Bone morphogenetic protein type 2 receptor gene therapy attenuates hypoxic pulmonary hypertension

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1Lang Research Laboratory, Royal Adelaide Hospital, 2Hanson Institute and 3University of Adelaide, Adelaide, South Australia, Australia; 4Department of Anesthesiology, University of Illinois at Chicago, Chicago, Illinois; 5Division of Human Gene Therapy, University of Alabama at Birmingham, Birmingham, Alabama; and 6Department of Medicine, University of Cambridge School of Clinical Medicine, Addenbrooke’s and Papworth Hospitals, Cambridge, United Kingdom

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REYNOLDS AM, Xia W, Holmes MD, Hodge SJ, Danilov S, Curiel DT, Morrell NW, Reynolds PN. Bone morphogenetic protein type 2 receptor gene therapy attenuates hypoxic pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 292: L1182–L1192, 2007. First published February 2, 2007; doi:10.1152/ajplung.00020.2006.—Idiopathic pulmonary arterial hypertension (PAH) is characterized by proliferation of pulmonary vascular endothelial and smooth muscle cells causing increased vascular resistance and right heart failure. Mutations in the bone morphogenetic protein receptor type 2 (BMPR2) are believed to cause the familial form of the disease. Reduced expression of BMPR2 is also noted in secondary PAH. Recent advances in the therapy of PAH have improved quality of life and survival, but many patients continue to do poorly. The possibility of treating PAH via improving BMPR2 signaling is thus a rational consideration. Such an approach could be synergistic with or additive to current treatments. We developed adenoviral vectors containing the BMPR2 gene. Transfection of cells in vitro resulted in upregulation of SMAD signaling and reduced cell proliferation. Targeted delivery of vector to the pulmonary vascular endothelium of rats substantially reduced the pulmonary hypertensive response to chronic hypoxia, as reflected by reductions in pulmonary artery and right ventricular pressures, right ventricular hypertrophy, and muscularization of distal pulmonary arterioles. These data provide further evidence for a role for BMPR2 in PAH and provide a rationale for the development of therapies aimed at improving BMPR2 signaling.

hypoxia; growth substances; endothelium

IDIOPATHIC PULMONARY ARTERIAL HYPERTENSION (PAH) is a fatal disease characterized by abnormal proliferation of pulmonary vascular endothelial and smooth muscle cells (16). The disease causes progressive dyspnea and right heart failure. Recent advances have identified the bone morphogenetic protein (BMP) pathway as having particular importance in PAH pathogenesis (3). Many cases of familial PAH have now been shown to be related to heterozygous mutations in the BMP receptor type 2 (BMPR2), a member of the transforming growth factor (TGF)-β superfamily of receptors (13). Decreased expression of BMPR2 has also been seen in secondary pulmonary hypertension, suggesting this pathway might also be important in the pathogenesis of a variety of common clinical situations in which pulmonary hypertension is a feature (1). The described BMPR2 mutations predict a downregulation of BMP signaling which, in turn, may lead to abnormal proliferative responses in pulmonary vascular cells. Ex vivo analysis of pulmonary smooth muscle cells from patients with familial PAH have confirmed abnormal proliferative responses to BMP ligands and TGF-β (13).

BMP signaling involves ligand-initiated heterodimerization of cell surface BMPR2 and BMPR1 (A or B); activation of the latter then phosphorylates a series of receptor-regulated (R-) SMAD proteins (e.g., Smad1, 5, and 8) that in turn complex with the common partner Smad (co-Smad, Smad4) and translocate into the nucleus to regulate target gene transcription (34). Dysfunctional SMAD signaling has been seen in PAH (35).

The importance of BMPR2 in pulmonary hypertension has recently been supported by transgenic mouse studies. West et al. (32) developed a mouse in which a dominant negative BMPR2 receptor is expressed under the control of a tetracycline-responsive, smooth muscle promoter cassette. When fed tetracycline, these mice developed pulmonary hypertension, and their propensity to do so was increased by mild hypoxia. Heterozygous BMPR2 knockout mice developed pulmonary hypertension in response to an inflammatory challenge mediated by intratracheal delivery of an adenoviral (Ad) vector carrying the gene for lipoxigenase, whereas genetically intact mice did not (24). Interestingly, however, these mice were relatively resistant to the pulmonary vascular remodeling effects of hypoxia (2), and a separate study has shown that mice deficient in one of the BMPR2 ligands, BMP4, were also resistant to hypoxia-induced pulmonary hypertension (4). Takahashi et al. (26) recently showed that hypoxia led to an early upregulation of the ligand BMP2, with a later downregulation of the BMPR2 receptor.

In the current study, we sought to further investigate the role of the BMPR2 pathway in the pathogenesis of pulmonary hypertension, and more specifically to determine whether this pathway could be exploited as a potential therapy. Thus, we developed Ad vectors containing the gene for wild-type BMPR2, validated the construct for functionality in vitro, and then determined the impact of vector administration on development of pulmonary hypertension and vascular remodeling in vivo. We used the rat model of hypoxic pulmonary hypertension in view of the indication from recent studies that interrelation between hypoxia and BMPR2 signaling exists (26, 32). For the in vitro studies, we have capitalized on our previously developed Ad vector targeting strategy whereby we...
link the Ad vector to a bispecific antibody that targets the virus to angiotensin-converting enzyme (ACE), a membrane-bound protease highly expressed on pulmonary endothelial cells. This strategy circumvents the relatively low levels of Ad receptors on pulmonary endothelium by providing an alternate means of viral attachment. The approach has been shown to substantially improve pulmonary vascular gene delivery and the effectiveness of vascular-directed gene therapy (12, 18, 20).

Herein we demonstrate that upregulation of the BMPR2 signaling pathway attenuates hypoxia-induced pulmonary hypertension in otherwise normal rats. These findings provide evidence for a role for the BMPR2 pathway beyond those cases directly related to genetic BMPR2 mutations. Importantly, the study demonstrates that upregulation of BMPR2 signaling could be developed as a potential therapeutic strategy for pulmonary hypertension, and, in principle, a gene therapy approach could be one means to achieve this.

METHODS

Viral construction. AdCMVBMPR2myc, a replication-incompetent serotype 5 adenoviral vector, was created by cloning the full-length human BMPR2 cDNA containing a myc tag at the COOH terminus into the plasmid pShuttleCMV, containing the cytomegalovirus promoter. Homologous recombination with plasmid pAdEasy1 (7) resulted in the generation of pAdCMVBMPR2myc. Virus was generated by standard techniques by Pac1 digestion of the plasmid and transfection into HEK-293 cells. Large-scale virus prep was generated in HEK-293 cells and then purified by cesium chloride centrifugation. Viral titers were determined by 50% tissue culture infective dose (TCID50) assay and particle titer by optical density at 260 nm (OD260). Control viruses contained the cytomegalovirus promoter driving expression of reporter genes luciferase (Luc) or green fluorescent protein (GFP).

Construction and validation of Fab-9B9 has previously been described (18, 20). Fab and MAb 9B9 were derivatized with the bifunctional crosslinker N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP; Pierce, Rockford, IL). SPDP was combined with 9B9 or Fab in PBS at a molar ratio of 4 SPDP:1 antibody and incubated shaking at room temperature (RT) for 30 min. The pH of Fab was lowered by adding 0.1 vol of 1 M sodium acetate, pH 4.5, and then the Fab was reduced by adding 1 mg of dithiothreitol (Bio-Rad, Hercules, CA). After a 5-min incubation, the reduced Fab was passed through a PD10 column (Pharmacia, Uppsala, Sweden), equilibrated in borate buffer, and then added immediately to the derivatized 9B9 and shaken at RT overnight. The conjugate mixture was purified by gel filtration (Superose 12 column; Pharmacia) in borate buffer, pH 8.5. Monomeric Fab and 9B9 were discarded, and fractions larger than 150 kDa were pooled by size exclusion chromatography (Superose 12; Pharmacia, Uppsala, Sweden), equilibrated in borate buffer, pH 8.5, and then electroblotted to a Hybond-ECL membrane (Amer sham Biosciences). After the electrotransfer, the membranes were blocked in 10% skim milk in Tris-buffered saline Tween 20 (TBS-T) and then incubated overnight at 4°C with rabbit anti-myc 1:1,250 (Sigma, St. Louis, MO). After being washed, the membrane was incubated with goat anti-rabbit horseradish peroxidase (1:50,000; Pierce Biotechnology), and reactive bands were detected by chemiluminescence (Amer sham).

In vitro subcellular localization of BMP2myc. Following overnight adherence in chamber slides (Nunc, Naperville, IL). NMuMG or HUVEC (4 × 103/well) were infected with Ad vectors at 100 pfu/cell. After 48 h, the cells were fixed using ice-cold methanol-5% acetone, block for 30 min in 3% goat serum, and then incubated for 1 h with a rabbit anti-myc antibody (1:500; Sigma) followed by detection using Alexa 594 goat anti-rabbit (1:1,000; Molecular Probes, Eugene, OR).

Animals. All animal protocols were reviewed and approved by the Animal Research Ethics Committee of the Institute of Medical and Veterinary Science (IMVS), Royal Adelaide Hospital, and The University of Adelaide. Experiments were conducted on 4-wk-old male Sprague-Dawley rats. The rats were housed in the IMVS animal care facility and fed food and water ad libitum.

Immunoblot for detecting BMP2myc transgene expression. To assess the ability of our newly derived vector to achieve expression of BMP2 receptors, 293T cells (2 × 106/well) were infected at a calculated dose of 200 plaque-forming units (pfu)/cell with either AdCMVBMPR2myc or control Ad vector (AdCMVLuc), as previously described (20). Forty-eight hours later, cell culture media was collected, and the transduced cells were lifted, washed, and lysed. Media or lysate samples (20 μl/lane) were loaded onto 12% polyacrylamide/SDS gels, electrophoresed, and then electroblotted to a Hybond-ECL membrane (Amer sham Biosciences). After the electrotransfer, the membranes were blocked in 10% skim milk in Tris-buffered saline Tween 20 (TBS-T) and then incubated overnight at 4°C with rabbit anti-myc 1:2,500 (Sigma, St. Louis, MO). After being washed, the membrane was incubated with goat anti-rabbit horseradish peroxidase (1:50,000; Pierce Biotechnology), and reactive bands were detected by chemiluminescence (Amer sham).
PNCA quantification by Western blot. Forty-eight hours after transfection, HMVEC-LB1 (see above) were lysed, and 20–30 μg of total protein per sample was electrophoresed and transblotted as described above. After the electrottransfer, the membranes were blocked in 10% skim milk and then incubated overnight at 4°C with mouse anti-PNCA (clone PC10; Dako Cytometries). Immunodetection was performed using ECL-Plus substrate (GE Healthcare), and densitometry measurements were made using a Typhoon 9410 Imager (Molecular Dynamics, Sunnyvale, CA). To confirm equal protein loading, blots were stripped with Restore Western stripping buffer (Pierce) and reprobed using an anti-actin antibody (Santa Cruz Biotechnology).

Immuno-detection of BMPR2myc transgene expression in the pulmonary vasculature. Rats were injected with 5 × 109 pfu of either AdCMVBMPR2myc or AdCMVLuc complexed to Fab-9B9 via the lateral tail vein. Three days later, the animals were killed and lungs perfused via a 20G catheter (Insite; BD, Sandy, UT) in the right ventricle and making a small slit in the left ventricle. The pulmonary vasculature was perfused with PBS/heparin (30 ml, 20 cmH2O) and then 30 ml of 10% neutral buffered formalin (ACE Chemical, Camden Park, SA, Australia). The lungs were inflated by tracheal instillation of formalin, and the trachea was tied off. After a 24-h fixation, the lung tissue was processed into paraffin blocks. Sections were cut at 5 μm and heat mounted (58°C for 1 h) on coated glass slides (Polysine, Lake Success, NY). The sections were deparaffinized with xylene and taken to H2O2. Slides were immersed in preheated Target Retrieval Solution (DakoCytomation, Carpinteria, CA) at 95°C for 30 min, cooled, and blocked with 2.5% horse serum for 30 min. Sections then incubated overnight at RT with the primary antibody for smooth muscle α-actin (Sigma) diluted 1:60,000 in 2.5% horse serum. The next day, slides were washed twice in PBS for 3 min followed by treatment using the Quick Kit (Vector Labs, Burlingame, CA) and diaminobenzidine (DAB) color detection systems (Vector Labs) according to the manufacturer’s protocol. Sections were counterstained with Hematoxylin Q5 (Vector Labs), dehydrated, cleared in xylene, and mounted with DePeX (BDH; Laboratory Supplies, Melbourne, Australia). For PCNA detection, we used mouse anti-PCNA (Dako) at 1:1,000 dilution (without counterstain), and for cleaved caspase-3, we used rabbit polyclonal antibody (Cell Signaling, Beverly, MA) at 1:1,000 dilution (counterstain methyl green). All slides were examined using an Olympus BH2 microscope (Japan) for bright-field microscopy.

To assess the degree of muscularization of small pulmonary arterioles, lung slides of four randomly chosen animals in each treatment group were examined. In each slide, 10 high-power (>200) fields were selected at random at the level of distal acini, and vessels having an approximately circular profile with their external diameters less than 50 μm were identified. From these, the number of completely muscularized vessels was counted. Measurement of muscle thickness was assessed using a calibrated eye piece and measuring the mean smooth muscle thickness of 30- to 50-μm vessels (4 rats/group × 15 vessels/rat). For PCNA and cleaved caspase-3 detection, 5 high-power fields were examined, and 15 vessels less than 50 μm/animal were counted.

Statistical analysis. Data are expressed as means ± SE. Multiple comparisons between group means were performed using the non-
parametric Kruskal-Wallis $H$ test. When an $H$ value indicated a significant difference, pairwise comparisons between group means were performed using the protected rank sum test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Immunoblot detection of BMPR2myc transgene expression. Western blot analysis verified transgene expression of myc-tagged BMPR2 (Fig. 1). A detectable immunoreactive band at the appropriate molecular weight was seen in the cell lysate of 293T cells infected with AdCMVBMPR2myc, but not in culture medium, indicating cell-associated protein. The lack of positive staining in cell lysate from the viral control (AdCMVLuc)-infected cells confirmed specificity.

In vitro subcellular localization of BMPR2myc. NMuMG cells and HUVECs transfected with AdCMVBMPR2myc were assessed by immunofluorescence staining to determine the expression pattern of BMPR2myc. These cells exhibited intense red staining of the plasma membrane (Fig. 2), demonstrating the cell surface trafficking ability of the newly synthesized BMPR2. The specificity of immunostaining was demonstrated by the absence of signal in sections processed after the omission of primary antibody and the lack of staining seen in uninfected cells or cells infected with an irrelevant virus (data not shown).

Functional assessment of the delivered bmp2myc gene. NMuMG cells transfected with a luciferase reporter plasmid driven by a SMAD-sensitive promoter (2GC2wt-Lux) demonstrated both basal and BMP-4-stimulated transcriptional activation of luciferase, indicative of constitutively active BMPRs (Fig. 3). A significant sixfold increase in luciferase activity was seen in cells infected with AdCMVBMPR2myc, which was further increased with addition of BMP-4, demonstrating delivery of functional BMPR2. Luciferase activity was not significantly different in cells infected with control Ad vector compared with uninfected cells.

Proliferation assays. The effect of BMPR2 overexpression on cell number was determined in NMuMG cultures (Fig. 4). Cells were infected with 200 pfu/cell, and viable adherent cells were counted 4 days after infection. Overexpression of BMPR2 significantly inhibited cell proliferation by 25 $\pm$ 2% compared with viral control infected cells, consistent with activation via endogenous BMPs as seen with activation of the luciferase construct in Fig. 3. Exogenous BMP-4 (50 ng/ml)
caused a trend toward further reduction in cell proliferation, although this did not reach statistical significance.

**Immunodetection of BMPR2myc in the pulmonary vasculature.** Pulmonary targeting (schema shown in Fig. 5) of Ad-mediated BMPR2myc transgene expression was confirmed by immunofluorescent staining of the myc tag. Rats that received the AdCMVBMPR2myc/Fab-9B9 combination were killed at day 3. These animals demonstrated positive green staining in small pulmonary vessels and alveolar capillaries confirming BMPR2myc transgene expression (Fig. 6). These results are in accord with our previously published data using reporter genes and the gene for endothelial nitric oxide synthase (eNOS) (12, 18, 20), and in some sections, stained structures containing red blood cells were apparent (e.g., Fig. 6). No staining was seen in uninfected rats (Fig. 6) or in sections incubated with no primary antibody or in rats injected with a targeted irrelevant virus (not shown). We also examined hematoxylin and eosin-stained sections to evaluate inflammation. No significant changes were seen in the lungs from the rats that received either AdCMVBMPR2myc/Fab-9B9 or control Ad/Fab-9B9 (data not shown).

**Effect of chronic hypoxia.** Lower body weights were observed following chronic hypoxia compared with the normoxic group (Table 1), an effect consistent with the well-known reduction in food and water consumption of animals during exposure to hypoxia (8). Hematocrit values were significantly elevated in hypoxia- and hypoxia/Ad-treated animals (Table 1).

**Table 1. Body weight, heart rate, and hematocrit for rats in each experimental group**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Body Wt, g</th>
<th>Heart Rate, beats/min</th>
<th>%Hematocrit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxic</td>
<td>334±9</td>
<td>291±17</td>
<td>46.8±0.5</td>
</tr>
<tr>
<td>Hypoxic</td>
<td>276±4*</td>
<td>278±19</td>
<td>60.2±0.7*</td>
</tr>
<tr>
<td>Hypoxic Control Ad</td>
<td>263±7*</td>
<td>236±20</td>
<td>57.2±1.2*</td>
</tr>
<tr>
<td>Hypoxic CMVBMPR2myc+Fab-9B9</td>
<td>272±7*</td>
<td>235±33</td>
<td>58.3±0.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P* < 0.05 for normoxia vs. all hypoxia treatments.
AdCMVBMPR2myc + Fab-9B9 treatment in chronic hypoxia significantly attenuated RVSP and mPAP changes compared with control Ad + Fab-9B9 animals (36.2 ± 2.2 mmHg vs. 42.2 ± 3.2 mmHg and 28.2 ± 3.3 mmHg vs. 36.4 ± 1.8 mmHg, respectively, \( P < 0.05 \); \( n = 6 \) except RVSP and mPAP for normoxia and hypoxia control Ad, \( n = 5 \). For hypoxia AdCMVBMPR2 myc RVSP, \( n = 6 \); mPAP, \( n = 4 \)).

**Effects on right ventricular hypertrophy.** As expected, chronic hypoxia induced marked right ventricular hypertrophy, as assessed by RV weight over S + LV compared with normoxic animals (0.52 ± 0.01 vs. 0.25 ± 0.01, respectively, \( P < 0.05 \), Fig. 7C). The degree of hypertrophy was unchanged in control Ad + Fab-9B9-treated animals (0.50 ± 0.01). Right ventricular hypertrophy was significantly reduced by BMPR2 gene delivery (AdCMVBMPR2myc + Fab-9B9) compared with control Ad + Fab-9B9 (0.43 ± 0.01 vs. 0.50 ± 0.01, respectively, \( P < 0.05 \), Fig. 7C), \( N = 6 \) for all groups.

**Effect on vascular muscularization.** To assess the effect of BMP2 transgene expression on hypoxia-induced vascular remodeling, 10 high-power lung fields were examined in four rats from each treatment group in which the number of completely muscularized vessels less than 50 \( \mu \)m were counted. Chronic hypoxia almost tripled the number of muscularized vessels compared with normoxia (56 ± 4 vs. 21 ± 1, respectively, \( P < 0.05 \), Fig. 8A). A similar effect was seen on smooth muscle layer thickness (Fig. 8B). This effect was unchanged by control Ad vector delivery, whereas targeted BMPR2 gene delivery significantly attenuated the hypoxia-induced increase in vascular muscularization by ~40% \( (P < 0.05) \). Lung tissue sections, representative of each treatment group, immuno-stained for smooth muscle \( \alpha \)-actin, are shown in Fig. 9, A–D.

**Effects on cellular proliferation.** We further assessed the sections for PCNA and found reduced cellular proliferation of both endothelial and smooth muscle cells with AdCMVBMPR2 gene delivery compared with the other hypoxic groups (Fig. 10, A and B, respectively).

**Effects on apoptosis.** We performed TUNEL assay on the sections from the rats exposed to 3 wk of hypoxia but saw very few positive cells and no obvious difference between groups. This might relate to rapid clearance of apoptotic cells. We did, however, detect a slight increase in cleaved caspase-3 in endothelial cells of animals treated with the AdCMVBMPR2myc vector (Fig. 11).

It has been shown in a rat model that pulmonary hypertension occurs following endothelial apoptosis induced by VEGF inhibition in combination with hypoxia (27). This treatment led to the emergence of apoptotic-resistant endothelial cell clones that proliferate abnormally leading to vascular obstruction. Under certain conditions, stimulation of the BMP2 signaling pathway may actually protect endothelial cells from apoptosis (28). It has been proposed that the different effects of BMP signaling in smooth muscle vs. endothelium (proapoptotic in the former and antiapoptotic in the latter) might underlie pulmonary hypertension pathogenesis. These different effects are, however, highly context specific, and studies have yielded apparently conflicting results. Endothelial apoptosis has previously been described within the first 24 h of hypoxia in the rat model (26); thus, we considered whether the BMP2 transduction may protect against this as a mechanism for the protective effect against pulmonary hypertension. To investigate this pathway, we first transduced human pulmonary microvascular cells (HMVC-LB1) in vitro. Cells were infected in complete medium and then quiesced in 0.1% FCS medium. Contrary to our hypothesis, we found that transduction with AdCMVBMPR2 actually led to increased apoptosis, compared with uninfected cells or control vector, rather than any protec-
tive effect. This effect was evident by an increase in 7-AAD staining 48 h after transduction and a reduction in PCNA (Fig. 12, A and B, respectively). To determine the effect of BMPR2 transduction in vivo, we first performed a time-course analysis of endothelial apoptosis in response to hypoxia. Rats were exposed to 10% oxygen as above, with groups killed at 14, 23, and 38 h. Lungs were harvested and stained for cleaved caspase-3, and positive endothelial cells were counted. Over this time period, we noted a peak in endothelial apoptosis around 14 h (Fig. 13A). Thus, in a subsequent experiment, rats were injected with targeted control Ad or AdCMVBMPR2myc (or uninjected) and exposed to hypoxia for 14 h, and endothelial cleaved caspase-3 was assessed. In this experiment, we found that BMPR2 transduction actually increased endothelial apoptosis relative to the control vector (Fig. 13B).

DISCUSSION

There have been major developments in the understanding of the pathogenesis of pulmonary hypertension in recent years and in the development of new therapies such as endothelin receptor antagonists, phosphodiesterase inhibitors, and new prostacyclin analogs, which are beginning to translate to improvements in survival (10, 23). Nevertheless, a large number

Fig. 9. Photomicrographs (×20 magnification) of smooth muscle α-actin-stained sections of normoxia lung (A), hypoxia lung (B), control virus + Fab-9B9 hypoxia lung (C), and AdCMVBMPR2myc + Fab-9B9 hypoxia lung (D). Arrows indicate fully muscularized small vessels.
of patients continue to be severely debilitated, with some still progressing to heart-lung transplantation. Rational combinations of currently available therapies may further improve the outlook, and trials of combined therapy are underway.

The results of the current study are significant in a number of respects. The data presented helps to broaden our understanding of the impact of BMPR2 signaling in pulmonary hypertension beyond the relatively rare setting of inherited genetic mutations in BMPR2. Evidence that BMPR2 might be important beyond familial PAH was first noted by the study of Atkinson et al. (1), where reduced expression of BMPR2 in secondary as well as primary pulmonary hypertension was noted. Similarly, in the hypoxic rat model of PAH, chronic hypoxia has been shown to decrease BMPR2 expression in the pulmonary vasculature (26). More importantly, the current data show that upregulation of BMPR2 signaling can achieve a therapeutic outcome, which broadens the therapeutic possibilities for PAH therapy. Currently available treatments do not specifically address BMPR2 signaling; thus, this new approach could potentially be pursued to achieve either synergistic or additive advances on current practice. Although we have used a gene-based approach herein, the data also provide a rationale for the development of conventional pharmaceuticals that may achieve the same end. Nevertheless, the study is novel in its use of a pulmonary vascular-targeted, gene-based approach to treatment delivery, and gene therapy for patients with a recognized BMPR2 mutation would be an attractive proposition.

The precise mechanism by which BMPR2 mutations lead to PAH remains unclear. In general, the initial hypotheses were that BMPR2 activation is proapoptotic or antiproliferative, and this is consistent with the hypothesis that loss of activity in this

Fig. 10. Quantification of PCNA positive cell nuclei in endothelial cells (EC; A) and vascular smooth muscle cells (SMC; B) of small (<50 μm) vessels; n = 4 animals/group, 15 vessels/animal were assessed. *P < 0.05 AdCMVMMPR2myc + Fab-9B9 vs. control Ad + Fab-9B9.

Fig. 11. Quantification of cleaved caspase-3-positive cells in endothelial cells of small (<50 mm) vessels; n = 2 animals/group, 15 vessels/animal. *P < 0.05 AdCMVMMPR2myc + Fab-9B9 vs. control Ad + Fab-9B9.

Fig. 12. Effect of BMPR2 gene delivery to HMVEC-LBl cells. A: apoptosis determined by flow cytometry for 7-amino-actinomycin D (7-AAD) + Fab-9B9. B: PCNA/actin ratio determined by Western blot. Data are means ± SE; n = 3 experiments. *P < 0.05 AdCMVMMPR2myc vs. control Ad.
pathway predisposes to abnormal proliferation as seen in PAH. The recent work by Yang et al. (35) indicates that the antiproliferative effect is mediated by SMAD signaling, at least in proximal pulmonary artery smooth muscle cells. This study also showed that there is a reduced proportion of cells expressing the activated form of SMAD1 (a downstream messenger for BMPR2 signaling) in the small pulmonary vessels of patients with both familial and idiopathic PAH (35). We have shown that our gene delivery approach does indeed upregulate SMAD transcriptional responses in vitro. However, in more distal pulmonary muscle cells, Yang et al. showed that BMP stimulation actually mediates smooth muscle cell proliferation, an effect mediated via p38 MAPK and ERK1/2 pathways. This finding is more difficult to reconcile with the pathological findings, and it is as yet unknown which pattern of response might best reflect the situation in the most distal small vessels, the primary sites of abnormality in PAH. Less is known about the role of BMPR2 expression in endothelial cells than smooth muscle cells in this process. Endothelial expression may be critical, particularly as in the human lung BMPR2 is predominantly expressed in the endothelium, with lesser amounts in the smooth muscle cells. Our current immunohistochemical studies of transduced rat lung and those previously published with reporter genes using the ACE-targeting approach (including electron microscopy) show that it is the pulmonary vascular endothelium that is predominantly being transduced (20). Recently, Teichert-Kuliszewska and colleagues (28) showed that BMP signaling via BMP2 and BMP7 actually protected endothelial cells from apoptosis induced by serum starvation in vitro. They also showed that downregulation of BMPR2 by small interfering RNA led to an increase in apoptosis. Thus, it appears that BMP signaling, in certain contexts, has differential effects on smooth muscle cells and endothelial cells, at least in vitro. From this, it has been proposed (but not shown) that BMPs may exert a prosurvival or antiapoptotic effect in the endothelium in vivo (25). If correct, loss of this effect could then lead to disordered vascular integrity and PAH development. In principle, support for this notion comes from studies of anti-VEGF treatment (which reduces endothelial survival): endothelial cell death is followed by proliferation of apoptosis-resistant endothelial clones with development of severe pulmonary hypertension in the rat hypoxia model (27). However, a stage of increased endothelial apoptosis has not been described in PAH patients. The effects of BMP signaling, like all TGF-β family members, are highly context specific. In our studies, we have shown both in vitro and in vivo that BMPR2 transduction led to an increase in endothelial apoptosis. The mechanism by which this then leads to a reduction in the pulmonary hypertensive response is unclear and requires further study of endothelially derived mediators such as endothelin. We have found no evidence for an endothelial protective effect.

Recently, McMurtry et al. (11) investigated nebulized delivery of BMPR2 using an Ad vector in the monocrotaline model of pulmonary hypertension and found no effect on established pulmonary hypertension in this setting. Their study differs from ours in a number of important respects. The mechanism of PAH development in their model is quite different than that for the hypoxia model, and responses to therapies may vary. Opposite effects of TIMP-1 gene delivery in the hypoxia model vs. monocrotaline model have previously been reported (29, 30). The method of gene delivery is also quite different, with airway epithelial and vascular smooth muscle cells being the major transduced cells via the airway route in contrast to endothelial cells via our ACE-targeted approach. The McMurtry group focused on established disease, whereas we have first investigated mechanisms in a preventive context. All pulmonary hypertension models are a compromise, and the hypoxia model we have used in the current study has acknowledged limitations with regard to its correlation to human PAH. Specifically, the plexiform lesions characteristic of idiopathic and familial PAH are not seen. Nevertheless, hypoxic pulmonary vasoconstriction and vascular remodeling are contributing factors in a broad range of conditions leading to PAH in clinical settings. The failure of nebulized BMPR2 gene therapy in the monocrotaline model does not exclude the possibility that endothelial-targeted BMPR2 therapy might have benefits in inflammatory conditions. The recent study by
Song et al. (24) showing that BMPR2 heterozygous mice are more susceptible to inflammatory stimuli provides a basis to hypothesize that BMPR2 therapy may have utility in inflammation-induced PAH.

The clinical application of gene therapy has been a major challenge. Vector technology continues to be a limiting factor, and advances in vectorology per se are a major focus of gene therapy research at present. The therapeutic gains that can be achieved by specifically targeted vectors have recently been demonstrated in a systemic hypertension model where vascular targeted eNOS gene delivery achieved a therapeutic impact, but untargeted vector did not (12). We have recently also shown that therapeutic gains are achieved with pulmonary vascular targeting of eNOS gene delivery compared with untargeted vector in the rat hypoxia pulmonary hypertension model (19). In the present study, we thus chose to use our current state-of-the-art technology to examine the effects of BMPR2 gene delivery. In the context of established pulmonary hypertension, the ACE-targeting approach could prove particularly useful as ACE is known to be upregulated in areas of vascular remodeling (15). However, considerable further refinement of the vector approach would be required for possible use in human disease. One of the drawbacks of the current system is the “two-component” approach (Ad + antibody) that makes clinical grade formulation difficult. Hence, a number of groups are working toward the generation of genetically modified vectors whereby the targeting ligand is incorporated into the vector particle (14, 33). In our current system, we have used a first-generation Ad that is well known to have proinflammatory effects due to low-level expression of viral genes within transduced cells and the stimulation of T cell–mediated cytotoxicity. This problem often leads to a relatively short duration of transgene expression. To circumvent this problem, helper-dependent Ads have been developed that lack viral sequences in their genome (22). It has recently been shown that these agents achieve longer transgene expression with less inflammatory response after vascular gene delivery (31). In other model systems, more than 2 yr of expression after a single injection has already been demonstrated (22). Despite the foregoing concerns, we did not note significant inflammation in our model, consistent with our previously published studies using reporter genes (18, 20). Thus, even at its current stage of development, our vector approach provides a useful strategy for the initial evaluation of candidate therapeutic genes for pulmonary vascular disease. The approach could also be used to assess the impact of other TGF-β family genes implicated in PAH, such as ALK1 (6).

In conclusion, the current work provides new insights into the pathogenesis of pulmonary hypertension, illustrates the usefulness of a novel platform technology for the evaluation of candidate therapeutic pathways, and specifically indicates the potential for BMPR2 modulation to be considered as a future therapeutic option for the development of new treatments for this devastating disease. With further vector refinement, a gene therapy approach is feasible.

**REFERENCES**


**DISCLOSURES**

S. Danilov has a financial interest in Mono-ACE, the company that makes the ACE-targeting antibody used herein.

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BMPR2 GENE THERAPY


