Overexpression of human bone morphogenetic protein receptor 2 does not ameliorate monocrotaline pulmonary arterial hypertension

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McMurtry MS, Moudgil R, Hashimoto K, Bonnet S, Michalakis ED, Archer SL. Overexpression of human bone morphogenetic protein receptor 2 does not ameliorate monocrotaline pulmonary arterial hypertension. Am J Physiol Lung Cell Mol Physiol 292: L872–L878, 2007. First published December 1, 2006; doi:10.1152/ajplung.00309.2006.—Pulmonary arterial hypertension (PAH) is associated with mutations of bone morphogenetic protein receptor 2 (BMPR2), and BMPR2 expression decreases with the development of experimental PAH. Decreased BMPR2 expression and impaired intracellular BMP signaling in pulmonary artery (PA) smooth muscle cells (PASMC) suppresses apoptosis and promotes proliferation, thereby contributing to the pathogenesis of PAH. We hypothesized that overexpression of BMPR2 in resistance PAs would ameliorate established monocrotaline PAH. Human BMPR2 was inserted into a serotype 5 adenovirus with a green fluorescent protein (GFP) reporter. Dose-dependent transgene expression was confirmed in PASMC using fluorescence microscopy, quantitative RT-PCR, and immunoblots. PAH was induced by injecting Sprague-Dawley rats with monocrotaline (60 mg/kg ip) or saline. On day 14, post-monocrotaline (MCT) rats received 5 × 10⁴ plaque-forming units of either Ad-human BMPR2 (Ad-hBMPR2) or Ad-GFP. Transgene expression was confirmed by fluorescence microscopy, quantitative RT-PCR of whole lung samples, and laser-capture microdissected resistance PAs. Invasive hemodynamic and echocardiographic end points of pulmonary hypertension were assessed on day 24. Endogenous BMPR2 mRNA levels were greatest in resistance PAs, and expression declined with MCT PAH. Despite robust hBMPR2 expression in all lung lobes and within resistance PAs of treated rats, hBMPR2 did not lower mean PA pressure, pulmonary vascular resistance index, right ventricular hypertrophy, or remodeling of resistance PAs. Nebulized intratracheal adenoviral gene therapy with hBMPR2 reliably distributed hBMPR2 to resistance PAs but did not ameliorate PAH. Decreased BMPR2 expression may be a marker of PAH but is not central to the pathogenesis of this model of PAH.

proliferation; apoptosis

PULMONARY ARTERIAL HYPERTENSION (PAH) is a syndrome characterized by proliferative remodeling and obliteration of resistance pulmonary arteries (PAs). The high pulmonary vascular resistance (PVR) eventually causes failure of the afterload-intolerant right ventricle, and, despite advances in therapy, PAH continues to result in high mortality rates (6, 26), with a recent estimate of 88% survival at 1 year in a French cohort of 674 patients (16). Despite the fact that only 20% of PAH patients have a significant vasoconstrictor component to their pulmonary hypertension, current therapy for PAH is largely limited to agents selected for their vasodilator properties, although it is now clear that some of these agents also have antiproliferative effects (11, 35). PAH therapies include prostacyclin and prostacyclin analogs (5), endothelin receptor blockers (9), phosphodiesterase 5 inhibitors (14), and calcium channel blockers (2). Recently, it has been appreciated that PAH is predominantly a disease of excess cell proliferation and impaired apoptosis (36), raising the possibility of therapies that target the proliferative remodeling of the resistance PAs, such as dichloroacetate (21), simvastatin (32), anti-survivin (20), or K⁺ channel replacement therapy (27).

A genetic cause for PAH had long been suspected based on the occurrence of familial PAH. A recent study indicates that in France, familial PAH accounts for 3.9% of all PAH (16). Familial PAH relates to mutations of bone morphogenetic protein receptor type 2 (BMPR2) and activin-like kinase type-1 (ALK-1) receptors (25). Mutations of BMPR2 occur in ~75% of patients with familial PAH (12, 17); however, they appear to be relatively rare in sporadic PAH (~10%) (25). A member of the transforming growth factor (TGF)-β superfamily, BMPR2, is crucial to embryologic growth and development (8). BMPR2 is a constitutively active serine-threonine kinase receptor that, in response to ligand binding (BMP2) (4, 7), forms heterodimers with any of three type 1 receptors (BMPR1A, BMPR1B, or ALK). This association results in phosphorylation of the intracellular portion of the type 1 receptor by BMPR2, initiating a cytosolic protein signaling cascade consisting of Smad proteins (30). Receptor-activated Smads (R-Smads), including Smad1, 5, and 8, complex with a common partner Smad (Smad4), permitting it to translocate to the nucleus where it can regulate gene transcription. The Smad DNA interaction is weak and requires coactivators or corepressors. In the nucleus, R-Smad/co-Smad complexes interact with genes that have a Smad-binding element (5-AGAC-3), altering pulmonary artery smooth muscle cell (PASMC) proliferation and apoptosis. BMPR2 receptors are expressed not only in human endothelium but also in human PASMCs where their activation by BMPs normally leads to phosphorylation of Smad1, decreased Bcl expression, and induction of apoptosis (37). This antiproliferative effect of BMPs, partially due to induction of PASMC apoptosis, is suppressed in PAH (37).

In support of the BMPR2 hypothesis of PAH, reduced pulmonary vascular BMPR2 expression has been described in human PAH (4), and PASMCs from patients with PAH proliferate abnormally (excessively) in response to BMP ligands (24). Mice with haploinsufficiency of BMPR2 have mild PAH (7, 34) and may be more susceptible to PAH when exposed to pulmonary vascular stressors (18, 31). Discoveries of related
TGF-β superfamily members that are associated with PAH and hereditary hemorrhagic telangiectasia, such as ALK-1 (33) and endoglin (10), have strengthened the case for BMPR2 playing an important role in the development of PAH. Interestingly, abnormalities on the TGF-BMP pathways have been described in tumors, like the juvenile colonic polyposis (29), or vascular lesions such as the coronary restenosis lesions postangioplasty (19). Monocrotaline (MCT) PAH, an animal model of PAH, is relatively irreversible, usually fatal, and recapitulates many features of human PAH. Based on the evidence implicating deficient BMPR2 in the development of PAH, we hypothesized that overexpression of BMPR2 in the pulmonary vascular tree using an adenoviral gene therapy approach would ameliorate or reverse established MCT PAH in the rat.

MATERIALS AND METHODS

Experimental protocols. All experiments were conducted with ethical approval from the University of Alberta Animal Policy and Welfare Committee. Adult male Sprague-Dawley rats (200–300 g) were used. Rats were injected with 60 mg/kg ip MCT or saline (vehicle) and 14 days later were randomized to receive either neutralized adenovirus coding for human BMPR2 (Ad-hBMPR2) or green fluorescent protein (Ad-GFP), each at 5 × 10⁹ plaque-forming units (pfu), n = 17 rats/group. This airway gene therapy was delivered to anesthetized, intubated rats via a nebulizer needle (Microsprayer; Penn Century, Philadelphia, PA), as previously described (27). Echocardiography and invasive hemodynamic measurements were performed 24 days following MCT injection (~10 days after inhalational therapy), and the rat was then killed.

Human BMPR2 adenovirus. A replication-deficient adenovirus encoding BMPR2 and/or GFP was generated as previously described (27) (Fig. 1). Briefly, sense and antisense primers of BMPR2 carrying a myc tag were synthesized, and PCR was performed on a cDNA template (synthesized by RT-PCR from mRNA obtained from a donor PA during cardiac transplantation). The BMPR2-myc was propagated with TA Cloning kit (Invitrogen, Burlington, ON) and was subsequently inserted into a pAdTrack-CMV. The resultant pAdTrack-CMV-BMPR2-myc construct was linearized with a Pmel restriction endonuclease digest and transformed, together with an adenoviral plasmid (Adeasy-1; Stratagene, La Jolla, CA), into bacterial BJ5183 cells [American Type Culture Collection (ATCC), Manassas, VA] that were plated on LB agar containing kanamycin. The selected colonies containing BMPR2-myc were isolated, amplified, purified, linearized (PacI endonuclease digest), and transfected into HEK-293 cells (ATCC) using LipofectAmine reagent (Invitrogen). Plates with complete cell lysis were collected and analyzed for BMPR2 expression with PCR and Western immunoblots. Purification of the adenovirus was done by stepwise, discontinuous CsCl gradient. The viral titer was determined by measuring pfu/ml using a plaque assay involving HEK-293 cells in DMEM growth medium solution studied in six-well plates.

Cell culture. Isolated PAs (4th to 5th division) were mechanically denuded of endothelium and digested by papain (1 mg/ml), DTT (0.5 mg/ml), collagenase (0.6 mg/ml), and bovine serum albumin (0.6 mg/ml; all from Sigma-Aldrich, Oakville, ON) for 20 min at 37°C. Cells were then cultured in medium supplemented with 10% fetal bovine serum (Sigma-Aldrich, Oakville, ON) and grown on 60-mm plates for 3 days at 37°C. PASMCs were exposed to 100 μl of 5 × 10⁶ pfu/ml Ad-hBMPR2 or Ad-GFP for 6 h. Cells were then washed free of virus and after 48 h in 10% fetal bovine serum were studied for transgene expression, using fluorescence microscopy and immunoblots.

Immunoblots. Protein expression in whole lungs was measured with immunoblotting using available antibodies, as previously described (23). The intensity of the bands was normalized to the intensity of a reporter protein (actin) using the Kodak Gel-doc system (Kodak, Toronto, ON).

Confocal microscopy. Fluorescence imaging of either plates of PASMCs or frozen sections of whole lung tissue embedded in optimum cutting temperature compound (OCT) and flash-frozen was performed using a Zeiss LSM 510 confocal microscope.

Echocardiography and hemodynamics. Right ventricular (RV) free wall thickness and PA Doppler signals were measured in the parasternal short axis view at the level of the aortic valve using a Sonos 5500 echo machine with a 15-MHz probe (Phillips, Markham, ON) under conditions of light anesthesia, as previously described (20, 21). For
invasive studies, rats were anesthetized with ketamine (60 mg/kg ip) and xylazine (20 mg/kg ip) and placed on a warmed surgical stage. Invasive left heart catheterization (carotid artery pressure and left ventricular pressure) and right heart catheterization (PA pressure) were performed as previously described using a micromanometer-tipped catheter (Millar Instruments, Houston, TX) (21, 27). Cardiac output was measured in triplicate by a validated thermodilution method using a thermistor probe (ADInstruments, Colorado Springs, CO) and 0.5-ml injections of iced saline. PVR index was calculated as (mean PA − left ventricular end diastolic pressure)/cardiac index.

Laser-capture microdissection. Lungs were inflated with and embedded in OCT, flash-frozen, and cut in 10-μm sections using a Leica CM 1850 cryostat (Leica Microsystems, Richmond Hill, ON). Histogène slides and dehydration/staining reagents were used as previously described (1, 3). Laser-capture microdissection was performed using the PixCell II system (Arcturus, Mountain View, CA). The cap containing the excised material (3 resistance PAs/cap, 5 caps/group) was then transferred to the ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA) for measurement of mRNA using quantitative RT-PCR (qRT-PCR).

qRT-PCR. RNA extraction was performed using RNeasy (Qiagen, Mississauga, ON) and picopure extraction kits (Arcturus). Samples were added to a microwell plate, along with TaqMan probes and reagents, and qRT-PCR was performed as previously described (21, 27). Except for 18S, all probes, including species-specific rat and human BMPR2 probes and housekeeping genes (SM22α and 18S), were custom designed using Primer Express software and purchased from Applied Biosystems. mRNA abundance, relative to a housekeeping gene and the expression in other samples, was expressed as $2^{-\Delta\Delta C_t}$, as previously described (27).

Drugs and statistics. Drugs were obtained from Sigma Aldrich (Oakville, ON) unless otherwise specified. Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Values are expressed as means ± SE. Kruskal-Wallis test or single measure ANOVA was used, as appropriate. Fisher’s probable least-significant difference test was used for post hoc analysis (StatView 4.02; SAS Institute, Cary, NC). P < 0.05 was considered statistically significant.

RESULTS

Heterologous expression of hBMPR2. Rat PASMCs incubated with Ad-hBMPR2 (Ad-GFP image not shown) were intensely fluorescent compared with controls, indicating efficient infection in vitro (Fig. 2A). qRT-PCR confirmed dose-dependent transgene expression (Fig. 2B). Immunoblots of protein extracted from the infected PASMCs showed robust expression of GFP and hBMPR2 protein, which were absent in control PASMC (Fig. 2C).

Laser-capture microdissection. qRT-PCR of laser-capture microdissected PAs (15/group) from the proximal PAs (extrapulmonary), midpulmonary artery (airway-associated), and distal PAs (intraparenchymal) demonstrated a gradient of rat BMPR2 mRNA expression, with the distal PAs having the highest expression levels. SM22α, a smooth muscle-specific reporter, was used to account for varying levels of muscularization of these arteries (Fig. 2D). The levels of endogenous rat BMPR2 expression in the distal (airway-associated) PAs were reduced in the rats treated with MCT, including both the GFP- and hBMPR2-treated rats (Fig. 2E). This also illustrates the species specificity of the BMPR2 primers.

Gene therapy. Nebulized gene therapy with Ad-hBMPR2 or Ad-GFP delivered transgene to the regions of the intraparenchymal resistance PAs, as shown by fluorescence confocal microscopy (Fig. 3A). qRT-PCR demonstrated robust and similar expression of hBMPR2 in all major lung lobes (Fig. 3B). hBMPR2 was expressed in the resistance PAs collected by laser-capture microdissection (Fig. 3C).

![Image](http://ajplung.physiology.org/File/obj_float/10.220.33.2/170417/1028012/anonymous/20070401_1028012_00003.jpg)
Invasive hemodynamic measurements. There were no deaths in the MCT and MCT+hBMPR2 and MCT-GFP groups. There was no significant difference between rats treated with Ad-GFP, Ad-hBMPR2, and control MCT-treated rats in mean PA pressure, mean systemic blood pressure, cardiac index, or PVR index (Fig. 4). In accordance with the absence of hemodynamic improvement, there was no difference between the Ad-GFP rats and the Ad-hBMPR2 rats in echocardiographic indices of pulmonary hypertension, including PA acceleration time, a measure of mean PA pressure, and RV free wall thickness (Fig. 5). Furthermore, Ad-hBMPR2 treatment did not reduce the hypertrophy of the right ventricle and distal resistance PAs caused by MCT (Fig. 5). Upon qualitative inspection, no marked intimal thickening or fibrosis was observed in either control or treated animals. Immunoblots of whole lung tissue confirmed that BMPR2 protein was overexpressed in Ad-hBMPR2-treated rats in vivo (Fig. 6).

Invasive Hemodynamics

![Invasive Hemodynamics](image)

Fig. 4. PA pressure as well as pulmonary vascular resistance index (PVRi) are not significantly improved by treatment with the hBMPR2 gene therapy. Similarly, there was no difference in mean systemic blood pressure or cardiac index (Ci).
DISCUSSION

We report the first attempt to restore BMPR2 expression in experimental PAH. Although the transgene was effectively delivered, via airway nebulization, this did not ameliorate MCT PAH. In addition to documenting the feasibility of augmenting receptor expression in vivo via an airway nebulization strategy, this work also demonstrates that BMPR2 expression is highest in the resistance PAs. Moreover, we show that BMPR2 expression in small resistance PAs is reduced in experimental PAH (Fig. 2D). This association supports a possible role for reduced BMPR2 expression and function in the development of both MCT PAH and human PAH. However, effective rescue of BMPR2, using gene therapy, was not beneficial.

Certain “malignant” BMPR2 mutations may be sufficient to cause PAH, with minimal requirement for a “second hit.” Recently, it was shown that smooth muscle cell-specific overexpression of a BMPR2 mutant from a family with a particularly malignant mutant results in PAH in transgenic mice (34). However, our results appear to indicate a lack of a causal role for BMPR2 deficiency in MCT PAH. These findings are consistent with the evolving notion that PAH usually occurs as a result of multifactorial insults, i.e., that a BMPR2 mutation or deficiency, while permissive, is not usually sufficient to produce PAH. Although some BMPR2 haploinsufficient mice do develop mild pulmonary hypertension (7, 34), other such mice do not (18). A recent report found that mice with BMPR2 haploinsufficiency had normal pulmonary hemodynamics and vascular structure (18). Even when challenged with chronic hypoxia, they did not develop more severe pulmonary hypertension than wild-type controls (18). However, these mice did manifest an exaggerated pulmonary hypertensive response to serotonin. Thus mice with BMPR2 haploinsufficiency (at least some strains) are more susceptible to PAH (18, 31), even though they do not spontaneously develop severe PAH.

The lack of therapeutic efficacy of receptor replacement therapy is also concordant with the literature showing the limited penetrance (15–20%) of BMPR2 mutations in most PAH families (25, 28). Indeed, although most mutations are thought to result in loss of BMPR2 function, it is estimated that the lifetime risk of developing PAH for an unaffected BMPR2 mutation carrier (in an affected family) is only 10% (25).

Are there other reasons why BMPR2 gene therapy was not therapeutically beneficial? An otherwise efficacious therapy might fail because it did not reach its intended target tissue or did not reach the target in an adequate dose. However, we show that the hBMPR2 transgene was expressed in the distal resist-

Fig. 5. Echocardiographic indices of PAH, including pulmonary artery acceleration time (PAAT) and right ventricular (RV) thickness, are not improved by treatment with the hBMPR2 gene therapy. Similarly, there was no significant improvement in RV hypertrophy, as measured by the right ventricle to left ventricle plus septum (RV/LV+S) weight ratio, or percent medial thickness.

Fig. 6. Immunoblotting of whole lung tissue shows that hBMPR2 protein was expressed in target lung tissues.
tance PAs throughout the lungs, a finding that is particularly robust as transgene expression was confirmed in isolated resistance PAs harvested by laser-capture microdissection. Underdosing of transgene is unlikely to have accounted for our negative therapeutic result, because the expression of hBMPR2 in the treatment group was similar to levels of rat BMPR2 in controls. Moreover, net BMPR2 expression was increased in the treatment group relative to levels seen in the control group (Fig. 6). We do not believe that the results of this study can be attributed to the experiment being underpowered, as there was no trend for benefit with the Ad-hBMPR2 treatment, even using very sensitive surrogate end points such as % medial thickness of resistance PAs.

While underdosing is unlikely to have accounted for our negative findings (based on the strong overexpression of hBMPR2 in the treatment group), it is possible that studying the animals only 10 days after the hBMPR2 therapy allowed insufficient time for beneficial changes to occur in the pulmonary vasculature. The choice of this time point reflected a compromise between the high spontaneous mortality that occurs in MCT PAH after 4 wk and the waning effect of adenoviral gene therapy after 14 days. A strength of our study is that we demonstrate that downregulation of endogenous BMPR2 expression occurs rapidly in MCT PAH (within 3 wk). This not only provided the rationale for replacement therapy but suggested that, if rapid downregulation of BMPR2 were causal, relatively brief replacement (10 days) should have been beneficial. The current study cannot exclude the possibility that benefit from BMPR2 gene therapy might have resulted from a different experimental design (e.g., of multiple rounds of therapy over a longer study time or prophylactic therapy). We elected not to do a prevention study because so many are positive and yet the opportunity to intervene before or concomitant with PAH does not occur clinically. An informal survey of PubMed reveals that there are approximately five times as many studies demonstrating prevention of PAH in an experimental model as showing any reversal of established disease over the last 2 years. Most PAH patients (75%) are New York Heart Association functional class III upon presentation and have been symptomatic for several years (16). For these reasons, there is a move away from prevention studies in PAH research. This decision to address the more rigorous standard (regression) also minimizes the number of animals killed.

The relative contribution of abnormal BMP signaling in endothelium vs. smooth muscle cells to the development of PAH is currently unknown (22). If the more important component of dysregulated BMP signaling occurs in the endothelium, it is possible that the therapy did not reach the necessary target. Further study is necessary to clarify this issue. The recent observation that BMP signaling in PA endothelial cells inhibits apoptosis (as opposed to inducing apoptosis in PASMCS) is intriguing, and it is in theory possible that if our transgene reached the endothelium (the virus reaches the PAs from the alveolar/adventitial, not the luminal, surface), its endothelial effects might have counteracted the effects on the PASMCS.

An additional possible reason that monogene hBMPR2 therapy did not ameliorate MCT-induced PAH is that other members of the BMP signaling cascade might also be abnormally expressed. Studies of human tissue comparing PAH patients with controls demonstrate hundreds of genes that are differentially expressed (13). If the distal effectors of the BMP signaling pathway are also reduced, such as Smads or transcription factors (15), correcting the proximal BMPR2 deficiency may not be sufficient. Finally, we acknowledge that the pathology and pathogenesis of MCT PAH is not identical to human PAH and that the failure of hMBPR2 therapy might be due to other model-specific factors.

We conclude that successful replacement of BMPR2, achieved using an aerosolized gene therapy approach, does not ameliorate MCT-induced PAH. Although this study does not exclude an important role for BMPR2 in the development of both animal and human PAH, it does suggest that BMPR2 deficiency by itself is not the central or most important molecular mechanism in this model.

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