Potential Interventions Against BMPR2-Related Pulmonary Hypertension

James West, PhD  
Vanderbilt University Medical Center  
Department of Medicine  
Nashville, Tennessee

James E. Loyd, MD  
Vanderbilt University Medical Center  
Department of Medicine  
Nashville, Tennessee

Rizwan Hamid, MD, PhD  
Vanderbilt University Medical Center  
Departments of Genetics and Pediatrics  
Nashville, Tennessee

For more than 60 years, researchers have sought to understand the molecular basis of idiopathic pulmonary arterial hypertension (PAH). Recognition of the heritable form of the disease led to the creation of patient registries in the 1980s and 1990s, and discovery of BMPR2 as the cause of roughly 80% of heritable PAH in 2000. With discovery of the disease gene came opportunity for intervention, with focus on 2 alternative approaches. First, it may be possible to correct the effects of BMPR2 mutation directly through interventions targeted at correction of trafficking defects, increasing expression of the unmutated allele, and correction of splicing defects. Second, therapeutic interventions are being targeted at the signaling consequences of BMPR2 mutation. In particular, therapies targeting cytoskeletal and metabolic defects caused by BMPR2 mutation are currently in trials, or will be ready for human trials in the near future. Translation of these findings into therapies is the culmination of decades of research, and holds great promise for treatment of the underlying molecular bases of disease.

Prior to development of cardiac catheterization in the late 1940s, primary pulmonary hypertension (PPH) was rarely suspected or confirmed prior to autopsy. PPH first became a clinical entity when investigators were able to measure pulmonary artery pressure and thus make the diagnosis of pulmonary hypertension for the first time in living patients.1,2 Cardiac catheterization also brought new understanding about the various causes of pulmonary hypertension and how these could be distinguished from one another. Physicians have long been fascinated by the many unique features of PPH, including its prevalence in healthy young women, its rarity, and its focal pathology within the pulmonary vascular bed, sparing the systemic circulation. Speculation by authorities about the origins of PPH favored abnormal vasoreactivity or microthrombosis, or both, until the past decade. Widespread interest for PPH among the general public and medical community was generated in the 1970s by the first large anorexigen epidemic related to aminorex/Menocil use in Europe,3 and the subsequent pivotal WHO meeting in 1973, which developed, in part, as a consequence.4

Dr David Dresdale described the first PPH family in 1951, including a mother, her son, and her sister.5 During the next 3 decades, several families (13) were reported in the US. In 1980, we met a young lady with PPH at Vanderbilt, and she described several young female family members with premature cardiorespiratory death, as young as age 23. That year, Dr John Newman joined the Vanderbilt pulmonary faculty and stimulated our investigation. We contacted the authors of the earlier reports, and they generously contacted the former families to join our developing study. We described 9 new cases, which occurred in 8 families during the interval after the original reports.6 The transmission pattern was readily apparent even then, as vertical transmission (highly indicative of a single dominant gene) and father to son transmission (excluding X or Y linkage) were evident in multiple families. Incomplete penetrance, with multiple skip generations, was apparent; this phenomenon is still not understood and confounds any attempt at disease prediction or genetic counseling.

In the mid-1980s, Dr Newman served as Vanderbilt principal investigator in the National Institutes of Health (NIH) natural history study of PPH and enrolled 11 patients from Vanderbilt.7 At semiannual NIH meetings for the study, the other investigators around the US learned of our growing interest in PPH families and encouraged PPH families at their centers to participate in our fledgling familial PPH registry. The NIH natural history study was the benchmark that defined its clinical features and identified a positive family history in 6% of the 187 patients enrolled.7

By the late 1980s, before any gene search, a major concern arose about the heterogeneity of the cohort. The pathologic literature in that era suggested that PPH was actually many different diseases, including plexogenic PPH, thromboembolic PPH, pulmonary veno-occlusive disease, isolated medial hypertrophy, and pulmonary capillary hemangiomatosis.8 That set of pathologic observations and the related notion that PPH was not a single disease entity imposed serious limitations on a gene search: it would be illogical to search for one gene as a cause of different diseases. This conundrum was resolved by a study of the breadth of the pathologic findings in autopsies within the same family.9 One family suffered 3 patients with very different autopsy manifestations, and thus we concluded that the

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Correspondence: j.west@vanderbilt.edu
various pathologic findings are not different diseases, but have a single basis, because they occurred in a family with vertical transmission indicating a single gene. With that information we concluded that all the PPH families in our cohort could be joined into one group to conduct a gene search, which would be complete within the decade.

The familial PPH registry continued to grow, and collaboration by geneticist John Phillips after he moved to Vanderbilt in 1985 provided direction to envision a gene search. He urged us to bank patient and family specimens in the hope that a gene search would eventually become feasible. Gene discovery methods improved in the 1990s, and Bill Nichols at Michigan conducted a PPH microsatellite marker search. Using the patient and family samples from our familial PPH registry, he identified linkage on chromosome 2q32 in 1997, and subsequently the underlying mutation responsible in most families was identified in bone morphogenetic protein receptor 2 (BMPR2) in 2000. Similar studies of linkage of PPH and discovery that BMPR2 is the gene of interest were also accomplished independently in a nearly identical time frame at Columbia-Presbyterian by a team led by Drs Robyn Barst, Jane Morse, and Jim Knowles. Mutation in BMPR2 is now known to be the basis for the vast majority of families with PAH, and more than 120 BMPR2 mutation families are known in the USA, with estimates of 500 families worldwide. Other genes related to TGF-β are less frequently responsible for the disease we now call hereditary pulmonary arterial hypertension (HPAH): ACVRL1 (ALK1) and endoglin, which more frequently cause hereditary hemorrhagic telangiectasia. More recently, an association between HPAH and SMAD 9 was reported. In summary, germline mutation between HPAH and SMAD 9 was demonstrated.14 In summary, germline mutation between HPAH and SMAD 9 was demonstrated.14 In summary, germline mutation between HPAH and SMAD 9 was demonstrated.14 In summary, germline mutation between HPAH and SMAD 9 was demonstrated.14

INTERVENTIONS AGAINST BMPR2 EXPRESSION, SPLICING, AND TRAFFICKING

Currently there is no therapy known to prevent, delay, or reverse the pulmonary vasculopathy of pulmonary arterial hypertension (PAH). Perhaps the greatest barriers to developing effective treatments are 2 closely related knowledge gaps: what is the exact role of BMPR2 in the pathologic signaling of PAH and what mechanisms cause the pulmonary vascular disease itself. Certain unique features of BMPR2-related PAH provide tantalizing clues into disease pathogenesis. For example, one of the most striking features of HPAH is its reduced penetrance; a mutation carrier has only a 20% chance of developing PAH. Thus nearly 80% of BMPR2 mutation carriers have no clinical symptoms but can produce offspring that are affected. The first clue toward understanding reduced penetrance came from the recognition that BMPR2 mutations can either be haploinsufficient (HI) or dominant negative. This distinction is important to understand, as it provides unique insights into disease pathogenesis and opens avenues toward better HPAH diagnosis and treatment. RNA studies have shown that some BMPR2 mutations produce stable transcripts, while others contain premature termination codons (PTC) and are rapidly degraded through the nonsense mediated decay (NMD) pathway. NMD is an mRNA surveillance system that degrades transcripts containing PTCs to prevent translation of unnecessary or harmful transcripts. HPAH patients with BMPR2 mutations that do not cause PTC and are therefore not subject to NMD (NMD-) have disease due to dominant negative effects of the mutated protein, while patients with mutations subject to NMD (NMD+) have disease due to functional HI of BMPR2 (NMD degradation of the mutated allele’s mRNA). Thus, it appears that HI is a heterozygous state in which the normal allele of BMPR2 has insufficient expression to maintain normal cellular function and prevent disease (this “threshold effect” is illustrated in Figure 1). Mutations that cause HI of BMPR2 are slightly more common (~55%-60% of HPAH). Thus, HI mutations teach us that total cellular BMPR2 mRNA or protein levels are important for disease penetrance. This observation in families fits with the broader observation that decreased BMPR2 expression is present in other human and experimental forms of pulmonary hypertension, though it remains unclear whether this is a precipitating event or a side effect. For example, decreased BMPR2 expression is present in pulmonary vascular tissue of patients with IPAH and in multiple experimental animal models of PAH, including those induced by monocrotaline, chronic hypoxia, and chronic systemic-to-pulmonary shunting. These studies thus clearly demonstrate that BMPR2 expression is important in many forms of PAH.

In studying HI NMD+ BMPR2 mutation carriers, we noticed that mutation carriers had variation in their total cellular BMPR2 levels. Analysis of 4 HPAH kindreds showed that, indeed, the expression of wild-type BMPR2 transcript was lower in affected patients compared to unaffected mutation carriers (P < .0005). This association of transcript levels with penetrance was not limited to a single type of NMD+ mutation since all 4 of the kindreds analyzed had different NMD+ mutations. These data strongly suggest that the level of expression of the wild-type (WT) (normal) BMPR2 allele predicts the clinical development of HPAH in individuals who carry HI BMPR2 mutations, and thus the expression of the WT BMPR2 allele may be a primary modifier of HPAH penetrance. These data also suggest that there is likely a cellular threshold for BMPR2 expression; ie, once the cell loses one BMPR2 allele secondary to an HI mutation, the cellular BMPR2 levels are determined by the expression of the remaining (normal, wild-type) allele (Figure 1). Thus decreased expression of the WT allele in an HI background (caused by the heterozygous NMD+ mutation) lowers total BMPR2 expression below a critical threshold needed for proper cellular function, thus causing disease. In contrast, higher expression of the WT BMPR2 allele (higher than the pre-
sumed threshold) with the same heterozygous NMD+ mutation may prevent clinical disease in the mutation carrier.\textsuperscript{28} Such modulation of disease penetrance by WT transcripts has until now been thought to be a rare phenomenon, being previously reported in only 3 genetic disorders: dominantly inherited erythropoietic protoporphyria, hereditary elliptocytosis, and autosomal dominant retinitis pigmentosa.\textsuperscript{29-32} One explanation for how levels of WT transcripts might affect HPAH penetrance comes from the finding that \textit{BMPR2} forms a heterotrimeric complex with \textit{BMPR1A} and \textit{BMPR1B}. The degree of deficiency of normal \textit{BMPR2} could affect the receptor complex stoichiometry, leading to decreased signaling and disease.\textsuperscript{28}

The molecular mechanisms behind this variability in \textit{BMPR2} expression are not known. It is unlikely to be related to \textit{BMPR2} promoter mutations (unpublished data) and more likely due to \textit{cis} (function of proximal regulatory regions) or a \textit{trans} (function of distal genes) effect. Our data showing that normal individuals have baseline variability in \textit{BMPR2} expression (unpublished data) certainly support this hypothesis.

\textit{BMPR2} expression may also help explain another interesting aspect of this disease: that females are 1.9- to 4.1-fold more likely to develop disease than males.\textsuperscript{33} It was recently shown that the \textit{BMPR2} promoter contains an evolutionarily conserved estrogen receptor binding site that responds to estrogen by suppressing \textit{BMPR2} expression.\textsuperscript{34} This observation provides one clue as to why females are more likely to develop PAH than males and further highlights the importance of \textit{BMPR2} expression levels in PAH pathogenesis.

Recent data, however, suggest that the eventual role of \textit{BMPR2} expression in HPAH is likely to be even more complex than levels of total WT \textit{BMPR2} expression. These data show that \textit{BMPR2} alternative splicing may play a role in HPAH pathogenesis—it may not be a simple question of total \textit{BMPR2} expression, but in fact the relative levels of alternatively spliced \textit{BMPR2} transcripts. Alternative splicing is a mechanism by which a single gene can generate multiple transcripts with likely different functions through internal deletion (“skipping”) of exons in various combinations. This mRNA processing can have clinical consequences and has been shown to play a role in many human diseases.\textsuperscript{35,36} In lung disease, alternative splicing has been shown to be important in chronic obstructive pulmonary disease (COPD), bronchopulmonary dysplasia (BPD), chronic interstitial lung disease, familial and sporadic interstitial lung disease,\textsuperscript{35-39} and cystic fibrosis.\textsuperscript{40,41} \textit{BMPR2} has 13 exons and is alternatively spliced to produce 2 primary transcripts: isoform A, which is the full length gene product containing all 13 exons of the gene and isoform B, a much rarer transcript missing exon 12.\textsuperscript{42-44} Several studies have hinted at the importance of isoform B in proper functioning of \textit{BMPR2} and in the development of PAH. Deletion of exon 12 is a common \textit{BMPR2} mutation found in HPAH patients, and previous

\textbf{Figure 1: Cellular levels of \textit{BMPR2} mRNA are determined only by the WT allele if one inherits a mutated allele that degrades the product transcribed from the mutated allele. In this case if the expression from the nonmutated allele is enough to reach a presumed threshold for \textit{BMPR2} mRNA expression, that patient will not get PAH (hypothesis from Hamid et al. \textit{Hum Mutat.} 2009).}
studies have shown that it can disrupt BMPR2 function in a dominant negative fashion.\textsuperscript{45-47} Furthermore, studies in mice have shown that overexpression of a BMPR2 transcript with an exon 12 deletion results in PAH.\textsuperscript{48} Interestingly, our data (unpublished, in review) suggest that cells from patients are more likely to have higher levels of isoform B relative to levels of isoform A (B/A ratio) compared to carriers. Thus the relationship between BMPR2 expression and PAH pathogenesis is complex and involves not only the expression of WT allele but also alternative splicing. The relative contributions of either of these mechanisms toward HPAH pathogenesis are currently not known; however, it is quite likely that there is some overlap at a molecular level.

These data then suggest several potentially novel approaches toward disease diagnosis and treatment. For example, could we use this information to design better diagnostic tools for HPAH patients? The fact that we cannot predict disease development in BMPR2 carriers with extensive family history of PAH results in significant physical, emotional, and economic burden. Mutation carriers do not know whether or when they will develop clinical disease. Moreover, at the time of diagnosis, 75% of subjects with HPAH already have symptoms in New York Heart Association functional class III or IV, and this more advanced symptom complex predicts poor survival despite available treatment. Thus, any approach that will predict which mutation carriers are likely to develop disease would be tremendously helpful both for those likely to develop disease and those likely to remain disease free. If ongoing studies prove that expression of the normal BMPR2 allele predicts disease development, this finding could possibly be used as a diagnostic tool to reassure some patients and provide optimal surveillance for those at higher risk.

These data also raise intriguing treatment possibilities—what if cellular BMPR2 expression levels could be increased over this critical threshold? Several approaches can now be used to identify drugs that will alter BMPR2 cellular expression. For example, the Connectivity Map database\textsuperscript{49} offers a novel way to identify and test drugs that may modulate BMPR2 expression. Furthermore, since drugs within the Map are already FDA-approved, the time frame from bench to bedside is significantly shortened, with obvious benefits to patients.\textsuperscript{50} Drugs that could upregulate BMPR2 expression could be potential PAH treatments, while it might be wise to avoid those that down-regulate BMPR2 expression, which could increase an individual’s risk of developing disease.

Modification of BMPR2 alternative splicing may also offer an approach to change disease course. Several studies, including our own, have shown that splicing is a dynamic process and can be altered by the environment, including exposure to drugs.\textsuperscript{51,52} This raises the intriguing possibility that we inadvertently make the disease worse by our selection of particular pharmacological agents. It is likely that BMPR2 alternative splicing is dynamic both in its response to medication (patient’s pharmacological milieu) and other environmental signals (such as hypoxia). A better understanding of the environmental determinants for alternative splicing could be highly relevant for clinicians if, in fact, the drugs we use favorably (or unfavorably) influence BMPR2 expression and thus cellular function in HPAH patients.

**THERAPEUTIC INTERVENTIONS AGAINST SIGNALING CONSEQUENCES OF BMPR2 MUTATION**

An alternative to targeting the expression and splicing of the BMPR2 receptor itself is to target therapies at the downstream signaling consequences of BMPR2 mutation. This will probably be necessary for most classes of NMD- (dominant negative) BMPR2 mutation. Moreover, the molecular pathways affected in most IPAH patients are nearly identical to those in HPAH patients.\textsuperscript{53} Since most IPAH patients lack identifiable defects in BMPR2, targeting downstream signaling will be required. In the 12 years since BMPR2 was identified as the primary heritable PAH gene, substantial progress has been made in understanding the signaling consequences of BMPR2 mutation in the pulmonary vasculature; some of these consequences are approaching readiness for therapeutic intervention in patients.

BMPR2 is a 1038 amino acid single pass transmembrane protein. Dimers of BMPR2 in combination with dimers of type 1 BMP receptors, usually BMPRIA or BMPRIB, bind to BMP ligand\textsuperscript{54} and signal directly through several different mechanisms (Figure 2). There may be alterations in signaling specificity based on whether the receptor complex is preformed or assembles in the presence of ligand,\textsuperscript{55} and the specific type 1 receptors present in the complex may be important, but these details are currently poorly understood. The receptor itself consists of 4 domains: an extracellular ligand binding domain, a short transmembrane domain, a kinase domain, and a long cytoplasmic tail. BMPR2 is highly homologous to other type 2 TGF-\(\beta\) superfamily receptors, with the exception of its cytoplasmic tail domain, which is both unique and highly conserved across species.

The most well studied method by which BMPR2 signals is through phosphorylation and activation of a type 1 receptor. The cytoplasmic tail domain is dispensable for this function; both tail domain mutations in BMPR2 and naturally occurring BMPR2 alternative splice isoform B, are capable of phosphorylating the type 1 receptor. On the other hand, the BMPR2 cytoplasmic tail appears to be indispensable for binding and activation of at least 3 targets related to regulation of intracellular trafficking and the cytoskeleton, probably also requiring a functional BMPR2 kinase domain. These targets are SRC, dynein light chain tctex-1 (DYNLT1), and LIM domain kinase 1 (LIMK1, Figure 2).

**BMPR2 SIGNALING THROUGH THE BMPRIA OR BMPRIB**

When phosphorylated by BMPR2, the type 1 receptor signals through 2 distinct pathways: it phosphorylates and activates SMAD transcription factors 1, 5, or 8, and under some circumstances also regulates the TGF-\(\beta\) activated kinase 1 and its binding protein (TAK1/TAB1) complex bridged by X-linked inactivator of apoptosis (XIAP).\textsuperscript{56}
When phosphorylated, SMAD1, 5, and/or 8 bind to the co-SMAD, SMAD4, and enter the nucleus to drive transcription. The most well-studied SMAD transcription targets regulate terminal differentiation of cells, a role for which BMP has been extensively studied in the developmental literature. The BMP pathway also suppresses inflammatory markers including interleukin-6 and STAT3 through SMAD-mediated mechanisms.57,58 SMAD proteins also regulate micro RNA (miRNA) splicing through a nontranscriptional mechanism, not requiring binding to SMAD4, which promotes miRNA processing by DROSHA.59 Micro RNAs are increasingly recognized as important signals in health and disease, including pulmonary hypertension. Thus, SMAD signaling regulates smooth muscle differentiation state60 in 2 ways: by direct transcriptional regulation of genes involved in maintaining a fully differentiated, contractile state (in particular, KLF genes62,63) and by regulating maturation of miRNAs, which further regulate differentiation state. In animal models, BMPR2 mutations affecting SMAD signaling drive smooth muscle from a contractile to a synthetic state,60 likely resulting in significant changes in cellular proliferation, extracellular matrix deposition, vascular inflammation, and mechanical stiffness.

In addition, decreased BMPR2 signaling through the SMAD transcription factors may cause increased TGF-β signaling. Both pathways compete for use of the same co-SMADs, SMAD4, and so reduced use of SMAD4 by BMP may result in increased availability for TGF-β signaling,68 although there are hints that reciprocal regulation between the BMP and TGF-β signaling pathways is more complex than this. TGF-β -signaling in the lung is generally regarded as profibrotic and proinflammatory, and increased TGF-β signaling contributes to remodeling in some animal models.69 Thus, decreased BMPR2 mediated signaling may contribute to remodeling by allowing an increase in TGF-β-signaling. There are potential interventions that could be targeted against defects caused by reduced BMPR2 signaling through BMPR1 receptors; however, such interventions are all currently at a very early stage of development. It would also be sensible to target the increased interleukin 6 and STAT3 signaling or to suppress increased TGF-β signaling. Inhaled miRNA targeting these or other BMPR2 pathways would also be logical. However, these approaches would first need to be vetted for safety and efficacy in robust animal models, and such highly targeted therapies are likely more than a decade away.

**BMPR2 SIGNALING THROUGH SRC, DYNLT1, AND LIMK1**

In 2003, Ora Bernard’s group in Australia found that the BMPR2 cytoplasmic tail physically interacted with and regulated LIMK1,70 and that when bound to BMPR2, LIMK1 function was reduced. BMPR2 mutation led to decreased LIMK1 binding and increased activity. The primary phosphorylation target for LIMK1 is the actin binding and reorganization protein coflin (CFL1); we have confirmed that BMPR2 mutation leads to increased CFL1 phosphorylation in lungs from BMPR2R899X mice, and this would have functional consequences to promote the pulmonary hypertension phenotype.

Also in 2003, Richard Trembath and Nick Morrell’s group found that BMPR2 colocalizes with, binds, and phosphorylates DYNLT1.71 These functions were disrupted by PAH-causing mutations within the cytoplasmic tail of BMPR2. DYNLT1 also physically interacts with both the mitochondrial membrane permeability protein VDAC172 and the Rho/RAC guanine nucleotide exchange factor ARHGEF2.73 We have found profound defects in energy metabolism in BMPR2 mutant mice, cells, and patients,74 and an increase in GTP-bound RAC1 in both whole mouse lung, vascular smooth muscle, and pulmonary microvascular endothelium with BMPR2 mutations.75 Especially because altered energy metabolism
and Rho-kinase signaling are increasingly recognized as important to PAH pathogenesis in humans, our observations strongly implicate DYNLT1 as a functionally important downstream target of BMPR2 mutations.

In summary, it seems that the BMPR2 cytoplasmic tail regulates multiple critical cytoskeletal functions. Expression array experiments in mice and cells cultured from mice with BMPR2 mutation specific to the cytoplasmic tail show changes in metabolic, cytoskeletal, adhesion, and microtubule-related genes. BMPR2 regulation of the cytoskeleton may explain the defects in endothelial barrier function, mitochondrial fission and fusion, and motility found in PAH.

Therapies targeted at the cytoskeletal or the metabolic defects are either currently in trials, or will be ready for human trials in the near future. Dichloroacetate is a direct inhibitor of pyruvate dehydrogenase kinase, a key enzyme regulating glycolysis, thus enhancing glucose oxidation, which has entered a Phase I trial for PAH at University of Alberta and Imperial College London. Because this addresses at University of Alberta and Imperial College London. Because this addresses metabolic, cytoskeletal, adhesion, and mitochondrial fission and fusion, and motility found in PAH.

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Our group used ACE2, which reversed the SRC and RAC1 defects, as well as many of the downstream metabolic consequences, and achieved reversal of elevated right ventricular systolic pressure in mice expressing the cytoplasmic tail domain mutant BMPR2R899X. Both of these drugs are currently in Phase I or II trials for non-PAH conditions, and so are readily translatable to clinical trials for human PAH.

CONCLUSION

The search for the molecular etiology of PAH has stretched over decades, kicking into high gear in 2000 with the discovery that BMPR2 was the most common heritable PAH gene. Now, therapies targeted at correcting the BMPR2 mutation itself or at correcting the downstream consequences of BMPR2 mutation are rapidly approaching human translation, with the promise of new, more effective treatments targeted specifically at the molecular defects that give rise to disease.

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