Pulmonary Hypertension in Transgenic Mice Expressing a Dominant-Negative BMPRII Gene in Smooth Muscle

James West, Karen Fagan, Wolfgang Steudel, Brian Fouty, Kirk Lane, Julie Harral, Marloes Hoedt-Miller, Yuji Tada, John Ozimek, Rubin Tuder, David M. Rodman

Abstract—Bone morphogenetic peptides (BMPs), a family of cytokines critical to normal development, were recently implicated in the pathogenesis of familial pulmonary arterial hypertension (PAH). The type-II receptor (BMPRII) is required for recognition of all BMPs, and targeted deletion of BMPRII in mice results in fetal lethality before gastrulation. To overcome this limitation and study the role of BMP signaling in postnatal vascular disease, we constructed a smooth muscle–specific transgenic mouse expressing a dominant-negative BMPRII under control of the tetracycline gene switch (SM22-tet-BMPRII<sup>delx4</sup> mice). When the mutation was activated after birth, mice developed increased pulmonary artery pressure, RV/LV+S ratio, and pulmonary arterial muscularization with no increase in systemic arterial pressure. Studies with SM22-tet-BMPRII<sup>delx4</sup> mice support the hypothesis that loss of BMPRII signaling in smooth muscle is sufficient to produce the pulmonary hypertensive phenotype. (Circ Res. 2004;94:1109-1114.)

Key Words: artery ■ bone morphogenetic peptide ■ hypertension ■ smooth muscle ■ vascular

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responsive luciferase construct. Every well also received a β-galactosidase expression plasmid, for normalization to transfection efficiency. Whereas transfection efficiency was only 15% to 20%, because the two plasmids were mixed and transfected together, cells expressing the reporter also expressed the mutant BMP, and thus the luciferase measurements were not significantly affected by transfection efficiency. Forty-eight hours after transfection, either recombinant BMPRII delx4 mutant gene is transcribed.

Results
Validation of Transgene Function and Tissue Distribution
To confirm that the BMPRII delx4 mutation encoded a dominant-negative receptor, we coexpressed BMPRII delx4 along with a second expression plasmid encoding a SMAD response element driving the luciferase reporter gene to assess BMP receptor activity. As Figure 2 shows, cells expressing the delx4 mutation had markedly reduced SMAD activity, indicative of a dominant-negative phenotype. To validate use of the SM22 promoter, SM22-tet-luciferase mice were generated and, as Figure 2 shows, when the double transgenic progeny were fed doxycycline, they demonstrated increased luciferase activity in both pulmonary (lung) and systemic (aorta) beds. Cellular distribution was evaluated by generating SM22-tet-lacZ transgenic mice. Figure 2 shows an example of lacZ immunostaining in these mice, which revealed immunoreactive protein in the media of the vasculature. However, the level of intensity of immunostaining was weak, particularly in small vessels, indicating that the 488-bp SM22-α promoter sequence produced a relatively low level of expression. Normal sized litters resulted from the SM22-rTA X TetO7-BMPRII delx4 cross with the expected proportion of double transgenic progeny. The resulting double transgenic mice, which we have termed SM22-tet-BMPRII delx4, were assayed at age 8 weeks for transgene expression using quantitative RT/PCR. As Figure 2 shows, dominant-negative BMPRII RNA was detected in the lungs of SM22-tet-BMPRII delx4 mice fed doxycycline from birth, with little expression detected in littersmates not fed doxycycline.

Phenotyping SM22-tet-BMPRII delx4 Mice
The cardiovascular phenotype of SM22-tet-BMPRII delx4 mice was evaluated using in vivo hemodynamic measurements. The Table shows average data for hemodynamic parameters. Heart rate and LV systolic pressure did not differ in doxycycline-fed and -unfed mice. RV and LV diastolic pressure was near zero in all mice (data not shown). As Figure 3 shows, right ventricular (RV) systolic pressure was nearly 2-fold increased in doxycycline-fed transgenic mice, compared with non–doxycycline-fed littersmates. Measurement of RV relative weight confirmed the presence of sustained pulmonary hypertension, with a 30% increase in RV/LV + septum weight in doxycycline-fed transgenics. There was a modest increase in hematocrit in doxycycline-fed transgenic mice, although not of a magnitude capable of increasing blood viscosity or pressure. To evaluate the effect of hypoxia, arterial blood gas studies in mice spontaneously breathing room air were performed. Arterial PO2 (PaO2) did not differ between groups (dox-, 74 ± 5 versus dox+, 78 ± 3 mm Hg; P = NS, n = 4), and PCO2 and pH were normal (data not shown). To evaluate the possibility that mice expressing BMPRII delx4 were more susceptible to the mild effects of hypoxia in Denver (PaO2 75 to 80 mm Hg versus 110 to 120 mm Hg at sea level), two pregnant double transgenic mice were placed into hyperbaric chambers that maintained normal sea level ambient pressure...
RV systolic (Denver), mm Hg 32.7

LV systolic, mm Hg 110

Heart rate, bpm 266±43
262±25

LV systolic, mm Hg 110±5
116±5

RV systolic (Denver), mm Hg 32.7±1.3
55.1±2.2*

RV systolic (sea level), mm Hg ND
44.0±2.0† (n=10)

Hematocrit, % 38.3±2.4
43.9±1.2‡

Values are mean±SEM. *P<0.01 from Denver−Dox; †P<0.01 from Denver+Dox; ‡P<0.05 from −Dox. ND indicates not determined.

Discussion

Reports that mutations in BMPRII were associated with selective pulmonary arterial hypertension in humans suggested a link between BMP signaling and the occurrence of the disease but did not establish whether mutations were sufficient to produce the disease phenotype or simply permissive. Further, as the mutations were somatic and BMPRII is ubiquitously expressed, they did not establish the relevant cell types in which abnormal BMP signaling leads to the disease phenotype.

The first goal of our study was to test the hypothesis that abnormal BMP signaling resulting from expression of a loss-of-function mutation was sufficient to produce the disease phenotype and, consistent with this hypothesis, SM22-

and PaO2 during pregnancy and weaning of 10 pups that were raised in the chambers until 8 weeks of age, when they were catheretered. The Table shows that in these doxycycline-fed transgenic mice, RV systolic pressure was intermediate to Denver altitude doxycycline-fed and control mice. RV/LV ratio was also intermediate, being 0.315 in sea level doxycycline-fed mice versus 0.360±0.013 in Denver altitude doxycycline-fed mice and 0.242±0.023 in control mice (P<0.01 all groups).

To evaluate structural abnormalities, we performed immuno-histochemistry, identifying muscular pulmonary arteries by α-SMC actin staining and assessing the degree of muscularization by quantitative morphometry. General aspects of lung development were unaffected by expression of the transgene, with normal-appearing alveoli and airways, indicating that unlike TNF-α transgenic mice and Fawn-hooded rats, which also develop pulmonary hypertension, loss of lung tissue and abnormal development are not the reason for the pulmonary hypertensive phenotype in these mice.15,16

Using quantitative morphometry, we found increased medial thickness of muscular pulmonary arteries in doxycycline-fed SM22-tet-BMPRIIΔdelx4 mice. In addition, there was increased muscularization of small pulmonary arteries at the level of alveolar ducts. These abnormalities recapitulate one of the primary lesions seen in humans with PAH and suggest that an effect of loss of SMC BMPRII signaling is the development of increased muscularization of pulmonary arteries, leading either directly or via associated vasoconstriction to increased pulmonary vascular resistance. Figure 4 demonstrates the morphological findings in SM22-tet-BMPRIIΔdelx4 mice.

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tet-BMPRIII delx4/H11001 mice developed pulmonary hypertension by 8 weeks of age. Like other type II receptors in the TGFβ superfamily, signaling function of BMPRII requires binding to ligand, dimerization with, and phosphorylation of a type I receptor and, finally, phosphorylation and activation of a signaling SMAD.17 The mutation we chose for generation of the transgenic has a premature stop codon with truncation early in the kinase domain. Functionally the mutation resulted in dominant-negative inhibition of receptor kinase activity (as assessed by the inability of BMP to activate SMAD signaling). Recently, it was also reported that the N-terminal portion of BMPRII binds to, and inactivates the cytoskeletal regulator, LIM-kinase.18 On dimerization with a type I receptor, BMPRII releases LIM-kinase, allowing it to phosphorylate its substrates. Although we did not test this property directly, overexpression of delx4/H11001 would also function as a dominant-negative in this regard, as endogenously expressed wild-type BMPRII that did not bind BMPRI (due to competition from BMPRII delx4/H11001) would continue to inactivate LIM-kinase. Therefore, although our studies support the hypothesis that loss-of-function of BMPRII produces pulmonary arterial hypertension, additional studies will be required to determine if defective receptor kinase activity, inactivation of LIM-kinase, or a combination of the two are responsible for the phenotype.

While our studies support the hypothesis that a mutation resulting in sufficient loss of BMPRII function is capable of producing the disease phenotype, they do not rule out the possibility that disease occurrence in individuals with less severe BMPRII mutations, such as haploinsufficiency, requires the presence of an environmental or genetic costimulus. Consistent with the hypothesis that mutations in BMPRII can synergize with environmental factors, SM22-tet-BMPRII delx4/H11001 mice raised in Denver developed more severe pulmonary hypertension than those raised at sea level PAO2, although significant pulmonary hypertension was seen even in the “low altitude” group. This suggests that, like humans with idiopathic PAH, even mild hypoxia exacerbates the phenotype of SM22-tet-BMPRII delx4/H11001 mice.19

The second goal of our study was to test the hypothesis that inducing abnormal BMPRII function selectively in smooth muscle was sufficient to produce the disease phenotype, and our findings support that hypothesis as well. BMPRII is expressed in a number of lung cell types, including smooth muscle, endothelium, epithelium, and macrophages.20 Prior studies showed that BMPs could control smooth muscle cell proliferation in vitro.21,22 Therefore, we chose to target the mutation to smooth muscle. Morphometric analysis showed that there was increased medial smooth muscle thickness and a tendency for distal muscularization of small pulmonary arteries. These findings recapitulate one of the pathological hallmarks of PAH. However, the magnitude of the change was small, relative to the large increase in pulmonary artery pressure and, unlike advanced PAH, no intimal lesions were seen. A potential explanation for this paradox is the possibility that a component of the pulmonary hypertension in 8-week-old SM22-tet-BMPRII delx4/H11001 mice was due to enhanced vasoconstriction. This is consistent with a two-phase hypothesis for the pathogenesis of PAH in which early
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Figure 4. Morphometric phenotyping of SM22-tet-BMPRII<sup>delx4+</sup> transgenic mice. A, Hematoxylin and eosin staining of lung from an SM22-tet-BMPRII<sup>delx4+</sup> transgenic mouse fed doxycycline from birth to age 8 weeks (20× objective). B indicates the lumen of a bronchus; AD, alveolar ducts. Remainder of the field shows individual alveoli. Lung morphology is grossly normal. B, Serial section stained with anti-SMC α-actin to identify small pulmonary arteries. Arrows point to the small, precapillary pulmonary arteries identified by α-actin staining that were used for morphometric analysis. C, Quantitative analysis of lung development. Number of alveoli per high power field and the number of α-actin-positive vessels per alveoli were compared between doxycycline-fed and -unfed transgenic mice. No difference was seen, confirming relatively normal lung development. D, Quantitative analysis of pulmonary artery muscularity. An increase in medial thickness of muscular pulmonary arteries from 46% to 54% of total vessel diameter was found (P<0.05). In addition, the number of fully muscularized (FM) precapillary pulmonary arteries and the proportion of precapillary pulmonary arteries that were fully muscularized (versus partially muscularized, PM) tended to be increased in doxycycline-fed transgenic mice (P<0.06).

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References


Disease is characterized by enhanced vasoconstriction and minimal remodeling and later disease by progressive remodeling and little vasoconstriction. Further studies will be needed to define the mechanisms underlying the increase in tone and determine if remodeling becomes more pronounced as the mice age.

Interestingly, even though the SM22 promoter we used resulted in expression in systemic as well as pulmonary smooth muscle, phenotypic abnormalities were only seen in the lung. Although we were not able to quantify the relative amount of mutant receptor protein expressed in systemic versus pulmonary resistance arteries, luciferase expression in the aortas of SM22-tet-luc mice suggested that, consistent with the literature on SM22 expression, the promoter was active in systemic arteries. Thus, it appears that under our experimental conditions, BMP signaling played a more significant role in maintaining normal tone and structure of the pulmonary than systemic circulation. It is possible that in other circumstances, such as systemic atherosclerosis, BMP signaling may modulate systemic vascular disease as well. Although we do not have an explanation for the pulmonary vascular specificity of the phenotype, it is consistent with the lack of systemic vascular disease in patients with pulmonary arterial hypertension and suggests that BMP signaling plays a unique role in controlling tone and structure in the pulmonary circulation.

Although our studies support the hypothesis that BMPRII mutations are sufficient to produce pulmonary arterial hypertension and that the mutation need only be expressed in smooth muscle to produce the phenotype. Furthermore, the lack of a major systemic vascular phenotype in these mice suggests that BMPRII signaling is uniquely important in the pulmonary circulation. SM22-tet-BMPRII<sup>delx4+</sup> mice should provide a useful model to study the molecular and physiological abnormalities in pulmonary arterial hypertension and facilitate the discovery and preclinical testing of potential new therapies.


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