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## Targeting Vascular Remodeling to Treat Pulmonary Arterial Hypertension

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### Abstract

Pulmonary arterial hypertension (PAH) describes a group of conditions with a common hemodynamic phenotype of increased pulmonary artery pressure, driven by progressive remodeling of small pulmonary arteries, leading to right heart failure and death. Vascular remodeling is the key pathological feature of PAH but treatments targeting this process are lacking. In this review, we summarize important advances in our understanding of PAH pathogenesis from genetic and epigenetic factors, to cell metabolism and DNA damage. We show how these processes may integrate and highlight exploitable targets that could alter the relentless vascular remodeling in PAH.

## **Pulmonary Vascular Remodeling in PAH – Many Problems, Few Solutions.**

Pulmonary hypertension (PH) is defined by a mean pulmonary artery pressure of greater than 25 mmHg at rest [1]. Diagnostic groups are further classified according to clinical, etiological and hemodynamic features (Table 1) **but share common symptoms of increased breathlessness on exertion, right heart failure and ultimately result in premature death** [1]. **Pulmonary arterial hypertension (PAH)** (see Glossary) is characterized by sustained vasoconstriction and a progressive obliteration of small resistance pulmonary arteries and arterioles through a process of intimal and medial thickening and the formation of **angioproliferative plexiform lesions** [2]. Pulmonary vascular insults cause **endothelial dysfunction** and apoptosis **thereby impairing endothelial-mediated suppression of quiescent smooth muscle cells** [3]. This, **coupled with clonal expansion of apoptosis-resistant endothelial cells,** promotes a **proliferative vasculopathy** that also involves a complex interplay with adventitial fibroblasts, perivascular inflammatory cells and the extracellular matrix [3]. Despite considerable advances in PAH treatment, this devastating disease still carries a prognosis worse than many cancers with a 3-year survival of 68-70% [4, 5]. Current therapies predominantly target **pulmonary vasoconstriction** rather than proliferative vascular remodeling and therefore new strategies are urgently required to directly address the pathological remodeling that underpins the disease.

**PAH constitutes diagnostic Group 1 (Table 1) and** is characterized by added hemodynamic criteria that define **pre-capillary pulmonary hypertension** with absence of left-sided heart disease (Group 2), lung diseases (Group 3) or **thromboembolic disease** (Group 4). However, Group 1 comprises patients with a wide range of conditions that are associated with the development of PAH and thus within this group, there may be considerable mechanistic heterogeneity at a molecular **and pathophysiological** level. Consequently, we limit this review by focusing on key pathogenic processes of **idiopathic PAH (IPAH) and its heritable form (HPAH), rare conditions with a combined** annual incidence of approximately 1-2 per million [4, 6].

The breadth of disruption of pulmonary vascular cell biology in PAH is becoming apparent. Recent studies of genetic and epigenetic factors, **DNA damage** and disordered metabolism have yielded important mechanistic insights into established disturbances of endothelial function and smooth muscle cell proliferation. **We discuss**

the central importance of **bone morphogenetic protein** receptor 2 (BMPR-II) signaling in PAH and focus on approaches to rescue the disease-associated suppression of this pathway. Other new genetic leads are explored and the rapidly expanding field of microRNA biology and its relevance to PAH is discussed. Furthermore, work on triggers for PAH development has added to our understanding of the role of inflammation and hypoxia in PAH and we highlight key recent findings in these areas. Although it is not yet clear how all of these problems combine temporally and mechanistically, these advances bring enormous potential for new therapeutic approaches that specifically target vascular remodeling, providing fresh hope of significant impact on disease progression and improved patient outcomes.

### **The Ups and Downs of BMPR-II Signaling in PAH.**

Since the identification of mutations in the gene encoding bone morphogenetic protein receptor 2 (BMPR2) in families with PAH [7, 8], BMPR2 has become the predominant genetic factor in heritable forms of the disease, with a large amount of evidence implicating it as a central molecular player in PAH. BMPR-II is a member of the transforming growth factor beta (TGF- $\beta$ ) receptor family that forms a dimer with an activin receptor-like kinase (ALK) [9]. There are multiple ALKs and each heterodimeric combination confers different ligand affinity [10]. Thus, differential expression in tissues may alter sensitivity to specific bone morphogenetic protein (BMP) ligands. Notably, the BMPR-II/ALK-1 heterodimer signals with relative specificity in response to BMP9 and BMP10 in human microvascular endothelial cells (ECs) [11] while BMPR-II/ALK-3 or -6 signals in response to BMP2 or BMP4 in smooth muscle cells [12]. Upon ligand binding, the receptor phosphorylates **SMAD proteins** (mothers against decapentaplegic homologs) that complex and translocate to the nucleus, regulating in turn the expression of target genes (e.g. inhibitor of DNA binding (ID) proteins) through SMAD binding elements (Figure 1) [13].

Mutations in BMPR2 have been found in >70% of patients with heritable disease [7, 8] and in up to 25% of patients with idiopathic disease [14]. On the one hand, reduced protein expression of BMPR-II is also found in patients with PAH devoid of BMPR2 mutations [15], emphasizing the importance of this signaling pathway in disease. But on the other hand, in HPAH, while transmission is autosomal dominant, only 20% of carriers develop disease, implying that other triggers are required [16]. The importance of environmental or host factors (Figure 2) in addition to impaired BMPR-II signaling is

supported by evidence from BMPR-II-deficient animal models. For instance, mice with heterozygous null or hypomorphic *Bmpr2* mutations do not develop spontaneous PAH, and only around 30% of mice with either Cre-dependent deletion of *Bmpr2* in ECs [17] or inducible overexpression of a dominant negative BMPR-II in smooth muscle cells [18] show evidence of elevated **right ventricular systolic pressure** (RVSP). In contrast, a more recent study used mice incorporating a heterozygous knock-in allele bearing the R899X stop mutation in exon 12, a *Bmpr2* mutation associated with human PAH, and these animals developed spontaneous elevation in RVSP by 6 months of age [19]. In this model, penetrance and disease severity were enhanced by additional knockout of the *Smad1* gene, suggesting that suppression of BMPR-II signaling might be sufficient to precipitate disease [19]. However, the explanation for the difference in disease susceptibility between mice with haploinsufficiency due to heterozygous null or R899X mutations in *Bmpr2* remains unclear and warrants further investigation. Interestingly, a transgenic rat with a heterozygous 140 base pair deletion in exon 1 of the *Bmpr2* gene has recently been described [20]. While not yet fully phenotyped, by 3 months of age these rats developed abnormal muscularization of small pulmonary arteries compared to wild-type littermates, although this was not associated with significant hemodynamic changes at that age [20]. These early remodeled vessels showed evidence of **endothelial-to-mesenchymal (EndoMT)** transition, with overexpression of relevant markers (such as Twist-1 and phosphorylated vimentin) by immunohistochemistry [20]. Evidence of EndoMT was also observed by staining human PAH lung sections and sections from **monocrotaline**-treated rats [20]. Another study demonstrated that siRNA knockdown of BMPR2 induced mesenchymal phenotypic changes in human pulmonary artery endothelial cells (PAECs) [21]. This was associated with increased expression of **high mobility group AT-hook 1** (HMGA1) and Slug, transcription factors that have been implicated in epithelial-to-mesenchymal transition and are overexpressed in PAECs from patients with BMPR2 mutations [21].

BMPR-II deficiency also impacts upon mechanisms involved in the reversal of vascular remodeling. In the knowledge that **hypoxia-induced pulmonary hypertension** in mice is ubiquitously reversible, one study demonstrated that EC-specific deletion of *Bmpr2* inhibited reversal of PH upon reoxygenation [22]. Mitochondrial dysfunction in BMPR-II deficient endothelial cells led to mitochondrial DNA damage and apoptosis, preventing regeneration of distal pulmonary arteries following hypoxia/reoxygenation [22]. Importantly, the sensitivity of PAECs to **mitochondrial-induced apoptosis** with

hypoxia/reoxygenation was confirmed in vitro in primary cells from patients with BMPR2 mutations [22]. While broader abnormalities in cellular metabolism -- including aerobic glycolysis, pentose phosphate pathway activation and abnormal fatty acid metabolism -- have been reported in PAH (Box 1), this finding of mitochondrial dysfunction in BMPR-II-deficient cells implies that genetic predisposition directly alters the capacity to repair a pulmonary vascular injury. Moreover, as will be discussed later, impaired BMPR-II signaling predisposes to pro-inflammatory phenotypes in pulmonary vascular tissue cells that may exacerbate the injury response and promote disease progression [22]. While targeting EndoMT, mitochondrial dysfunction and inflammation may be possible, the pleotropic effects of suppressed BMPR-II signaling in PAH stress the importance of restoring this pathway as a therapeutic objective.

### The Sexy Side of BMPR-II.

An important host factor associated with disease susceptibility is sex, with a female to male ratio of approximately 2.5:1 in recent IPAH cohorts. [4, 6]. The mechanisms underlying this predisposition are becoming clearer and link closely to BMPR-II signaling. For example, estrogen has been shown to suppress BMPR-II signaling [23] with sex-specific consequences for in vitro PASMC phenotypes [24]. Female, but not male human PASMCs, proliferate in response to the same dose of key mitogens (e.g. serotonin (5HT), platelet-derived growth factor (PDGF)), suggesting heightened sensitivity to pro-proliferative stimuli [24]. In this study, sex differences were attributed to suppression of SMAD activation upon stimulation, in addition to a reduction in basal expression of BMPR-II pathway components including BMPR-II, SMAD1/5/8, and the ID proteins, ID1 and ID3 [24]. These in vitro findings are supported by the in vivo observation that the aromatase inhibitor **anastrozole** reduced progression of established PAH in female rats and mice [25]. Translation of these pre-clinical studies into human trials is eagerly anticipated, particularly in view of recent evidence, from a small case-control study, that male patients with PAH exhibit significantly higher levels of circulating estradiol when compared to healthy controls [26]. However, there is an apparent paradox in these findings, as male sex is a predictor of worse survival in patients with PAH, a finding associated with poorer right ventricular function [27, 28]. Possible explanations for this include the relative deficiency in male patients of dehydroepiandrosterone [26], a precursor of testosterone and estrogen that is protective in rodent PH models [29]. Alternatively, these findings could be linked to the differential

effects of male and female sex hormone metabolite profiles on the pulmonary vasculature and right ventricle (reviewed in [30]).

### **More BMPR-II –Amplifying the Signal for Therapeutic Benefit.**

The evidence implicating BMPR-II deficiency in PAH pathogenesis has driven efforts to enhance or restore the BMPR-II signaling. Moreover, therapeutic agents targeting this mechanism are progressing into human trials (Figure 1). One study performed a high throughput screen of FDA-approved drugs using a luciferase reporter assay and identified **FK506 (tacrolimus)** as an activator of BMPR-II signaling [31]. FK506, administered via continuous subcutaneous infusion by osmotic pump, prevented the development of PAH in mice with endothelial deletion of *Bmpr2* and furthermore, reversed established PAH in two **rat models** [31]. Indeed, there are also initial reports of positive clinical responses from compassionate use of FK506 in patients with severe PAH but these beneficial effects will require verification in ongoing clinical trials [32].

Other work has demonstrated direct amplification of BMP signaling using the endothelial selective ligand, BMP9 [19]. In vitro, BMP9 reduced tumor necrosis factor (TNF $\alpha$ )-induced EC apoptosis and inhibited endothelial monolayer permeability induced by endotoxin in both pulmonary artery ECs, as well as in **blood outgrowth ECs** [19]. In rodent models, BMP9 reversed the spontaneous development of PAH in mice with heterozygous R899X mutations and reversed established PAH in rat models [19]. Earlier work from the same group demonstrated that the lysosomal inhibitor chloroquine could increase the cell surface expression of BMPR-II in ECs, and could be used as a strategy to restore BMP9-BMPR-II signaling in cells with BMPR2 mutations [33]. Although EC apoptosis and monolayer permeability are enhanced by BMPR-II haploinsufficiency [34], it was notable that the in vitro benefits conferred by BMP9 were preserved in cells bearing BMPR2 mutations, implying that reduced signaling can be directly augmented by BMP9 without restoration of receptor expression [19].

Augmentation of the pathway via suppression of negative regulation is another emerging strategy. The downstream effectors of BMPR-II signaling, SMAD proteins, are negatively regulated by ubiquitination and proteasomal degradation [35]. Recent evidence in rodent models of PAH and in explanted lung tissue from patients with or without BMPR2 mutations has demonstrated upregulation of SMURF1, a ubiquitin ligase that can target SMAD proteins and BMP receptors, [36, 37]. Indeed, genetic deletion of **Smurf1** protected mice from the development of experimental PAH while

nebulized administration of a micro-RNA (miR-140-5p), shown to target and repress SMURF1, attenuated PAH in rat models [36]. As small molecule inhibitors of SMURF1 are now available, these may potentially offer another mode of enhancing BMPR-II signaling [38].

Finally, a different group described an additional mechanism to restore BMPR-II signaling via activation of the transcription factor, **Forkhead box O1 (FoxO1)** [39]. This master-regulator of cellular proliferation was shown to be downregulated in PAH in human lungs, a finding that could be replicated through exposure of PASMCs to inflammatory cytokines (**TNF $\alpha$ , IL-6**) and growth factors (**PDGF, insulin growth factor 1 (IGF-1)**) ex vivo [39]. **Pharmacological** inhibition of FoxO1 induced proliferation of PASMCs and furthermore, smooth muscle-specific FoxO1 deletion in mice led to a spontaneous PH phenotype [39]. Conversely, activation of FoxO1 with the chemotherapeutic agent, **paclitaxel**, suppressed PAH PASMC proliferation and reversed in vivo PH models [39]. Although the activation of FoxO1 was associated with restoration of BMPR-II signaling in animal models, suppression of in vitro PASMC proliferation was not dependent upon an intact BMPR-II pathway, implying that paclitaxel might have therapeutic benefits even in the presence of BMPR2 mutations.

**These strategies to target one of the key pathways implicated in PAH pathogenesis offer real potential to translate into disease-modifying treatments, even for patients with BMPR2 mutations. However, given diversity of the molecular mechanisms involved and the limited pool of patients for large randomized trials, work to predict those likely to respond to specific strategies should be encouraged.**

### **Beyond BMPR2 – Other Genetic Regulators of IPAH.**

Analysis of candidate BMP/SMAD pathway genes have revealed rare mutations in PAH patients including SMAD1, SMAD4 and SMAD9 (reviewed in [14]). Next generation techniques such as **whole exome sequencing** have identified mutations in other genes providing insight into other potentially important regulators of PAH pathogenesis. For example, a study of a large family with autosomal dominant HPAH revealed a shared mutation in exon 3 of CAV1 [23]. The product of this gene, Caveolin-1, is a structural component of specialized plasma membrane microdomains, caveolae, which are critical for the maintenance of endothelial function and permeability [40]. **Interestingly, pulmonary artery ECs from mice with heterozygous null Bmpr2 mutations have caveolar**

trafficking defects and these have been linked to the impaired barrier function exhibited by these cells [41].

In a further study of HPAH patient exome sequences, Ma et al. identified a coding variant in the potassium channel, subfamily K, member 3 (KCNK3) [42]. Expression of KCNK3 and its current was subsequently found to fall in rat smooth muscle cells, freshly isolated during development of monocrotaline-induced PH, while pharmacological inhibition of KCNK3 induced pulmonary vascular cell proliferation in vivo in rats and increased right ventricular systolic pressure [43]. By contrast, activation of KCNK3 attenuated PH development but was unable to reverse the disease, perhaps due to disease-mediated reduction in channel expression [43].

In a study of IPAH patients with no known PAH-linked mutations, candidate genes associated with genetic variants were prioritized on the basis of putative relevance to pulmonary biology, human disease and known expression in heart and lung tissue [44]. This study reported **TopBP1** as a candidate gene of interest, given its role in the DNA damage response, an emerging area of PAH biology (Box 2). Mutations in this gene may contribute to disease penetrance in BMPR2 mutation carriers or alter disease susceptibility in the context of environmental triggers (Figure 2).

Given the rarity of IPAH, genome-wide association studies have been limited by insufficient sample sizes. To improve power, one study employed a case-control approach in two independent cohorts of patients without detectable BMPR2 mutations and identified two polymorphisms downstream of the CBLN2 gene that were associated with IPAH [45]. How the protein product, Cerebellin-2, contributes to PAH pathogenesis remains unclear, but may involve inhibition of PASMC proliferation [45].

While there is huge potential to discover rare genetic variations associated with PAH through the analysis of coding sequences, and now, whole genome sequencing with next generation sequencing, interpretation of these data remains difficult. Indeed, large patient numbers are required to identify rare causal variants with sufficient power. The successful use of these technologies in this rare disease will therefore require broad international collaborations.

### **The Role of microRNAs in PAH.**

Beyond the genetic factors involved in PAH, a broad disruption of transcriptional regulation is now also evident. The expression of numerous micro-RNAs (miRs) is altered in the circulation and lung tissue of patients with PAH [36, 46, 47] and, as

mentioned, miRs can affect BMPR-II signaling [36] and DNA repair mechanisms in PAH (Box 2) [48].

Most studies have focused on individual miRs and cell-specific responses in PAH. However, an elegant study using a network-based bioinformatics approach has shown evidence of a coordinated regulation of miR biology in PAH [49]. This study identified members of the miR-130/301 family, as key regulators of other miRs and consequent cell phenotypes in PAH [49]. The authors proposed that miR-130/301 represses **peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ )**, a target of BMP signaling [50], and confirmed this in vitro by forced expression of miR-130a in human ECs and PASMCs [49]. PPAR $\gamma$  might in turn regulate both a STAT3-miR-204-SRC pathway implicated in PASM C proliferation and apoptosis resistance [51] and an apelin-miR-424/503-FGF2 pathway, to maintain PAEC homeostasis and suppress PASM C proliferation [52]. Moreover, upregulation of the miR-130/301 family was demonstrated in rodent models and lung sections and plasma from patients with PAH [49]. In addition, factors capable of inducing its upregulation in vitro included hypoxia and inflammatory cytokines (IL-1 $\beta$  and IL-6), known triggers or drivers of PAH.

The explosion in miR biology opens many potential therapeutic avenues and a variety of miR mimics or inhibitors have shown efficacy in animal PAH models (Table 2). However, target specificity, cellular penetration, degradation and possible hepatotoxicity represent significant hurdles that need to be overcome for these molecules to translate into therapeutic interventions in humans.

### **Disease Drivers in PAH.**

While the described disturbances in BMPR-II pathway regulation, metabolism and DNA damage/repair mechanisms are fundamental in PAH pathophysiology, the triggers and drivers of disease (both in the presence and absence of genetic predisposition) remain unclear. Recent studies have provided insight into the involvement of hypoxia and inflammation as potential environmental mediators or drivers of PAH.

### **Hypoxic Signals Triggering PAH.**

Ambient hypoxia is widely used as a stimulus for a proliferative vasculopathy in animal PH models; it also triggers reversible pulmonary vascular remodeling in humans following ascent to high altitude [53, 54]. Alveolar hypoxia is likely to be an integral pathogenic factor in Group 3 patients (Table 1) but **relative pulmonary arterial hypoxia**

due to progressive **mixed venous hypoxemia** in IPAH may also be an important driver of this disease. Cellular oxygen-sensing is multi-layered and tissue-specific but the hypoxia-inducible transcription factors, **HIF-1 $\alpha$**  and **HIF-2 $\alpha$** , are key regulators of hypoxic adaptation in pulmonary vascular cells (reviewed in [55]). Expression of HIF- $\alpha$  subunits is tightly controlled by oxygen-sensitive **prolyl hydroxylase domain-containing enzymes (PHDs)** which, in the presence of oxygen, hydroxylate and target HIF- $\alpha$  subunits for ubiquitination by the **von-Hippel Lindau protein (VHL)** and proteasomal destruction [56]. It has long been known that heterozygosity of either Hif1a [57] or Hif2a [58] protects mice from hypoxia-induced PH but the mechanism has been unclear. The dominant regulation of metabolic genes by HIF-1 $\alpha$  is likely to be important given the metabolic changes favoring aerobic glycolysis in pulmonary vascular cells (Box 2) and normoxic stabilization of HIF-1 $\alpha$  has been described in **human pulmonary vascular lesions** [59]. However, recent evidence has highlighted the importance of endothelial HIF-2 $\alpha$  in animal models of PH.

One study reported that mice harboring a Phd2 (**Egln1**) deletion in endothelial and hematopoietic cells (Tie2-Cre expression) developed a spontaneous and fatal PH phenotype with impressive pulmonary vascular remodeling and hemodynamic changes [60]. As expected, both HIF- $\alpha$  isoforms were upregulated in normoxic PHD-2 deficient ECs, but double knockout studies (**Phd2 and Hif1a or Hif2a**) indicated HIF-2 $\alpha$  was the key mediator of the phenotype [60]. Several pathways implicated in PH were altered in PHD-2 deficient mice including downregulation of Bmpr2 and Cav1 and could be rescued by deletion of Hif2a [60]. The proposed mechanism involved HIF-2 $\alpha$ -mediated upregulation of chemokine CXCL12, a proliferative stimulus for PSMCs. Similar findings were independently obtained using an alternative mouse line (Cdh5-Cre expression) to conditionally delete Phd2; however, **this paper focused on hypoxic induction of the vasoconstrictor endothelin-1 (Edn1) as a potential mechanism, showing HIF-2 $\alpha$ -dependent increases in lung expression of Edn1 in PHD-2 deficient mice** [61]. Interestingly, PAH has also been described in humans with rare gain-of-function mutations in HIF2A [62]. However, PAH was not reported as a cause of death in a small study of patients with HIF activation due to mutations in the VHL gene (**Chuvash polycythemia**) [63], even though such patients do present heightened pulmonary vasoconstriction in response to hypoxia [64].

There are several alternative mechanisms for the regulation of PAH by HIF-2 $\alpha$ . It has been recently demonstrated that the protection against hypoxia-induced PH mediated by EC deletion of Hif2a **in mice** is associated with reduced expression of arginases and that deletion of endothelial Arg1 reproduced a protective phenotype [65]. Moreover, the endothelial NO pathway is strongly implicated in PAH and may represent an important therapeutic target [66]. Thus, diversion of L-arginine from nitric oxide synthase through increased arginase activity may be a credible disease driver. Notably, a further target of HIF-2 $\alpha$  is the transcription factor, POU5F1 (or OCT4), which can upregulate miR-130/301 **in ECs and PASMCs exposed to** hypoxia, as discussed earlier [49]. Finally, through elegant linkage analysis and congenic breeding of a rat strain known to be resistant to hypoxia-induced PH (the Fisher 344 strain), the **Slc39a12** gene was identified as a novel HIF-activated regulator of hypoxia-induced pulmonary vascular remodeling [67]. The upregulation of the protein product, zinc transporter (ZIP12), in hypoxic pulmonary vascular tissue was conserved across several species and confirmed in IPAH tissue, while mutation of Slc39a12 attenuated hypoxia-induced pulmonary hypertension in rats [67]. In light of these multiple strands of evidence for HIF-pathway activation in PAH, it may be important to consider screening for PH in patients treated with PHD inhibitors **(e.g. Roxadustat)**, which are progressing in clinical trials to ameliorate **renal anemia** [68].

### **Inflammation and Cytokines – Potential Targets in PAH.**

Perivascular inflammation is a characteristic feature of PAH [2] and many inflammatory cell-types are mooted to have roles in disease pathogenesis [19, 69]. Elevated inflammatory cytokine levels are described in IPAH patients [70, 71] but whether inflammation is a trigger for disease in susceptible patients or a consequence of established disease is not clear. There is increasing evidence that genetic predisposition to PAH through BMPR-II deficiency involves a pro-inflammatory phenotype. **Human PAECs transfected with BMPR2 siRNA exhibited enhanced expression of IL-6 and IL-8 compared to control cells [22] and BMPR2<sup>+/-</sup> human PASMCs released more IL-6 in response to endotoxin (LPS) [72]. BMPR-II deficient mice showed increased expression of IL-6 and KC (murine IL-8) in lung tissue in response to LPS and these findings were mirrored in murine PASMCs ex vivo [72]. Of note, murine PASMCs were isolated from haploinsufficient BMPR-II mice with no**

evidence of PH but exposure of such mice to LPS for 6 weeks was sufficient to induce disease supporting the hypothesis that inflammation plays an initiating role in PAH, at least in the context of BMPR-II deficiency [72]. BMPR-II deficient human and murine PASMCs and *Bmpr2* haploinsufficient mouse lungs demonstrated superoxide dismutase (SOD3) deficiency, implicating impaired handling of reactive oxygen species in the mechanism underlying the pro-inflammatory phenotype in these models [72]. Indeed, both in vitro inflammatory responses due to BMPR-II deficiency and the development of PH in BMPR-II deficient mice following chronic LPS exposure could be attenuated by tempol, an antioxidant [72]. These data imply that antioxidant supplementation and targeting specific cytokines (e.g. IL-6) might represent strategies to prevent the development of PAH in patients with BMPR2 mutations.

How BMPR-II regulates increased inflammatory cytokine expression is not fully understood but recent work has implicated the eukaryotic translation initiation factor (eIF2) as a translational regulator of TNF-induced granulocyte macrophage colony-stimulating factor (GM-CSF) release from human PAECs [73]. In this study, BMPR-II deficiency was associated with sustained activation of p38 mitogen-activated protein kinase (MAPK) following TNF stimulation which indirectly led to de-phosphorylation of eIF2 and thus, enhanced GM-CSF translation [73]. In human IPAH there was evidence of higher GM-CSF expression in diseased lung tissue, while administration of GM-CSF in mice worsened PH in chronic hypoxia, consistent with a role for this cytokine in disease progression [73]. The importance of wider translational repression by eIF2 deserves exploration given the observation that mutations in this pathway -- specifically loss-of-function homozygous and compound heterozygous mutations in *EIF2AK4*, the gene encoding eIF2 $\alpha$  kinase 4 -- have been documented in other conditions of aberrant pulmonary vascular remodeling such as **pulmonary veno-occlusive disease** and **pulmonary capillary hemangiomatosis** [74, 75].

Associated with the enhanced GM-CSF signaling described above, an increase in perivascular cells expressing macrophage markers was reported [73] suggesting that macrophage-derived cytokines such as leukotriene B<sub>4</sub> [76] and IL-6 [70, 77] may be implicated in PAH. Given the potential for biological targeting of specific cytokines that may be pathogenically over-expressed in PAH, the role of macrophage and tissue-derived cytokines in vascular remodeling is of particular interest.

Our group explored the role of TNF-related apoptosis-inducing ligand (TRAIL) in PAH, a TNF family member. Expression of TRAIL was upregulated in lung tissues from rodent

models of PH [78], in human lung sections and in PSMCs isolated from PAH patients [79]. Despite its known role of inducing apoptosis in transformed or malignant cells TRAIL acted as a PSMC mitogen in vitro [80]. Furthermore, blockade of TRAIL by either genetic deletion in mice or by administration of an anti-TRAIL polyclonal antibody in rats, prevented development of PH [80]. Moreover, polyclonal antibody targeting of TRAIL reversed established disease in mouse PH models [80]. Of note, osteoprotegerin (OPG), a binding partner for TRAIL -- best known for its role in bone formation -- is similarly of mechanistic and therapeutic interest [78]. In fact, levels of OPG are also increased by suppressed BMPR-II, serotonin and inflammatory stimuli (e.g. IL-1) [79], and serum levels correlated with markers of disease severity (right atrial pressure and cardiac index) and predicted survival in PAH patients [81]. In addition, OPG, like TRAIL, drives proliferation of human PSMCs in vitro [79, 80]. Thus, studies investigating the therapeutic potential of targeting OPG in rodent models are currently underway [82].

### **Concluding Remarks**

Recent advances have revealed a wide range of biological disruption in PAH including metabolic, inflammatory and epigenetic abnormalities (Figure 2, Box 3). Understanding how all these processes integrate to promote the aberrant proliferative remodeling characteristic of PAH will be key to the generation of successful anti-remodeling therapies. Other questions also remain (see Outstanding questions), in particular how best to increase the success rate of translating pre-clinical discoveries to the bedside, which has been a significant challenge in PH [83]. It is noteworthy that the majority of studies in this review highlighting new therapeutic targets have incorporated both in vitro evidence from patient cells and often more than one animal model of disease, hopefully strengthening the translational potential of these discoveries.

Clear hubs for new therapies to target certainly exist. As highlighted above, novel strategies to enhance BMPR-II signaling (Figure 1) may be among the first of a new generation of drugs for PAH that specifically target known defective molecular regulators of pulmonary vascular remodeling. In addition, our increased understanding of metabolic abnormalities and inflammation has led to clinical trials targeting the metabolic switch to a pro-proliferative glycolytic phenotype in pulmonary vascular cells

(e.g. **Dichloroacetate**, NCT01083524) and pro-proliferative cytokine pathways (e.g. anti-IL-6 antibody, **Tocilizumab**, NCT02676947).

However, as different clinical groups, sexes and individuals are likely to have different drivers of disease, molecular phenotyping of patients with a precision medicine approach, akin to that used in cancer, may be required to identify patients most likely to respond to specific treatments. For example, in the context of reduced BMPR-II signaling (Figure 1), an individual with high SMURF1 protein expression or activity may respond better to a SMURF1 inhibitor rather than a treatment aiming to augment BMPR-II expression. To open the door for precision medicine, we need to integrate complex multi-omic datasets and generate a more comprehensive understanding of the heterogeneity in disease mechanisms, a strategy that will require a strong international collaboration. Nonetheless, for this devastating condition characterized by relentless vascular remodeling, the emerging opportunities described above provide hope of novel anti-remodeling treatments that will significantly improve patient outcomes in PAH.

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**TABLE 1. Clinical Classification of Pulmonary Hypertension.**

WHO group	Classification	Hemodynamics	Sub-classification
1	Pulmonary arterial hypertension	Pre-capillary mPAP $\geq 25$ mmHg PAWP $\leq 15$ mmHg	Idiopathic Heritable Drugs and toxins induced Associated with: Connective tissue disease HIV infection Portal hypertension Congenital heart disease Schistosomiasis
1'	PVOD and/or PCH	Pre-capillary	
2	Pulmonary hypertension due to left heart disease	Post-capillary mPAP $\geq 25$ mmHg PAWP $> 15$ mmHg	Systolic dysfunction Diastolic dysfunction Valvular disease Outflow/inflow tract obstruction Congenital cardiomyopathies
3	Pulmonary hypertension due to lung disease and/or hypoxia	Pre-capillary	Chronic obstructive pulmonary disease Interstitial lung disease Sleep-disordered breathing Alveolar hypoventilation Chronic exposure to high altitude Developmental lung diseases
4	Chronic thromboembolic pulmonary hypertension	Pre-capillary	Thromboembolic disease
5	Pulmonary hypertension with unclear multifactorial mechanisms	Pre-capillary or combined pre- and post-capillary	Hematological disorders e.g. Splenectomy Chronic hemolytic anemia Myeloproliferative disorders Systemic disorders e.g. Sarcoidosis Pulmonary histiocytosis Metabolic disorders e.g. Glycogen storage disease Gaucher's disease Others e.g. Tumors Chronic renal failure

WHO, World Health Organization; mPAP, mean pulmonary artery pressure; PAWP, pulmonary artery wedge pressure; PVOD, pulmonary veno-occlusive disease; PCH, pulmonary capillary hemangiomas.

**Table 2. Therapeutic Manipulation of microRNAs in Animal Models.**

microRNA	Target	Evidence from miR-based therapies in animal models	Ref
miR-17	p21	Intravenous anti-miR-17 treatment reduced RVSP and remodeling in hypoxic mouse and MCT rat models.	[84]
miR-20a	SMAD5	Prophylactic intraperitoneal injections of anti-miR-20a prevented RVH and remodeling in hypoxic mouse model.	[85]
miR-21	BMPR2, WWP1	Intratracheal anti-miR-21 (prophylactic or treatment) reduced RVH and remodeling in hypoxic mouse model.	[86]
miR-26b	CTGF, CCND1	Intratracheal treatment with mir-26b mimic reduced remodeling in MCT rat model.	[87]
miR-27b	PPAR $\gamma$	Intravenous anti-miR-27b treatment reduced RVSP, RVH and remodeling in rat MCT model.	[88]
miR-29	PPAR $\gamma$	Intravenous anti-miR-29 injection attenuated RVSP and remodeling in Bmpr2 <sup>R899X</sup> (Doxycycline-induced transgene expression) model.	[89]
miR-34a	PDGFRA	Nebulized treatment with miR-34a mimic reversed PH in hypoxic rat model.	[90]
miR-96	5-HT <sub>1B</sub>	Prophylactic intravenous administration of miR-96 mimic to female mice attenuated RVSP, RVH and remodeling in hypoxic mouse model.	[91]
miR-126	SPRED-1	Intravenous treatment with miR-126 mimic improved RV function and CO but had no significant effect on RVSP or pulmonary vascular remodeling in rat MCT model.	[92]
miR-130/301	PPAR $\gamma$	Intrapharyngeal (mouse) or intraperitoneal (rat) injections of miR-130/301 inhibitor reversed PH in SuHx mouse model and rat MCT model.	[49] [93]
miR-140-5p	SMURF1	Nebulized miR-140-5p mimic (prophylactic or treatment) reduced RVSP, RVH and remodeling in rat MCT and SuHx models.	[36]
miR-145	KLF4	Prophylactic subcutaneous anti-miR-145 attenuated RVSP and remodeling in hypoxic mouse model.	[94]
miR-193	IGF-1R ALOX5/ 12/15	Intratracheal treatment with miR-193 mimic reduced RVSP, RVH and remodeling in hypoxic mouse and MCT rat models.	[95]
miR-199a-5p	SMAD3	Intravenous anti-miR-199a-5p treatment reduced RVSP and RVH in rat MCT model.	[96]
miR-204	SRC	Nebulized treatment with miR-204 mimic reduced mPAP and remodeling in rat MCT model	[51]
miR-210	ISCU1/2	Intravenous anti-miR-210 (prophylactic or treatment) reduced	[97]

		RVSP and remodeling in SuHx mouse model.	
miR-223	PARP-1	Nebulized treatment with miR-223 mimic reduced RVSP, RVH, remodeling and mortality in MCT rat model.	[48]
	IGF-1R	Prophylactic adeno-associated virus transduction of miR-223 improved cardiac output in hypoxic mouse model.	[98]
miR-424/503	FGF2, FGFR1	Intranasal delivery of lentiviral miR-424 and miR-503 reduced RVSP and remodeling in MCT rat model (prophylactic or treatment) and SuHx models (treatment).	[52]

5-HT1B, 5-hydroxytryptamine receptor 1B; BMPR2, bone morphogenetic protein receptor type 2; WWP1, WW domain containing E3 ubiquitin protein ligase 1; CTGF, connective tissue growth factor; CCND1, cyclin D1; FGF2, fibroblast growth factor 2; FGFR1, fibroblast growth factor receptor 1; IGF1R, insulin-like growth factor 1 receptor; ALOX5, arachidonate 5-lipoxygenase; ISCU1/2, iron-sulfur cluster assembly proteins 1 and 2; KLF4, kruppel-like factor 4; p21, cyclin-dependent kinase inhibitor 1; PARP-1, poly(ADP-ribose) polymerase 1; PDGFRA, platelet-derived growth factor receptor alpha; PPAR $\gamma$ , peroxisome proliferator-activated receptor gamma; SMURF1, SMAD-specific E3 ubiquitin-protein ligase 1; SPRED-1, sprouty related EVH1 domain containing 1; SRC, proto-oncogene tyrosine-protein kinase; RVSP, right ventricular systolic pressure; RVH, right ventricular hypertrophy; mPAP, mean pulmonary artery pressure; MCT, monocrotaline; SuHx, SU5416 and hypoxia; CO, cardiac output.

### **Box 1. Metabolic Reprogramming in PAH.**

Abnormalities in cellular metabolism are well described in PAH, with evidence of aerobic glycolysis, pentose phosphate pathway activation, mitochondrial dysfunction, altered fatty acid metabolism and insulin resistance in disease [99] (and reviewed in [100, 101]). Mitochondrial hyperpolarization [102] and the shift towards aerobic glycolysis (or Warburg effect [103]) has highlighted parallels between the biology of pulmonary artery smooth muscle cells (PASMCs) from PAH patients and cancer cells [100]. Recent work has increased our understanding of potential mechanisms behind these metabolic phenotypes.

One study found evidence implicating the mitochondrial de-acetylase Sirtuin 3 in PAH [104]. PASMCs deficient in SIRT3, had reduced mitochondrial respiration and increased membrane potential (suppressing mitochondrial-induced apoptosis) [104]. These cells also displayed activation of nuclear factor of activated T cells (NFAT), signal transducer and activator of transcription 3 (STAT3) and hypoxia inducible factor-1alpha (HIF-1 $\alpha$ ), all key transcription factors linked to PAH pathogenesis [105-107]. As an upstream regulator of mitochondrial function linking to several key pro-proliferative and anti-apoptotic pathways, SIRT3 may therefore constitute a promising therapeutic target. Interestingly, a polymorphism associated with reduced SIRT3 function was over-represented in 49 IPAH patients [104]. However, this finding requires replication in other patient cohorts and the same SNP was not identified in a genome-wide association study [45]. While this study implies that metabolic abnormalities predate the development of PAH, other work has linked changes in vascular stiffness, a consequence of extracellular matrix (ECM) remodeling, with metabolism. Another study found activation of mechanosensitive transcriptional coactivators YAP (Yes-associated protein 1) and TAZ (transcriptional coactivator with PDZ-binding motif) in diseased human tissue and animal models of PH, promoting a feedback loop of ECM stiffening [93]. **YAP/TAZ** activation also reprogrammed metabolism in PAECs and PASMCs in vitro enhancing glycolysis and glutaminolysis, key processes in the maintenance of pro-proliferative cell phenotypes [49].

Beyond the pulmonary vasculature, the failure of the right ventricle (RV) to adapt and sustain its function in the face of rising pulmonary vascular resistance may also occur due to metabolic dysfunction. Using human RV tissue obtained at autopsy, direct evidence has been provided of a failure of long-chain fatty acid oxidation in myocardial cells, preventing subsequent utilization by the mitochondria. Consequent accumulation

of fatty acids was associated with evidence of elevated ceramide, a marker of lipotoxicity [108]. Visualization of lipid accumulation in the RV myocardium was also evident using magnetic resonance spectroscopy on living patients implying this process is not simply an end-stage observation [108].

### **Box 2. DNA Damage in PAH.**

The hypothesis that DNA damage may lead to somatic mutations triggering changes in cell phenotype in PAH is not new with descriptions of clonal endothelial cell expansion in plexiform lesions and chromosomal abnormalities in explanted lung tissue [109, 110]. However, recent evidence reporting increased susceptibility to DNA damage [111] and impaired DNA repair mechanisms [48, 112] highlight the importance of these processes in PAH and potentially provide insight into how apoptosis-susceptible, apoptosis-resistant and hyperproliferative cell phenotypes arise with pulmonary vascular remodeling.

Significantly more DNA damage was observed in pulmonary artery endothelial cells from PAH patients compared to controls, but higher levels of damage were also demonstrated in peripheral blood mononuclear cells (PBMCs) implying that the damage is not confined to pulmonary vascular tissues [111]. Intriguingly a similar degree of damage was observed in PBMCs from the unaffected relatives of PAH patients and these cells showed higher sensitivity to mutagens [111]. These findings suggest that damage precedes the development of disease and may be determined by currently unknown genetic factors.

DNA damage response mechanisms are also dysregulated in PAH. In PASMCs, excess damage was associated with **poly(ADP-ribose) polymerase-1 (PARP-1)** activation, promoting survival and proliferation [113]. Inhibiting PARP-1 pharmacologically or with the micro-RNA, miR-223, restored PASMC susceptibility to apoptosis, reduced proliferation and reversed established PH in experimental models [48, 113]. A further gene implicated in DNA repair, the breast cancer 1 (BRCA1) gene, was downregulated in PAECs isolated from patients with PAH and associated with increased apoptosis susceptibility [112]. Interestingly, BRCA1 binds to the BMPR2 promoter and also has a SMAD binding element in its own promoter suggesting potential interaction with the BMPR-II pathway that could lead to a cycle of negative regulation and thus endothelial dysfunction in PAH [112].

### **Box 3. The Clinician's Corner**

Reversal of aberrant pulmonary vascular remodeling in pulmonary hypertension remains a holy grail in the field. While currently available drugs have improved survival, these treatments predominantly target pulmonary vasoconstriction and are not specifically designed to modify molecular abnormalities associated with remodeling.

Defective BMPR-II signaling, whether through genetic mutation or suppression of the pathway by non-genetic factors, is an important contributor to IPAH development. Strategies to enhance BMPR-II signaling represent important and novel therapeutic opportunities to dampen or reverse pulmonary vascular remodeling.

An understanding of the mechanisms underpinning metabolic and inflammatory abnormalities in PAH has increased over recent years and has strengthened efforts to modify these abnormalities with drug treatments including **small molecules targeting mitochondrial enzymes (e.g. dichloroacetate)** and biologic **agents targeting pro-inflammatory cytokines (e.g. tocilizumab)**.

Oxygen-sensing pathways are heavily implicated in PAH pathogenesis. Drugs that activate these pathways may be used long term in renal anemia, and the development of PAH as a potential complication should be considered.

## Glossary

**Anastrozole**, a drug use to reduce conversion of androgens to estrogens.

**Angioproliferative plexiform lesions** are a disorganized growth of endothelial cells characteristic of PAH. Other histological changes include thickening of intimal and medial layers, muscularization of distal pulmonary arteries and vascular occlusion.

**Bone morphogenetic proteins** are members of the TGF- $\beta$  superfamily and are known to regulate embryonic patterning and organogenesis. BMPs also have a wide range of roles as endocrine mediators of cardiovascular, metabolic and hematopoietic functions.

**Blood outgrowth endothelial cells** are cultured from human peripheral blood samples. These cells develop a typical cobblestone morphology characteristic of endothelial cell monolayers and express mature endothelial cell surface markers.

**Chuvash polycythemia** is an autosomal recessive form of familial erythrocytosis endemic to Chuvashia that is cause by a mutation in the VHL gene.

**Dichloroacetate (DCA)** is a pyruvate dehydrogenase kinase inhibitor that inhibits glycolysis (typically enhanced in PAH tissue and the right ventricle) and promotes oxidative phosphorylation.

**DNA damage.** DNA is vulnerable to damage from multiple endogenous and exogenous insults including oxidative stress, radiation and inflammation. While multiple repair pathways exist, errors can occur during repair leading to sequence alterations, deletions and translocations.

**Endothelial dysfunction** describes an imbalance of vasoactive mediators released from the pulmonary vascular endothelium resulting in vasoconstriction and failure to suppress smooth muscle cell proliferation.

**Endothelial-to-mesenchymal transition** is a process by which endothelial cells acquire a mesenchymal phenotype in association with expression of smooth muscle cell histological markers and genes.

**Forkhead box O1 (FoxO1)** belongs to the forkhead family of transcription factors and regulates a wide variety of genes involved in metabolism, inflammation and cell division.

**HIF-1 $\alpha$  and HIF-2 $\alpha$ .** Hypoxia-inducible factor (HIF) consists of a beta subunit and one of 3 known alpha subunits. HIF-1 $\alpha$  and HIF-2 $\alpha$  regulate overlapping sets of target genes, many of which are involved in cellular adaptation to hypoxia.

**High Mobility Group AT-hook 1 (HMGA1)** is an architectural factor that binds to DNA and alters the chromatin structure, thereby regulating transcriptional activity of a wide variety of genes.

**Hypoxia-induced pulmonary hypertension.** Exposure to ambient hypoxia induces pulmonary vascular remodelling in humans and many other species and is a common experimental method of inducing PH in rodents. Hypoxic exposure for 2 weeks produces a reversible form of PH in mice.

**ID proteins** are targets of BMP-SMAD signaling. They prevent basic helix-loop-helix (bHLH) domain-containing transcription factors from binding to DNA via their own bHLH domain.

**Idiopathic PAH** was previously termed primary pulmonary hypertension and describes pre-capillary disease in the absence of conditions known to associate with the development of PAH or a family history of the disease.

**Mitochondrial-induced apoptosis.** Mitochondrial membrane permeability is an important regulator of apoptosis and factors inducing mitochondrial dysfunction, loss of membrane potential and mitochondrial DNA damage can initiate this pathway.

**Mixed venous hypoxemia** refers to a relative reduction in the oxygen content of mixed venous blood with low levels indicating more severe disease in PAH.

**Monocrotaline-induced PH.** Monocrotaline is a toxic plant alkaloid that induces PAH in rats over 2-3 weeks following injection, alongside an inflammatory response.

**Paclitaxel** is a chemotherapeutic agent that promotes microtubule stability and suppresses tumor cell proliferation.

**Peroxisome proliferator-activated receptor- $\gamma$**  is a transcription factor with downstream targets involved in glucose homeostasis and vascular remodeling.

**Poly [ADP-ribose] polymerase 1 (PARP-1)** is an enzyme involved in the repair of single-stranded DNA breaks.

**Prolyl hydroxylase domain-containing enzymes (PHDs)** are oxygen sensitive 2-oxoglutarate dependent enzymes responsible for the hydroxylation and subsequent degradation of hypoxia-inducible factor alpha subunits.

**Pre-capillary pulmonary hypertension** is an elevation mean pulmonary arterial pressure (greater than 25 mmHg at rest) associated with a significant increase in pulmonary vascular resistance (greater than 3 Wood units) and in the absence of an elevated pulmonary artery wedge pressure (i.e. less than or equal to 15 mmHg). Post-capillary pulmonary hypertension is characterized by an elevated wedge pressure.

**Proliferative vasculopathy.** In pulmonary hypertension, small pulmonary arteries remodel through an imbalance of proliferation and apoptosis. All layers of the vessel are affected with key changes comprising intimal hyperplasia, medial hypertrophy and adventitial fibrosis.

**Pulmonary arterial hypertension** encompasses a group of conditions with a common hemodynamic phenotype of increased pulmonary arterial pressure and elevated pulmonary vascular resistance. Clinically the condition is characterized by progressive breathlessness, fatigue, syncope, right heart failure and premature death.

**Pulmonary capillary hemangiomatosis** is a rare cause of pulmonary hypertension, distinct from IPAH, and due to alveolar capillary proliferation.

**Pulmonary vasoconstriction** matches ventilation and perfusion in healthy lungs, but PAH is characterized by sustained and severe vasoconstriction that contributes to the abnormal hemodynamic profile.

**Pulmonary veno-occlusive disease** occurs due to remodeling and occlusion of the pulmonary veins and venules and leads to severe and progressive pulmonary hypertension.

**Rat models** of pulmonary hypertension include the monocrotaline (MCT) and SUGEN/hypoxia (SuHx) models. SUGEN drug 5416, or SU5416, is VEGF receptor kinase inhibitor that induces endothelial cell apoptosis and, in combination with ambient hypoxia (10% oxygen), PAH develops over a 3-week period, with pulmonary vascular remodeling that resembles human pathology progressing over the proceeding 3-8 weeks.

**Renal anemia** occurs in the context of chronic kidney disease and is often treated by administration of erythropoietin (EPO). EPO is a HIF target gene and therefore inhibitors of PHD enzymes, that regulate HIF degradation, are in clinical trials to treat renal anemia.

**Right ventricular systolic pressure (RVSP)** is often reported in animal studies as it is not possible to advance the right heart catheter into the pulmonary artery to measure pulmonary arterial pressure.

**SLC39A12** is the gene that encodes the zinc transporter, ZIP12.

**SMAD proteins** are a family of structurally related signaling proteins involved in BMP signaling.

**Tacrolimus**, or FK506, is a calcineurin inhibitor that is commonly used as an immunosuppressant following transplantation.

**Tocilizumab** is an anti-IL-6 antibody currently used in rheumatoid arthritis.

**TopBP1** is the gene that encodes DNA topoisomerase 2-binding protein 1, a protein involved in DNA replication and repair.

**Thromboembolic disease** is an important cause of pulmonary hypertension that results from failure of thrombus resolution within the pulmonary circulation. Often patients will have a history of acute pulmonary embolism.

**von-Hippel Lindau protein** forms part of a ubiquitin ligase complex which ubiquitinates hydroxylated HIF- $\alpha$  subunits, marking them for proteasomal destruction.

**Whole exome sequencing** is a strategy used to find variants in the coding region of genes.

**YAP/TAZ** are transcription factors in the Hippo signaling pathway and have roles in the regulation of cell proliferation, apoptosis and metabolism.

## Figure Legends

### Figure 1 – BMPR-II Dysfunction in PAH and Proposed Therapeutic Intervention

Heterozygous gene mutations in families identified BMPR2, and therefore dysfunctional BMPR-II signaling, as crucial factor in the pathogenesis of PAH. Although the proportion of patients with PAH who harbor mutations is small, accumulating evidence points to reduced BMPR-II expression via a variety of mechanisms as a common molecular occurrence in PAH. This has led to a number of recent studies focused on identifying potential therapies that can augment BMPR-II signaling with the aim of identifying putative treatments. 1) Activation of BMPR2 gene expression using Paclitaxel via the activation of the transcription factor FoxO1. 2) Direct amplification of BMPR-II signaling in endothelial cells via administration of the endothelial selective ligand BMP9. 3) Tacrolimus (FK506) was demonstrated in a high throughput screen to activate downstream BMPR-II regulated genes. Treatment with FK506 prevented the development of PAH in mice with endothelial deletion of Bmpr2 and reversed established PAH in two rat models. 4) SMURF1, a ubiquitin ligase that targets the BMP receptors and downstream SMAD intracellular signaling proteins, upregulated in PAH. Nebulized administration of miR-140-5p, shown to target and repress SMURF1, attenuated PAH in rat models suggesting SMURF1 inhibitors may be a potential therapy to restore BMPR-II signaling and treat PAH. Inhibition of the lysosomal degradation of BMPR-II by chloroquine (or hydroxychloroquine) also restores BMPR-II signaling in vitro and in preclinical models. **BMPR2, type 2 bone morphogenetic protein receptor; BMP, bone morphogenetic protein; FKBP12, 12-kDa FK506 binding protein; SMAD, mothers against decapentaplegic homologs; SMURF1, SMAD-specific E3 ubiquitin-protein ligase 1; BRE, BMP response element.**

### Figure 2 – Pulmonary Vascular Remodeling and PAH Pathogenesis

Although the exact trigger for the development of PAH is unclear there are a number of known risk factors and drivers of disease pathogenesis. These include host factors, including gene mutations such as in BMPR2, and gene variants in other pathways, and gender. Other acquired factors such as somatic mutations, DNA damage, exposure to hypoxia or drugs and toxin are also known important disease modifiers. **Injury to a healthy vessel (left image)**, particularly early endothelial cell viability and loss of barrier integrity, is commonly thought to be the earliest manifestation of disease with alterations

in local shear stress and infection likely confounding factors. At a cellular level (center image) the most common downstream molecular consequences in IPAH are altered signaling through the BMP/TGF beta signaling pathways, altered miRNA expression, release of growth factors such as PDGF and cytokines such as IL-6 perhaps driving mitochondrial dysfunction and metabolic changes related to glycolysis. As a consequence of this perturbed signaling there is an increase in endothelial cell dysfunction and increased apoptosis eventually resulting in the emergence of an apoptotic resistant, pro-angiogenic endothelial cells. Increased proliferation of smooth muscle cells and adventitial fibroblasts drives both inward and outward vascular remodeling, assisted by altered extracellular matrix breakdown and deposition. In combination with the sustained pulmonary vasoconstriction also seen in PAH, these progressive processes drive the pulmonary vascular remodeling in resistance pulmonary arteries and generates the concentric and plexiform lesions characteristic of PAH (right image). Largely unchecked this pulmonary vascular remodeling increases pulmonary vascular resistance and right ventricular afterload, eventually leading to right heart failure and death. BMP, bone morphogenetic protein; TGF, transforming growth factor; EC, endothelial cell; SMC, smooth muscle cell; ECM, extracellular matrix; RV, right ventricle; PDGF, platelet derived growth factor.