression in the Bmp4 lacZ/H11001 mutants was associated with a dramatic decrease in phospho-Smad1/5/8 and Id1 expression in the hypoxic pulmonary vasculature. Therefore, we postulate that BMP4 plays a dominant role in regulating hypoxia-induced BMP signaling in the lung. In support of this, a reduction in hypoxic BMP4 expression in Bmp4 mutant mice attenuated pulmonary VSMC proliferation and delayed vascular remod-

Figure 5. Attenuation of hypoxia-induced peripheral vascular muscularization in Bmp4lacZ/H11001 mice. A, Degree of peripheral muscularization in wild-type and Bmp4 mutant mice (data represented as mean±SEM; wild type: normoxia, n=11, 3 weeks hypoxia, n=7, and 5 weeks hypoxia, n=8; Bmp4lacZ/H11001: normoxia, n=10, 3 weeks hypoxia, n=9, and 5 weeks hypoxia, n=7). Two-way ANOVA, P<0.05: *for all degrees of muscularization in wild-type normoxia vs hypoxia, **3-week hypoxic wild type vs Bmp4lacZ/H11001, ***5-week hypoxic wild type vs Bmp4lacZ/H11001. B through E, Representative immunofluorescence staining of lung sections for von Willebrand Factor (red) and α-actin (green) and merged (yellow) under normoxic conditions (B and D) and on exposure to hypoxia for 3 weeks (C and E) in wild-type (B and C) and Bmp4lacZ/H11001 mutant (D and E) mice. Small arrowheads indicate representative partially muscularized arteries; larger arrowheads, fully muscularized arteries; and small arrows, representative nonmuscularized arteries. F through I, Immunohistochemical peroxidase-based staining for α-actin demonstrating normal alveolar structure under normoxic conditions (F and H) and on exposure to hypoxia for 3 weeks (G and I) in wild-type (F and G) and Bmp4lacZ/H11001 mutant (H and I) mice. Images taken at ×200 magnification.

Figure 6. Hypoxia-induced pulmonary VSMC proliferation is attenuated in Bmp4lacZ/H11001 mutant. A, Proliferation index of VSMCs in wild type and Bmp4 after 4-day chronic hypoxia. B, Percentage of vessels with >1 proliferating VSMC (data represented as mean±SEM; n=6 for all 4 groups). Student t test, P<0.05: *wild-type normoxia vs hypoxia for both indices, **hypoxic wild type vs Bmp4lacZ/H11001. C through F, Representative immunofluorescence staining of lung sections for PCNA (red) and α-actin (green) in normoxic (C, D, G, and H) and after 4 days of exposure to hypoxia (E, F, I, and J) in wild-type (C through F) and Bmp4lacZ/H11001 mutant (G through J) mice. Arrowheads indicate PCNA-positive VSMCs; small arrows, PCNA-positive airway cells.
eling associated with chronic hypoxia. This delay in pulmonary vascular responses to hypoxia correlates with the kinetics of \( \text{Bmp4} \) expression during the progression of this disease. At stages where protection from the development of PH is greatest, we see the largest difference in \( \text{Bmp4} \) mRNA levels between wild-type and \( \text{Bmp4}^{lacZ/-} \) mutant mice. Furthermore, these changes in VSMC proliferation after 4 days of hypoxia are not associated with physiological changes in RV pressure, suggesting that the dominant effect of the \( \text{Bmp4}^{lacZ/-} \) mutation is on vascular remodeling rather than hypoxic vasoconstriction. This indicates that BMP4 plays a critical role in regulating the early phases of hypoxic pulmonary vascular remodeling.

These findings were unexpected, as previous studies demonstrated that loss of normal BMP-RII signaling is associated with aberrant pulmonary vascular remodeling in patients with FPPH\(^7–9\) and that interference with BMP-RII signaling in VSMCs gives rise to spontaneous PH in mice.\(^{12,13}\) As BMP-RII is 1 of the type II receptors responsible for mediating BMP4 signaling,\(^{10,11}\) we predicted that a reduction in BMP4 expression would exacerbate rather than attenuate vascular remodeling in hypoxic PH. However, these findings in hypoxic PH may bear no relation to the functional effects of \( \text{Bmp2} \) mutations in patients with FPPH, which, by definition, excludes states of chronic hypoxia. Although this is possible, an alternative explanation is that our findings suggest that the dominant effects of BMP4 on the pulmonary vasculature are mediated through BMP-RII–independent signaling pathways. Our observation that \( \text{Bmp2} \) mRNA is upregulated in the hypoxic lung and that BMP2 can exert an opposite and growth inhibitory effect on VSMCs suggest that BMP2 could be signaling through BMP-RII and that this may be opposing the effects of BMP4 under normal physiological conditions. Recent studies using human PASMC cultures indicate that alterations in BMP-receptor stoichiometry associated with \( \text{Bmp2} \) mutations can give rise to a switch from a growth inhibitory to a mitogenic response to BMP2 in patients with sporadic PPH.\(^{25}\) Additionally, there is recent in vitro evidence indicating that interference with normal type II–receptor usage can lead to a net gain in signaling by certain BMP ligands through the use of other alternative type II receptors.\(^{26}\) These findings suggest that aberrant vascular remodeling could result from alterations in the balance in cellular responses to different BMP ligands through multiple type II–receptor complexes, rather than a global loss of BMP responsiveness in patients with FPPH.

Although multiple mechanisms could be involved in regulating BMP4 expression in the hypoxic lung,\(^4,27–29\) our in vitro studies suggest that BMP4 secretion could be directly regulated by hypoxia in ECs. Given the critical role of ECs in the regulation of vascular development and remodeling,\(^{30}\) it is reasonable to propose that EC BMP4 could play a role in regulating these pulmonary vascular responses in hypoxia. The mitogenic effects of EC-derived BMP4 on VSMC proliferation are consistent with this hypothesis but are in contrast with earlier studies demonstrating growth inhibitory effects of BMPs on cultured VSMCs.\(^{31–33}\) This suggests that the cellular origin of VSMCs may play a critical role in defining the type of response to BMP4 in vitro and that discrepancy with previous reports is likely to reflect differences in cell-culture conditions and passage number in our primary cultures.\(^{35,36}\) In addition to these effects, we demonstrate that BMP4 induces migration of cultured VSMCs. Previous studies indicate that increased migration of VSMCs plays an important role in systemic vascular remodeling following
inhibited VSMC migration,38 indicating that BMP signaling may play a role in regulating VSMC migration in other vascular beds.

Our in vivo studies show that there is a marked reduction in hypoxia-induced Smad1/5/8 phosphorylation and expression of Id1 in the pulmonary vasculature of Bmp4−/− mutant mice. As Id1 is a direct target of Smad-dependent BMP signaling,22 this suggests that EC-derived BMP4 could be exerting both autocrine and paracrine effects in the hypoxic pulmonary vasculature. Furthermore, Id1 plays a critical role in promoting cellular proliferation in a variety of cell types and has been shown to mediate BMP-dependent EC migration.22,39 On this basis, we propose that BMP4-dependent activation of the Smad/Id1 axis may be involved in promoting hypoxic vascular remodeling.

Taken together, our findings indicate that hypoxia-induced BMP4 expression by ECs promotes VSMC proliferation and early remodeling events in hypoxic PH. Although further studies are needed to establish the signaling pathways mediating these events, these findings are of importance as chronic hypoxia associated with parenchymal lung disease is the most common underlying cause of PH in humans.1 Hence, selective inhibition of BMP4 may present a useful therapeutic strategy to attenuate aberrant pulmonary vascular remodeling in patients with hypoxia-induced PH.

Acknowledgments

This research was funded through the Vanderbilt University Interdisciplinary Discovery Grant program. We thank John Newman for helpful advice, Brigid Hogan for helpful advice, Brigid Hogan for helpful advice, Brigid Hogan for helpful advice, Brigid Hogan for helpful advice, and John Penn for use of hypoxia chambers.

References


Bone Morphogenetic Protein 4 Promotes Pulmonary Vascular Remodeling in Hypoxic Pulmonary Hypertension

David B. Frank, Amir Abtahi, D.J. Yamaguchi, Suzanne Manning, Yu Shyr, Ambra Pozzi, H. Scott Baldwin, Joyce E. Johnson and Mark P. de Caestecker

Circ Res. 2005;97:496-504; originally published online August 11, 2005;
doi: 10.1161/01.RES.0000181152.65534.07

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/97/5/496

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2005/08/11/01.RES.0000181152.65534.07.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Supplementary data

#1 Isolation and characterization of conditionally immortalized PMECs. Pulmonary microvascular endothelial cells were isolated as previously described. Over 95% of these cells express both endothelial-specific markers, VEGFR-2 and CD-31. Immortalized endothelial cells were maintained in EGM-2 media (Clonetics) supplemented with IFN-gamma and were switched from 33°C to 37°C and allowed to grow for at least 4 days in the absence of IFN-gamma, demonstrating an endothelial, cobblestone appearance. Capillary-like formation was analyzed as described. Briefly, 96 well plates were coated with 50 µl of Matrigel, and incubated 30 min at 37°C. Serum starved immortalized endothelial cells (grown at 37°C for 4 days) (1 × 10⁴) were plated over solidified Matrigel in 200 µl serum free medium. Capillary-like structures (determined by the ability of endothelial cells to sprout, branch and form ring-like structures) were recorded hourly for a period of 10 hours, and representative images taken 3 hours after plating are shown.

#2 RV pressure studies and harvesting of RV and lung tissue samples. Closed chest measurements of RVSP and heart rate were obtained in lightly anaesthetized (ketamine/xylazine) mice by inserting a trans-diaphragmatically pressure gauge needle into the right ventricle via an upper abdominal incision. The chest cavity was opened, and blood samples collected by cardiac puncture for measurement of hematocrit. The left lung was clamped, excised, and snap frozen in liquid nitrogen for subsequent RNA and protein analysis, and the trachea then cannulated, the right lung inflated with 10% formalin at 23 cm of water. The heart was then excised, and the free right ventricular wall was removed from the left ventricle and septum. After drying heart
sections overnight at 55 °C, right ventricular mass was expressed as the ratio of the right ventricular dry weight divided by the left ventricle plus the septum.

#3 Vessel morphometry. Medial wall thickening was analyzed using a blind-coded analysis by a trained pulmonary pathologist (J.J.), as previously described. Briefly, muscularized arteries were defined by location adjacent to an accompanying airway and the presence of two circumferential elastic laminae. Using elastin-stained tissue sections, external arterial diameters (ED) were determined by measuring the trans luminal distance between external elastic laminae. If the vessel was oval, measurements are taken at the widest diameter perpendicular to the long axis, and only those cross-sections of vessels with the widest diameter less than twice the shortest diameter were included in the studies. The distance between the internal and external elastic laminae was measured on each side of the artery lumen at the same point where the ED measurement is assessed (M1 and M2). Percent media of each vessel was assessed as [M1+M2/ED] X 100. All arteries meeting these criteria were measured. The accompanying airway for each artery was defined as the one within the same broncho-vascular connective tissue and of approximately the same diameter, and its type was noted (small bronchiole (B), terminal bronchiole (T), or respiratory bronchiole (R)).

References


**Supplementary Table 1.** Primers and PCR products.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmpr2</td>
<td>ggcagcaagcacaatca</td>
<td>tgcctctttgcaaggt</td>
<td>770bp</td>
<td>4</td>
</tr>
<tr>
<td>Acvr2</td>
<td>cggggataaggagctgtgc</td>
<td>gaacaagtcaggagttagg</td>
<td>525bp</td>
<td></td>
</tr>
<tr>
<td>Acvr2b</td>
<td>cgggagtgcatctactaca</td>
<td>cctcatggatgctccatcg</td>
<td>440bp</td>
<td></td>
</tr>
<tr>
<td>Bmp2</td>
<td>ttttgggacagctgctcag</td>
<td>tctctctaatgggcaactt</td>
<td>249bp</td>
<td></td>
</tr>
<tr>
<td>Bmp4</td>
<td>ggctcccaagaatcatggac</td>
<td>aagcagcttctacttggtc</td>
<td>467bp</td>
<td></td>
</tr>
<tr>
<td>Bmp5</td>
<td>gttgtggggctgttc</td>
<td>ttccctctcacaata</td>
<td>421bp</td>
<td>5</td>
</tr>
<tr>
<td>Bmp6</td>
<td>agtcctttcttctcgag</td>
<td>cctcagccgcttcac</td>
<td>578bp</td>
<td>5</td>
</tr>
<tr>
<td>Bmp7</td>
<td>gacgcaaccccaagtgg</td>
<td>tctggagctggtgctttg</td>
<td>376bp</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>aacggccagtgctcataatgtg</td>
<td>aagggggcccagactcattg</td>
<td>372bp</td>
<td>6</td>
</tr>
</tbody>
</table>

**Supplementary Table 1.** BMP ligand and receptor primer sequences used in quantitative RT-PCR.
**Supplementary Table 2.** Multivariate statistical data analysis on 3-week hypoxic mice.

<table>
<thead>
<tr>
<th>Variable</th>
<th>RVSP (mm Hg)</th>
<th>RV/LV+S (Ratio)</th>
<th>Hematocrit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED*</td>
<td>P-value</td>
<td>ED*</td>
</tr>
<tr>
<td>Normoxia vs. Hypoxia</td>
<td>15.32</td>
<td>&lt;0.0001</td>
<td>0.092</td>
</tr>
<tr>
<td>Wild type vs. <em>Bmp4</em>^lacZ/+</td>
<td>-6.27</td>
<td>0.0037</td>
<td>-0.037</td>
</tr>
<tr>
<td>Male vs. Female</td>
<td>-2.27</td>
<td>0.4096</td>
<td>-0.0119</td>
</tr>
<tr>
<td>Weight (Continuous)</td>
<td>-0.05</td>
<td>0.8709</td>
<td>-0.0034</td>
</tr>
</tbody>
</table>

**Supplementary Table 2.** Multivariate analysis using the General Linear model to adjust for collective variables in RVSP and RV/LV+S. ED* = Estimated Difference
Supplementary Figure 1. **A,** Immortalized endothelial cells were switched from 33C to 37C and allowed to grow for at least 4 days in the absence of IFN-gamma. Note the typical cobblestone shape of endothelial cells grown as indicated above. **B,** Endothelial cells [grown as indicated in (A)] were serum starved for 24 hours and then plated over solidified Matrigel. Capillary-like structures (determined by the ability of endothelial cells to sprout, branch and form ring-like structures) were recorded hourly for a period of 10 hours, and representative images taken 3 hours after plating is here shown.