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Rescuing the BMPR2 signaling axis in pulmonary arterial hypertension

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Abstract

Pulmonary arterial hypertension (PAH) is a lethal disorder characterized by pulmonary arterial remodeling, increased right ventricular systolic pressure (RVSP), vasoconstriction and inflammation. The heritable form of PAH (HPAH) is usually (>80%) caused by mutations in the bone morphogenic protein receptor 2 (*BMPR2*) gene. Existing treatments for PAH typically focus on the end-stage sequelae of the disease, but do not address underlying mechanisms of vascular obstruction and blood flow and thus, in the long run, have limited effect because they treat the symptoms rather than the cause. Over the past decade, improved understanding of the molecular mechanisms behind the disease has enabled us to consider several novel therapeutic pathways. These include approaches directed toward *BMPR2* gene expression, alternative splicing, downstream BMP signaling, metabolic pathways and the role of estrogens and estrogenic compounds in BMP signaling. It is likely that, ultimately, only one or two of these pathways will generate meaningful treatment options, however the potential benefits to PAH patients are still likely to be significant.

Introduction

Pulmonary arterial hypertension (PAH) has fascinated physicians and scientists for more than a century, even before the first clinical description in 1950, when the development of cardiac catheterization made central hemodynamic measurement available for routine clinical care [1]. During the epoch before effective therapy was found, nearly 20 years ago, it was a frustrating and depressing endeavor to provide care for PAH patients, for patients and providers alike. The tragic consequence of a lethal disease, especially in young women who are otherwise well, often creates lasting memories for clinicians and families. In this light, it is wonderful to have a broad and growing spectrum of effective therapies for current PAH patients; but overall the cup still remains half empty.

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None of the current treatments even approaches a cure nor do any of them correct the central underlying pathology – the obstructive pulmonary arterial disease. In addition, currently available treatments entail many significant burdens, including substantial expense. We believe that the best route to develop novel and highly effective therapy is through better understanding of the pathogenesis to target the origins of disease.

Despite substantial progress in understanding PAH during the past two decades, it seems that many of the most important questions remain unanswered. Although we have developed remarkable understanding of the genetic underpinnings of PAH, there is still little clarity about why the primary focus of disease occurs in only the smallest pulmonary arteries. Additional efforts are also needed to understand the additional triggers, which can provoke onset of disease in bone morphogenic protein receptor 2 (*BMPR2*) mutation carriers, and why disease occurs differentially by gender (in 42% of females and 14% of males) [2]. Similarly, no real understanding exists to date about why PAH is distributed equally across all human age groups.

Perhaps new understanding will arise from the recent discoveries showing that genes outside the transforming growth factor (TGF)-β pathway can contribute to a disease that appears phenotypically identical, including mutations in *CAV1*, *KCNK3* [s1]and *CBLN2* [3–5]. Our belief that the most effective therapy will address the disease at its origin, the *BMPR2* mutation and its consequences, will only be affirmed when development of relevant agents are identified and proven. We can envision fixing the deregulated BMP signaling in heritable PAH (HPAH) patients by approaches that focus on: (i) upstream elements of the signaling; (ii) the downstream elements of the signaling; or (iii) a combination of (i) and (ii) (Figure 1).

Approaches directed toward the upstream elements of signaling

An approach directed at the upstream element of BMP signaling would focus on *BMPR2* expression and its effects on downstream signaling (Figure 1). Are there ways we can alter *BMPR2* expression to compensate for the effects of the *BMPR2* mutations on the BMP pathway? There are two types of *BMPR2* mutations found in HPAH patients: mutations that cause premature termination codons (PTC) resulting in the activation of the nonsense-mediated decay (NMD) pathway [6]; and mutations that do not (also known as NMD^{-}). NMD^{+} mutations cause disease owing to functional haploinsufficiency, whereas NMD^{-} mutations cause disease owing to dominant-negative effects. We have shown that, in kindreds with NMD^{+} mutations, affected family members had lower levels of wild-type (WT) nonmutated or normal *BMPR2* transcripts compared with unaffected relatives (who had higher levels of the WT-*BMPR2* allele) (Figure 2a). This association of transcript levels with penetrance was not limited to a single NMD^{+} mutation but seen in all types of NMD+ mutations. Thus, the levels of the normal (nonmutated) *BMPR2* allele were important in disease pathogenesis and, at least in haploinsufficient patients, there was a likely cellular threshold for *BMPR2* expression, which separated normal status from clinical disease (Figure 2a) [7]. We have since replicated these findings in NMD^{-} patients as well (unpublished data), suggesting that modification of function of the mutated *BMPR2* allele by the normal or WT allele could be an important predictor of disease penetrance and

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pathogenesis (Figure 2a). These findings thus suggest that approaches that could upregulate cellular *BMPR2* expression might be beneficial in HPAH. In patients with NMD^- mutations that result in PTC we could use drugs that promote ribosomal read-through of PTCs [8], whereas in patients who do not have PTC (NMD^+ mutations) new bioinformatics tools such as the Connectivity Map (cMap) database could be used to identify drugs that increase total cellular *BMPR2* expression [9]. The cMap database is a particularly novel way to identify and test already FDA-approved drugs that can upregulate *BMPR2* expression, thus significantly shortening the timeframe from drug discovery to bedside application [10]. This approach recently determined that tacrolimus increases BMPR2 signaling, and is now in a small clinical trial in human patients [11] (Figure 1). Another mechanism to mitigate the effects of NMD^- *BMPR2* mutations could be with the use of chemical chaperones. NMD^- mutations cause the BMPR2 proteins to be misprocessed in the endoplasmic reticulum and Golgi with resultant failure of trafficking to the cell surface. In addition, this misprocessing can result in activation of the unfolded protein response pathway and apoptosis. Chemical chaperones can aid correct [s2]folding of mutant BMPR2 proteins thus restoring BMP signaling pathways [12] (Figure 1).

BMPR2 alternative splicing can also be used to modulate downstream BMPR2 functions (Figure 1). *BMPR2* has 13 exons and is alternatively spliced to produce two 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 [13] (Figure 2b). Exon 12 is important for proper functioning of BMPR2 and exon 12 mutations are common in HPAH and have been shown to disrupt BMPR2 function in a dominant-negative fashion. Our data show that affected *BMPR2* mutation carriers were more likely to have higher levels of isoform-B relative to isoform-A, and this imbalance had detrimental downstream signaling consequences [14]. We have further found that the *BMPR2* isoform ratio, in part, is controlled by a combination of exonic splice enhancers and specific splicing factors such as ASF/SF2 *BMPR2* [s3]that bind to these enhancers (Figure 2b). splicing is a dynamic continuous process that ensures appropriate signaling through the BMP pathway under varying cellular and tissues conditions. Our recent unpublished data show that titrated enforced expression of transgenic isoform-B in tissues of interest (lung and vascular) in mice results in recalibration of the endogenous *Bmpr2* splicing. This leads to dramatically increased levels of endogenous isoform-A and decreased levels of endogenous isoform-B, in an attempt by the splicing machinery to balance the ratios toward baseline. This, in part, was a result of increased expression of a splicing factor that does not bind to a specific splice enhancer but interacts with eukaryotic translation initiation factors. These data indicate that regulation of *BMPR2* splicing and the resultant effect on downstream BMP signaling are active ongoing processes, probably more complex than previously understood with many key players yet to be discovered. Nevertheless, key steps that can be targeted have been identified. It is well known that various steps in splicing are affected by the cellular environment and importantly by exposure to pharmacological agents. Our data raise the intriguing possibility that it might be possible use the known splicing modification techniques, in particular pharmacological agents, to alter HPAH course.

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Approaches directed toward the immediate downstream elements of signaling

There are two main ways in which BMPR2 signals inside the cell: through the SMAD family of transcription factors that directly regulate expression of a host of other genes involved in organism development; and through regulation of the cytoskeleton [15–17] (Figure 1). Many of the mutations found in patient families, however, leave SMAD signaling intact [18,19], and so targeting the defects in the cytoskeleton will probably be more broadly applicable therapeutically. These cytoskeletal defects are shared by most idiopathic patients as well [20].

What does this mean, functionally? Broadly, suppression of the BMP pathway appears to be a normal consequence of injury in the adult pulmonary vasculature; it has been seen as an immediate consequence of a great many different kinds of insult. Altered cytoskeletal regulation caused by suppression of the BMP pathway results in a number of changes that are an adaptive response to acute injury. These include: reduction in cell– cell junctions, allowing increased recruitment of inflammatory cells [21]; decreased planar polarity, facilitating processes needed for vascular repair [22]; and altered intracellular trafficking, needed to meet the altered metabolic demands of cells undergoing injury response [23,24]. In healthy individuals, BMP signaling recovers after the acute injury phase, allowing resolution of the healing program. When the injury response does not properly terminate, however, gradual loss of patency in the pulmonary vasculature can result.

At least two strategies have been proposed to attack the defects in signaling at the level of cytoskeletal defects, with success in rodents. Novartis scientists showed that use of a small molecule inhibitor of the interleukin (IL)-8 receptors CXCR1 and 2 reversed vascular leak and pulmonary hypertension in *Bmpr2* mutant mice [25]. Increasing angiotensin-converting enzyme (ACE)2 activity has reversed established PAH in hypoxic mice, monocrotaline-treated rats and *Bmpr2* mutant mice, using either small molecule agonists or recombinant ACE2 [15,26] (Figure 1). ACE2 converts the eight amino acid peptide angiotensin to the seven peptide Ang(1-7). Ang(1-7) signals through the Mas[s4] receptor, and directly corrects several of the cytoskeletal alterations caused by *Bmpr2* mutation [15].

Because recombinant ACE2 has been shown to increase cell–cell adhesion, it is currently in human clinical trials for acute lung injury (ClinicalTrials.gov identifier: NCT01597635), and so is probably the drug in this class closest to readiness for human trials in PAH (Figure 1). However, there are many potential mechanisms for intervention against this immediate downstream consequence of *Bmpr2* mutation, and further testing will be needed to identify the treatment with the greatest efficacy and specificity at this level of signaling.

Mixed effects related to functional crosstalk between BMP and estrogen signaling in PAH

It is well known that most PAH subtypes demonstrate elevated female prevalence among adult patients, and recent publications have begun to shed light on that discrepancy using the assessment of human subjects, as well as *in vitro* and animal model approaches [27–29]. In

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human PAH there is evidence of elevated circulating estrogens and skewed metabolism of estrogens that could result in enhanced functional estrogenic activity among those with and without *BMPR2* gene mutations [30–32]. Of note, a growing body of literature from the study of a variety of noncardiopulmonary conditions demonstrates a complex interaction between BMP signaling and estrogenic signaling (Figure 1). In breast cancer cells, for example, there is functional crosstalk between the BMP system and the actions of the estrogen receptors. BMPs can directly inhibit estrogen-induced breast cancer cell proliferation by inhibiting the expression of estrogens as well as via changing p38 phosphorylation. By contrast, estrogens reduce BMP-induced Smad [s5]signaling by downregulating *BMPR2* gene and protein expression in breast cancer cells [33].

In PAH recent data suggest that enhanced estrogenic activity contributes by manipulation of BMP signaling in the susceptible host, whereas altered BMP signaling modifies estrogenic activity. In humans, we demonstrated that female *BMPR2* mutation carriers with PAH had a significantly lower ratio of 2-hydroxyestrogens (2-$OHE_{1/2}$) to 16α-hydroxyestrone (16α-OHE_1) compared with unaffected female *BMPR2* mutation carriers [31]; and we found the same in a comparison of male *BMPR2* mutation carriers with PAH versus healthy male controls [32]. 16α-OHE_1 and other ‘16-estrogens’ possess estrogenic activity similar to estradiol, whereas ‘2-estrogens’ antagonize estrogenic activity. Subsequent *in vitro* studies of normal pulmonary microvascular endothelial cells demonstrated that estradiol, and ‘16-estrogens’ including 16α-OHE_1, directly reduce *BMPR2* gene expression [34]. Although the mechanisms behind this reduction require further exploration, this effect occurred at least in part via direct estrogen receptor alpha (ERα) binding to the *BMPR2* gene promoter in a manner similar to that seen in breast cancer cells [34]. However, highlighting the crosstalk in wild-type pulmonary microvascular endothelial cells transfected with *BMPR2* mutations showed there was profound dysregulation of ERα trafficking with movement of bound ERα to the cell surface instead of the nucleus.

Using *Bmpr2* mutant mice, we further explored the association of 16α-OHE_1 with PAH. Control mice and *Bmpr2* mutant mice were exposed to 16α-OHE_1 delivered by osmotic pumps. 16α-OHE_1 suppressed whole lung Bmpr2 protein levels threefold in the control mice. There was a corresponding reduction in BMP signaling, with a twofold drop in SMAD 1/5/8 phosphorylation in those mice. However, *Bmpr2* gene expression was unchanged in this model. The significance of this finding is an active area of investigation, with several possibilities including lysosomal regulation of *BMPR2* by estrogens via a nontranscriptional mechanism [35] or alternative splicing of *BMPR2* with the generation of alternative isoforms of *BMPR2* not differentiated using the gene expression techniques employed in this particular experiment (Figure 2b). Both of these explanations are plausible, because it is known that BMPR2 protein levels are tightly regulated via lysosomal degradation, and that *BMPR2* has multiple alternatively spliced variants of variable activity that can only be detected by specific gene expression assays [14,36] (Figure 2b). Consistent with human PAH, 16α-OHE_1-exposed animals also had a reduction in patent vessels, whereas disease penetrance was doubled among the Bmpr2 mutant mice.

Studies to date of PAH and the intricate relationship between BMP signaling and estrogenic activity suggest a complicated interrelationship, although much work remains to be done. In

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addition, it is important to recognize that estrogenic activity can be protective for other types of pulmonary hypertension, as reported in a recent comprehensive review by Umar *et al.* [37]. Regardless, the gender disparity in PAH, in concert with the developing focus on the modulation of BMP signaling, provides additional opportunities for synergy in the assessment and treatment of underlying defects associated with PAH.

Approaches directed at correcting metabolic defects

Alterations of the cellular metabolic program are increasingly recognized as a major contributing pathogenic mechanism in a variety of complex diseases, including PAH. Several groups have provided compelling evidence for an increase in glycolysis despite adequate oxygen supply (also known as the Warburg effect) in experimental and human PAH, as well as upregulation of glutamine metabolism, changes in fatty acid metabolism and disruption of other major metabolic pathways [38,39]. These are very similar to changes in cellular metabolism that have been identified in cancer. The metabolic changes in cancer are thought to enable malignant cells to avoid apoptosis, to proliferate rapidly and to maintain the ability to make all of the necessary building blocks for rapid growth. The current thinking is that these same metabolic alterations permit a similar set of changes in the pulmonary vasculature and the right ventricle in PAH, which are ultimately maladaptive and lead to disease. This places metabolic changes in a causative role for PAH.

What drives these widespread changes in cellular metabolism in PAH? In cancer, such changes are related to mutations or changes of function in oncogenes (e.g. Myc) and/or tumor suppressor genes [e.g. superoxide dismutase (SOD)2] as the inciting event [40,41]. Indeed, some of these same pathways have been identified as being altered in PAH [38,42]. However, these are probably secondary events in the pathogenesis of PAH and perhaps should be thought of as disease-sustaining as opposed to primarily causative changes. In PAH loss of function in the BMP pathway has the strongest evidence in humans for being primarily causative, either through mutations in *BMPR2* or through functional loss of BMP signaling. Although not classically thought of as a key regulator of metabolism, the BMP pathway could very reasonably be described as such.

A rapidly developing volume of literature places BMP signaling at the center of controlling the metabolic behavior of brown adipose tissue at the cellular and whole organism levels, with decreased BMP signaling leading to a decreased ability to generate heat and an increased susceptibility to obesity [43]. This is in line with the changes in multiple metabolic pathways at the cellular and whole organism level that have been demonstrated downstream from *BMPR2* mutations associated with PAH [23,44]. Evidence for similar metabolic changes has been found in human PAH patients, both in HPAH patients with known *BMPR2* mutations and in idiopathic PAH (IPAH) patients, the majority of whom have decreased *BMPR2* function [45].

Targeting the downstream metabolic consequences of impaired BMP signaling might prove to be a more therapeutically tractable strategy than targeting the BMP receptors themselves. There are many drugs available with some effect to modulate molecular metabolism. Some are used for specific metabolic effects (e.g. dichloroacetate, trimetazidine), some have

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metabolic effects that are well defined but less well understood at the mechanistic level (e.g. metformin) and some have metabolic modulatory effects as a side-effect largely unrelated to the originally identified mechanism of drug action (e.g. ranolazine) (Figure 1). Additionally, future therapeutics will probably exploit the full complement of pharmacological, cell-based and surgical therapies from such diverse research disciplines such as diabetes, obesity and cancer and bring them to bear in the treatment of PAH [46].

Concluding remarks

BMP signaling is impaired in most IPAH and HPAH. We can try to rescue BMP signaling upstream, at the level of the receptor and its ligands; we can rescue immediately downstream at the level of defective cytoskeletal signaling; or we can rescue the most important functional consequences, which appear to include metabolic derangements. Increased estrogenic activity appears to worsen BMPR2 expression as well as to exacerbate directly the metabolic defects caused by suppressed BMP signaling. Although no treatment targeting any of these pathways is currently approved for patients, trials in humans are underway or in development for all of these. Within the next few years, we expect new, much more effective, therapies for PAH to be available, based on the deeper understanding of the molecular etiology that has emerged from the combined efforts of the global PAH research community.

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Highlights

- BMPR2 signaling is an important factor in PAH
- This is an article that was commissioned for *Drug Discovery Today: Disease Mechanisms*
- This article is part of a themed section on pulmonary disease

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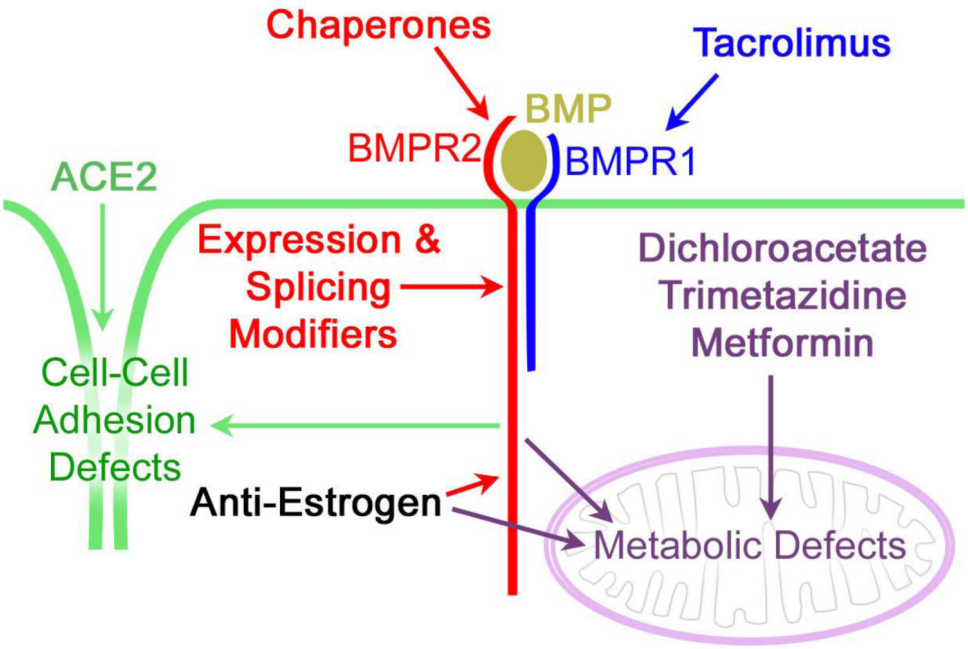

Figure[s11] 1.

Mechanisms of modulation of bone morphogenic protein receptor 2 (BMPR2) signaling.

Abbreviation: ACE, angiotensin-converting enzyme.

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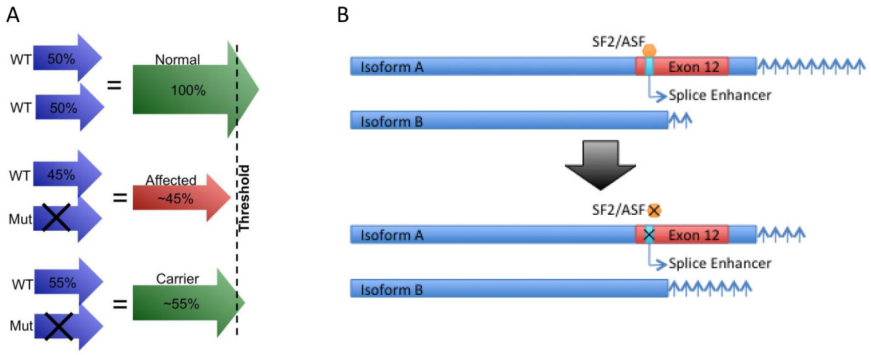

Figure[s12] 2.

Affect of bone morphogenic protein receptor 2 (*BMPR2*) expression and splicing on cellular BMPR2 levels, signaling and disease penetrance. **(a)** In mutation carriers higher levels of the nonmutated wild-type (WT) *BMPR2* allele protect against clinical disease. **(b)** Exon 12 inclusion (full-length *BMPR2/isoform-A*) or exclusion (*isoform-B*) is controlled by splice enhancers in exon 12 and the splicing factors such as SF2/ASF [s13]that bind to them. Mutation in the splicing enhancer, which prevents binding of the splicing factor (donated by X), or decreased levels of the factor itself (donated by X) results in exon 12 exclusion from the transcript leading to significantly more isoform B in the cell relative to the full-length *isoform-B* and the resultant disruption of BMP signaling.