BMP type II receptor as a therapeutic target in pulmonary arterial hypertension

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Abstract Pulmonary arterial hypertension (PAH) is a chronic disease characterized by a progressive elevation in mean pulmonary arterial pressure. This occurs due to abnormal remodeling of small peripheral lung vasculature resulting in progressive occlusion of the artery lumen that eventually causes right heart failure and death. The most common cause of PAH is inactivating mutations in the gene encoding a bone morphogenetic protein type II receptor (BMPRII). Current therapeutic options for PAH are limited and focused mainly on reversal of pulmonary vasoconstriction and proliferation of vascular cells. Although these treatments can relieve disease symptoms, PAH remains a progressive lethal disease. Emerging data suggest that restoration of BMPRII signaling in PAH is a promising alternative that could prevent and reverse pulmonary vascular remodeling. Here we will focus on recent advances in rescuing BMPRII expression, function, or signaling to prevent and reverse pulmonary remodeling in PAH and its feasibility for clinical translation. Furthermore, we summarize the role of described miRNAs that directly target the BMPR2 gene in blood vessels. We discuss the therapeutic potential and the limitations of promising new approaches to restore BMPRII signaling in PAH patients. Different mutations in BMPR2 and environmental/genetic factors make PAH a heterogeneous disease and it is thus likely that the best approach will be patient-tailored therapies.

Keywords Endothelial cell · Vascular smooth muscle cell · Signal transduction · Inflammation · Vascular remodeling and autophagy

Abbreviations
ACE Angiotensin-converting enzyme
ACVRII Activin type II receptor type
ACVRL1 Gene encoding activin receptor-like kinase
ALK Activin receptor-like kinase
BMP Bone morphogenetic protein
BMPRI Bone morphogenetic protein type I receptor
BMPRII Bone morphogenetic protein type II receptor
BMPR2 Gene encoding bone morphogenetic protein receptor II
Cav1 Caveolin-1
cGMP Cyclic guanosine monophosphate
DN Dominant negative
EC Endothelial cell
EndMT Endothelial-to-mesenchymal transition
FKBP12 FK-binding protein-12
GDF Growth and differentiation factor
GDF-2 Gene encoding BMP9
HMGA1 High-mobility group protein
iTOP Induced transduction by osmocytosis and propanebetaine
MCT Monocrotaline
mPAP Mean pulmonary arterial pressure
miRNA MicroRNA
NMD Non-sense mediated decay
PAEC Pulmonary arterial endothelial cells
PAH Pulmonary arterial hypertension
PASMC Pulmonary arterial smooth muscle cells
PGI2 Prostacyclin
PKG Protein kinase G
PTC Premature termination codon

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Introduction

Pulmonary arterial hypertension (PAH) is a chronic disease characterized by a progressive elevation in mean pulmonary arterial pressure (mPAP >25 mmHg) leading to right heart failure and death [1]. PAH is characterized by abnormal remodeling of small peripheral lung vasculature resulting in progressive occlusion of the artery lumen. In addition, at late stages, so-called plexiform lesions are found, which are complex vascular formations originating from abnormal endothelial cell (EC) proliferation and vascular smooth muscle cell (SMC) hypertrophy [2]. The basic pathogenic mechanisms underlying this disease include vasoconstriction, intimal proliferation, and medial hypertrophy. These processes are accompanied by illicit recruitment of inflammatory cells which release factors enhancing cell proliferation and elastin fibers degradation [3, 4] (Fig. 1).

More than 70% of patients with familial PAH and 20% of idiopathic PAH show heterozygous mutations in the bone morphogenetic protein type II receptor (BMPRII) [5–8]. BMPRII is a transmembrane serine/threonine kinase receptor of the bone morphogenetic protein (BMP) pathway which is essential for embryogenesis, development, and adult tissue homeostasis. Upon BMP-induced heteromeric complex formation of BMPRII with BMP type I receptor (BMPRI), BMPRII activates BMPRI by phosphorylation. Thereafter, the activated BMPRI propagates the signal into the cell through phosphorylation of the SMAD1/5/8 transcription factors.

In PAH, over 300 mutations have been found in the BMPR2 gene. These mutations target sequences that encode the ligand binding and kinase domain and the long cytoplasmic tail; the mutations compromise BMPRII function [9]. Although the BMPRII pathway is essential for vascular homeostasis and there is a strong correlation between BMPR2 mutations and PAH, the incomplete penetrance of BMPRII mutations (20–30%) suggests that other genetic and environmental factors contribute to the disease. Among them, BMPR2 alternative splicing plays a role in PAH penetrance. One BMPR2 splice variant lacks exon 12, which is the largest exon of the gene and encodes the cytoplasmic tail. It has been shown that carriers of this variant are more prone to develop PAH through a dominant-negative effect (DN) effect on wild-type BMPRII [10]. Furthermore, there are mutations in other genes in the BMP pathway, which further strengthens the notion of a causal role for this pathway in PAH [11]. Moreover, the co-existence of modifier genes, infections, toxic exposure, inflammation, or alterations in estrogen metabolism has been described [11–14] and some of them were found to down-regulate BMPRII expression. For example, pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and Interleukin 6 induce the expression of miRNAs that inhibit BMPRII expression [15]. Furthermore, BMPRII is essential for maintaining the barrier function of the pulmonary artery endothelial cell lining and BMPRII deficiency increases endothelial inflammatory responses thereby contributing to adverse vascular remodeling [16–18].

Current therapeutic options for PAH are limited and focused mainly on reversal of pulmonary vasoconstriction and proliferation of vascular cells through targeting of
prostacyclin (PGI₂), endothelin, or nitric oxide pathways [19]. Although these treatments can relieve disease symptoms and slow down its progression, PAH remains a progressive lethal disease. Abundant research over the past decade has improved our understanding of the molecular mechanisms underlying PAH progression revealing novel potential therapeutic interventions [20–22]. Among them there are several anti-proliferative strategies including cell cycle inhibitors (e.g., mTOR inhibitor rapamycin) and anti-apoptotic drugs (e.g., surviving inhibitors) [23]. Furthermore, based on the fact that Rho and ROCK mediate smooth muscle cell proliferation in a serotonin-BMPR-dependent pathway, Rho-kinase inhibitors have been also considered [23, 24]. Although several drugs with possible benefit in PAH have been identified, only very few have been approved for use in the clinic due to toxicity or lack of clinical efficacy. This review will focus on recent advances on the rescue of BMPRII expression, function, or signaling to prevent and reverse pulmonary vascular remodeling in PAH. We will discuss data on the in vitro efficacy of the different approaches together with the physiological outcomes in pre-clinical models and their feasibility for clinical translation.

**BMP signaling in vascular biology and PAH**

BMPs belong to the multifunctional transforming growth factor-β (TGF-β) family of secreted dimeric cytokines. The effects of BMPs are highly dependent on cellular context [25]. In general, BMPs control cellular proliferation, differentiation, and apoptosis, and play an important role in embryonic development and maintaining tissue homeostasis [26]. Therefore, perturbation of BMP signaling may lead to skeletal diseases, vascular diseases, and cancer [27]. BMPs can be subdivided into four subgroups based on their sequence similarity and cell surface receptor affinities: BMP2/4, BMP5/6/7/8, BMP9/10, and growth and differentiation factor (GDF)-5/6/7 [28, 29]. BMPs signal via hetero-tetrameric combinations of type I receptors (activin receptor-like kinase (ALK)1, ALK2, ALK3, or ALK6) and BMP type II receptors (BMPRII) and activin type II receptor (ACVRIIA) or ACVRIB complexes [30, 31]. Both, type I and type II receptors have a similar structure encompassing a short extracellular domain, a single transmembrane domain and an intracellular domain with intrinsic serine-threonine kinase activity. In the vascular endothelium, BMP signaling is mainly activated by BMP2, 4, 6, 9, and 10 [32]. BMP2 and BMP4 bind preferentially to the BMPRII in complex with ALK3 or ALK6. BMP6 binds to the ACVRIIA-ALK2 complex, while BMP9 and BMP10 bind to BMPRII or ACVRII in combination with ALK1 or ALK2. Whereas BMPRII is a specific receptor for BMPs, ACVRIIA and ACVRIB can also interact functionally with other ligands, such as activins, myostatin, and nodal. Interestingly, ALK2 and 6 are widely expressed in various cell types, while ALK1 has a more selective expression pattern and is mainly restricted to ECs. After BMP binding and receptor complex formation, the type II receptor kinase phosphorylates the type I receptor on serine and threonine residues in the glycine-serine rich (GS)-domain causing its activation and subsequent phosphorylation of the receptor-associated R-SMAD1, 5, and 8 effector proteins. The R-SMADs that are activated by TGF-β type I and activin type I receptor (i.e., ALK5 and ALK4, respectively) are SMAD2 and SMAD3 and these are distinct from BMP R-SMADs. Activated R-SMAD 1, 5, or 8 forms a heterooligomeric complex with common mediator co-SMAD4. This complex translocates to the nucleus and regulates the expression of target genes by binding to specific enhancers/promoters upstream of these target genes [30, 33, 34] (Fig. 2). Besides canonical BMP receptor/SMAD signaling, activated BMP receptors can initiate non-SMAD signaling pathways such as ERK, JNK, p38 MAP kinases, and the phosphatidylinositol 3 kinase (PI3K)/AKT pathways [35–37]. These non-SMAD pathways are also important for diversifying and modulating the canonical SMAD signaling pathways that are activated by the BMP receptors [38, 39]. In addition, BMP activity is also regulated by several extracellular modulators, including BMP binding proteins NOGGIN, CHORDIN, and FIBULINS. Co-receptors such as ENDOGLIN, BETAGLYCAN, and DRAGON family members may also modulate the interactions between BMPs and BMP receptors [40, 41]. Moreover, intracellular kinases/phosphatases and other binding proteins have been identified as regulators of the trafficking, subcellular localization, stability, and function of BMP receptors and SMADs [26].

Genetic depletion of different components of the BMP signaling cascade leads to embryonic death due to cardiovascular malformations and abnormal vascular remodeling. BMP signaling plays an important role in vasculogenesis (de novo formation of blood vessels from undifferentiated mesodermal cells) and angiogenesis (formation of new blood vessels from the existing vasculature). In this light, it is not surprising to discover that, besides PAH, dysfunction of BMP signaling has been found to be associated with other vascular diseases including hereditary hemorrhagic telangiectasia, cerebral cavernous malformation, atherosclerosis, and vascular calcification among others [42]. Furthermore, BMPRII downregulation has been found to be involved in pancreatic and lung fibrosis [43, 44].

Blood vessels are composed of three layers: the tunica adventitia consisting of fibroblasts and associated collagen fibers; the tunica media composed of SMCs; and the tunica intima consisting of ECs coating the interior surface [45, 46].
ECs, SMCs, and fibroblasts have been found to play a role in the pathogenesis of PAH. Abnormal EC proliferation resulting in the formation of plexiform lesions has been frequently described in many PAH cases [47]. In addition, pulmonary arterial SMCs show an increased proliferation and decreased apoptosis leading to vessel wall thickening and vascular remodeling (Fig. 1). The close interaction between ECs and SMCs has been found to be involved in vessel formation and maintenance. For instance, endothelial derived factors, like endothelin and angiotensin II, affect SMCs which increases vascular tone. Similarly, nitric oxide and PGI2 secreted by ECs modulate the vasodilator response of SMCs [48]. In particular, PGI2 has been found to be reduced in PAH patients [49].

BMP signaling is known to control cell migration, proliferation, and apoptosis in ECs and SMCs [45]. BMP9 and BMP10 are present in the circulation and play an important role in the vasculature. Their associated receptors BMPRII and ALK1 and co-receptor ENDOGLIN are predominantly expressed on ECs [50, 51] and together can modulate the ability of ECs to migrate and proliferate [27]. Furthermore, BMPRII is also expressed in vascular SMCs where it has been shown to be necessary for the control of proliferation and differentiation [52]. Besides mutations in the BMPR2 gene, mutations in the genes of other BMP signaling components (such as GDF-2, ACVR1, ENDOGLIN, and SMAD8) have also been linked to PAH development [11, 53–59]. This association reinforces the importance of BMP signaling in the control of vascular homeostasis, and suggests that there is a causal link between perturbation of canonical BMP/SMAD signaling and PAH. In support of this view, recent, new DNA sequencing techniques helped to identify new gene mutations associated to PAH [Caveolin-1 (CAV1), KCNK3, and EIF2AK4] [60, 61] (Fig. 2).

The abnormal vascular remodeling that characterizes PAH involves an accumulation of α-smooth muscle, actin-expressing mesenchymal-like cells indicating that the endothelial-to-mesenchymal transition (EndMT) may be involved in the pathogenesis of the disease [62]. In addition, BMPRII reduction in pulmonary artery endothelial cells (PAECs) has been found to promote the trans-differentiation of epithelial cells into motile mesenchymal cells via the transcription factors high-mobility group protein (HMGA)1 and its target SLUG [63].
Animal models of PAH

PAH has a complex etiology and pathobiology with many factors contributing to its development [64]. A variety of pre-clinical rodent models have been used to study the underlying pathophysiological mechanisms and to test novel therapeutic strategies for PAH. A proper model should be reproducible, inexpensive, and faithfully reproduce (in a defined period) the basic features of PAH such as complex destructive neointimal lesions and right ventricular (RV) dysfunction and failure. To date, there is no model that recapitulates all of the pathophysiological mechanisms and the clinical course of human PAH. For instance, in the chronic hypoxic exposure or monocrotaline (MCT)-induced rat models, pulmonary hypertension rarely develops with the same severity observed in humans perhaps due to the absence of obstructive intimal lesions in the peripheral pulmonary arteries [65, 66]. Furthermore, the chronic hypoxia model does not lead to RV failure, while MCT injection causes myocarditis affecting both ventricles and causing liver and kidney damage [67]. These limitations may explain why it is difficult to translate the reversal of PAH in animal models by several experimental compounds into therapies for PAH patients.

In recent years, second-generation animal models have been established based on the combination of multiple triggers: MCT plus pneumonectomy, MCT plus chronic hypoxia, and SU5416 plus chronic hypoxia. To circumvent the problem of the embryonic lethality of BMPR2 knock-out mice, switchable rodent models have been developed, by means of BMPR2 conditional knock-out, whereby the mutation can be activated after birth [68–70]. Moreover, genetic rodent models have been developed including overexpression of interleukin-6. These new models closely mimic the features and the severity of human PAH although not completely [71]).

Restoring BMPRII signaling as a therapeutic strategy

While hereditary PAH have been linked to heterozygous mutations in the BMPR2 gene, non-genetic forms of PAH show a reduction in BMPRII levels and activity [9]. Consistent with this, heterozygous BMPR2 deletion in PAECs and pulmonary artery smooth muscle cells (PASMCs) mimics the PAH phenotype [69, 72]. Furthermore, mice expressing a dominant-negative BMPRII (lacking an intracellular domain) in vascular SMC, develop vascular lesions in the lungs [68, 72].

There is strong evidence suggesting that BMPRII signaling has a protective role in the vascular wall by promoting the survival of PAECs, inhibiting PASMCs proliferation and triggering anti-inflammatory responses [17, 73, 74]. Based on this, modulation of BMPRII signaling is considered a promising therapeutic approach for PAH. Importantly, the rescue of BMPRII expression may not exclusively benefit PAH patients but also patients suffering from pancreatic and lung fibrosis where BMPRII deficiency has been implicated [43, 44]. BMPRII restoration can be targeted at different levels: genetic-based therapies, transcriptional and translational regulation, protein activity, and processing as well as SMAD downstream signaling modulation [27, 75, 76] (Fig. 3).

Genetic-based therapies

Exogenous BMPR2 gene delivery

One strategy to treat PAH patients is to rescue BMPRII expression through gene therapy targeting ECs. In preclinical models, this was explored by Reynolds et al., who administrated a vector inducing BMPRII expression via tail-vein injection. The BMPR2 encoding virus targets the pulmonary endothelium by binding to the highly expressed pulmonary endothelial angiotensin-converting enzyme (ACE) using a bi-specific conjugate antibody. This BMPR2 adenoviral vector restored BMPRII protein levels in human microvascular PAECs and attenuated the PAH phenotype in a chronic hypoxia model and MCT-treated rats [77, 78]. Furthermore, BMPRII overexpression in lung tissue was shown to reverse the imbalance between BMPRII and TGFβ signaling thus restoring normal levels of pSMAD 1/5/8 and the activation of PI3K and p38 MAP kinase [79]. In contrast, BMPRII administration via an aerosol route targeting PASMCs did not improve the PAH phenotype when tested in the MCT model [80]. The later result highlights the importance of BMPRII signaling in ECs but not SMCs. However, further investigations will be required to elucidate precisely how spatio-temporal control BMPRII overexpression might provide therapeutic benefit in the context of BMPR2 mutations. It should be noted that adenoviral vectors are only capable of transient gene expression since the delivered gene is not integrated into the host chromosome. Stable integration can be achieved and lentiviral vectors are potentially an attractive vehicle to deliver longer term transgene expression since they integrate into the genome and they can infect non-proliferating cells, when compared to retroviral vectors. An important potential limitation of this approach is that integrating vectors may generate gene mutations upon insertion and newer advances regarding self-inactivating vectors are needed [81, 82]. Adeno-associated virus and helper dependent adenoviral vectors (the latest generation of recombinant adenoviral vectors) are a promising alternative since they deliver longer durations of transgene expression when compared
to the first-generation vectors. Moreover, they show neither long-term adverse effects in liver nor an immunological response [83, 84]. Taken together, exogenous BMPRII delivery is a possible therapy for PAH, but further improvements in vector technology are required to translate this approach to the clinic for the treatment of pulmonary vascular disease.

**Transcriptional regulation**

**miRNA targeting BMPRII**

In recent years, there has been an increasing interest in the role of epigenetics in the development of PAH [85, 86]. Epigenetics refers to heritable changes in gene expression that do not involve alterations in the DNA sequence. miRNAs are small non-coding RNAs that negatively, post-transcriptionally regulate the expression of target genes by interfering with both the stability of the target transcript as well as its translation. miRNAs have emerged as essential players in the development (and diseases) of the cardiovascular system. They also play an important role in vascular remodeling [87, 88]. miRNAs are expressed in the vasculature and are essential for the regulation of vessel function. Many miRNAs control proliferation, differentiation, and apoptosis of ECs and SMCs by targeting components of the TGF-β/BMP signaling pathways. Several miRNAs, such as miR-145, miR-21 and the miR17/92 cluster, have been associated with the disrupted BMPRII pathway in PAH and can explain the incomplete penetrance of BMPR2 mutations [89–91]). Figure 4 and Table 1 provides an overview of currently described miRNAs that target BMPR2.

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expression in vascular cells. In addition, Table 1 shows a list of other miRNAs that are predicted to target BMPR2 in silico.

Currently, there are different technologies to inhibit aberrantly overexpressed miRNAs including the use of antisense oligonucleotides, masking, sponges, erasers, or decoys [92, 93]. In addition, administration of miRNA mimics can enhance the expression of downregulated miRNAs [94]. These strategies are still under development and more research is needed to establish how modulation of miRNA function could offer therapeutic benefits in a clinical setting while avoiding off-target effects, especially in the liver, where systemically administrated miRNAs or modulating compounds preferentially accumulate [95].

The delivery routes mostly used to target lung disease are local intranasal and intra-tracheal administration. These naked miRNAs are directly delivered into the lung with minimal systemic side effects [96]. Nevertheless, this method remains ineffective and challenging due to the complexity of the lung [97]. Recent advances in delivery strategies, such as the use of liposomes, nanoparticles, or virus, combined with improvements in chemically modifying miRNAs, represent promising strategies to improve lung miRNA delivery [98]. More than 20 miRNAs are currently in clinical trials, several in Phase III stage, highlighting the potential of miRNA therapeutics to restore BMPRII in PAH [99]. To date, the potential for miRNAs as a therapeutic tool is relatively limited. Further studies focusing on the specificity, safety, efficiency, and stable systemic delivery of miRNAs into target cells or tissues will improve the process of translating these findings to the clinic.

**Translational regulation**

**Read-through premature STOP codons**

Most BMPR2 mutations (~70%) are non-sense mutations (frame-shift deletions and insertions) generated by the insertion of a premature termination codon (PTC) resulting in truncated reading frames which produce non-functional proteins [7]. To prevent formation of truncated proteins, mutated transcripts are directly degraded through non-sense mediated decay (NMD) resulting in insufficient amounts of the functional protein, which is produced only by the wild-type allele (haplo-insufficiency) [100, 101]. NMD usually does not completely reduce the levels of mutated transcripts and as a result truncated proteins persist, and may exert a DN effect [102].

An approach aimed to correct these types of mutations consists on the induction of PTC read-through. Read-through of truncated mutations by aminoglycoside antibiotics, such as Gentamicin, has been extensively studied and has reached the clinical trial stage for genetic disorders such as cystic fibrosis [103] and Duchenne muscular dystrophy [104–110]. Aminoglycoside antibiotics bind to the decoding site of ribosomal RNA and eliminates the PTC
by incorporating an amino acid to generate full-length proteins [111]. Importantly, this read-through function of aminoglycosides does not affect normal translation because of the presence of upstream and downstream regulatory sequences around a normal termination codon that ensure optimal efficiency of termination [112]. Gentamicin treatment has been tested in lymphocytes derived from two PAH patients with PTC mutations [113, 114]. The results demonstrated increased amounts of full-length BMPRII protein, a reduction in the mutated BMPRII product and enhanced BMPRII downstream signaling. Although aminoglycosides are commonly used in the clinic to treat infections and are safe when administered directly to the lung by inhalation, several side effects of long-term treatments and/or high concentrations of the drug have been shown. Recently, a high-throughput screening for compounds that suppress non-sense mutations identified a new small molecule named Ataluren (PTC124) which mediates read-through of premature stop codons without acute side effects [115]. Aldred et al. have demonstrated that after Ataluren treatment, BMPRII protein levels were normalized and BMP-dependent phosphorylation of the downstream target R-SMADs was increased in PAECs and PASMCs from PAH patients. In addition, the hyperproliferative phenotype of these cells was reversed even in the presence of significant non-sense mediated mRNA decay. Although, further studies, including animal models, are required to explore the relevance of Ataluren in vivo in a PAH context, the compound has emerged as a promising therapeutic strategy for a subset of PAH patients.

### Table 1 miRNA targeting BMPRII expression

| MicroRNA | Cell type | Function | Model | Expression | References |
|----------|-----------|----------|-------|------------|------------|
| miR-17/92 EC | Interleukin-6 modulates the expression of the BMPRII through a novel STAT3–microRNA Cluster 17/92 pathway | PAEC | ? | Brock et al. [13] |
| SMC | Inhibition of miR-17 enhances BMPRII expression and improves heart and lung function in experimental PH | PASMDC | Hypoxia-induced PH mice | Pullamsetti et al. [164] |
| miR-20A SMC | Treatment with antagomiR-20a restores functional levels of BMPRII in pulmonary arteries and prevents the development of vascular remodeling | PASMDC | Hypoxia-induced PH mice | Brock et al. [165] |
| miR-21 EC | Hypoxia and BMPRII signaling independently upregulate miR-21. In a reciprocal feedback loop, miR-21 downregulates BMP receptor type II expression | PAEC | Several rodent models of PH miR-21-null mice | Parikh et al. [166] |
| SMC | BMPRII was downregulated in PASMCs overexpressing miR-21 | PASMDC | Hypoxia-induced PH mice | Yang et al. [167] |
| miR-125 EC | Inhibition of miR-125 resulted in upregulated BMPRII expression accompanied by increased proliferation of EC | PAEC | Hypoxia-induced PH mice Plasma PAH patients | Huber et al. [168] |
| miR-143/145 SMC | miR-145 expression is increased in primary PASMCs cultured from patients with BMPRII mutations and in the lungs of BMPRII-deficient mice | PASMDC | Hypoxia-induced PH mice BMPRII R899X knock-In Mice miR-145 knock-Out Mice Lung tis-sue PAH patients | Caruso et al. 2012 [169] |
| miR-302 SMC | Inhibition of miR-302 by BMP4 increases BMPRII expression and facilitates the BMP signaling pathway | PASMDC | ? | Kang et al. 2012 [170] |
| miR-181c cardiac | Increased miR-181c expression in human cardiac samples from individuals with ventricular septal defects (VSD) was correlated with downregulated BMPRII levels | Human VSD cardiac samples | # | Li et al. [171] |
Protein processing regulation

Rescue of BMPRII trafficking

30% of BMPR2 mutations are missense mutations leading to single amino acid substitutions in a conserved domain affecting the overall function of the protein [7]. Mutations resulting in the substitution of cysteine residues in the ligand binding and kinase domains disrupt protein folding and trafficking of BMPRII to the cell surface leading to retention of the mutant receptor in the endoplasmic reticulum (ER) [113, 116]. A potentially promising therapeutic strategy to increase the expression of BMPRII at the plasma membrane is to enhance the activity of chaperones which facilitate protein folding and trafficking. This can be done by means of chemical chaperones such as sodium phenylbutyrate (4-PBA), probenecid, and tauroursodeoxycholic acid (TUDCA). These have been shown to improve protein trafficking via several distinct mechanisms [117–126]. Different groups have demonstrated that treatment with chemical chaperones can partially restore cell surface expression of BMPRII in ECs. As a result, BMP-induced SMAD 1/5/8 phosphorylation and the expression of the target gene ID1 is restored [127–129]. These agents are showing promise in clinical trials for other diseases caused by misfolded proteins, such as cystic fibrosis. Since the currently used chemical chaperones are federal drug administration (FDA) approved drugs, there is an immediate translational potential to treat PAH patients [118, 130–133]. However, further in vivo studies are required to test the viability of this approach.

Even though chemical chaperones have the potential to rescue the BMPRII mutants which are retained in the ER, it remains to be investigated whether the amount of BMPRII reaching the plasma membrane is enough to induce a clinically relevant effect. Also, BMPRII with a protein-folding defect expressed at the cell surface may have a dominant-negative activity and adverse effects on BMP signaling [127]. Moreover, patients harboring missense mutations that affect the activity of the receptor (kinase domain) may not benefit from this therapeutic strategy. Further research, taking this mutation variability into account, is required to determine which patients might benefit from this approach.

Inhibition of lysosomal degradation

The deciphering of mechanisms which regulate cell surface expression levels of BMPRII are of potential clinical importance, particularly those mechanisms that prevent its rapid turnover and thereby restore downstream BMPRII signaling and function. In this context, several studies focused on the potential of targeting the degradation of BMPRII by preventing lysosomal degradation [134]. Durrington et al. have demonstrated that after Kaposi sarcoma-associated herpesvirus infection, BMPRII is ubiquitinated by K5 (membrane-associated RING E3 viral ubiquitin protein ligase) leading to lysosomal degradation in primary cultured pulmonary vascular cells [134]. In addition, cells treated with the lysosomal inhibitor concanamycin A exhibit increase levels of BMPRII. Furthermore, through siRNA screening of the NEDD4-like family E3 ubiquitin protein ligases, it was found that knockdown of ITCH expression resulted in increased BMPRII protein levels [134]. Whether ITCH ubiquitimates BMPRII, leading to lysosomal degradation, has yet to be investigated. Satow et al. have demonstrated that BMPRII is degraded via the proteosomal pathway in HEK 293T cells, when it is associated with Dullard phosphatase [135]. This might suggest that more than one mechanism accounts for BMPRII proteasome-mediated degradation. It is noteworthy that Satow et al. used a BMPRII overexpression system, whereas Durrington et al. studied the degradation of endogenous BMPRII [134]. Different membrane trafficking pathways such as endocytosis, phagocytosis, micropinocytosis, and autophagy, use lysosomes for the digestion of diverse macromolecules [136]. Caveolae-mediated endocytosis affects multiple cellular signaling pathways by the redistribution of transmembrane receptors and receptor-ligand complexes [137–139]. BMPRII localization has been found to be regulated by CAV1 in vascular SMC [137]. Recently, ELAFIN (endogenous serine protease inhibitor) treatment has been shown to prevent and reverse PAH in the SU-hypoxia rat model. This occurs via elastase inhibition and by promoting the interaction of BMPRII with CAV1. Interestingly, when ELAFIN was combined with BMP9, there was enhanced co-localization of CAV1 and BMPRII on PAEC surfaces, which led at an increase in BMP9-dependent SMAD1/5 phosphorylation and induction of ID1 [137]. Furthermore, transgenic mice overexpressing human ELAVIN in the cardiovascular system (by placing ELAVIN expression under the control of the pre-proendothelin-1 promoter), exhibited reduced SMC proliferation and medial/intimal thickening after carotid artery wire injury [140] and were protected from hypoxic pulmonary hypertension [141]. In agreement with this, peptidyl trifluoromethylketone serine elastase inhibitors such as M249314 or ZD0892, have been used to prevent and reverse PAH in the MCT rat model [142]. However, the clinical use of these compounds was not pursued due to hepatotoxicity. ELAFIN has been shown to inhibit myocardial ischaemia-reperfusion injury induced during coronary artery bypass graft surgery [143]. Even though ELAFIN infusion was safe and resulted in >50% inhibition of elastase activity in the first 24 h, myocardial injury was not reduced after 48 h. Based on the biology
of ischemia–reperfusion injury and PAH, we believe it is worth testing whether ELAFIN together with BMP9 could reverses PAH in patients.

Interestingly, autophagy has also been found to be involved in PAH. Autophagy (literally, “self-eating” in Greek) is a highly regulated catabolic process that involves sequestration and lysosomal degradation of cytosolic components such as dysfunctional organelles, misfolded proteins, lipid droplets, and invading pathogens [144]. Autophagy can be considered to be a general housekeeping mechanism maintaining the integrity of intracellular organelles and proteins. It is also triggered during development, differentiation, infection, and stress conditions. Thus, autophagy can be activated in the presence of damaged organelles, protein aggregates, intracellular pathogens, hypoxia, amino acid starvation, reactive oxygen species, and DNA damage [145]. Long et al. have shown that in rats suffering from PAH induced by MCT treatment, there is increased autophagy together with a decrease of BMPRII protein expression [146]. Moreover, inhibition of autophagic degradation by the lysosomal inhibitors chloroquine and hydroxychloroquine [147] prevents the development of PAH as well as its progression. The authors demonstrated that chloroquine and ATG5 (an autophagy protein involved in the elongation and closure of the autophagosomal membrane) knockdown inhibited proliferation and increased apoptosis of PASMCs and these effects correlated with increased levels of BMPRII via lysosomal inhibition. Although autophagy seems to be involved in the degradation of BMPRII, the exact mechanism by which this takes place has yet to be elucidated. Chloroquine and hydroxychloroquine have been widely utilized in malaria prophylaxis [148]. They have also been used to treat rheumatoid arthritis and lupus erythematosus (as anti-inflammatory agents) [148]. Since inflammation is thought to be a crucial second hit in PAH [149], these drugs might be effective at inhibiting PAH progression by impairing the degradation of BMPRII as well as inhibiting the inflammatory response. However, it is important to keep in mind that since lysosomal degradation is a ubiquitous cellular mechanism for regulating protein processing, this approach can lead to widespread and non-specific off-target effects independent of BMPRII signaling. Therefore, an improved understanding of the molecular mechanisms underlying BMPRII turnover is required for the development of more directed interventions.

**BMPRII signaling regulation**

**Delivery of exogenous BMP ligand**

As mentioned previously, BMP signaling in the vascular endothelium is mainly activated by BMP2, 4, 6, 9, and 10 [32]. In particular, BMP9 and BMP10 appear to play an important role in the vasculature due to their presence in the circulation and based on the fact that they are known to signal through receptors expressed on the endothelium, such as ALK1 and BMPRII or ACVRIIB. Therefore, the stimulation of BMP signaling with exogenous recombinant ligand is an interesting approach for PAH treatment [11, 150]. Long et al. have shown that BMP9 prevents apoptosis and enhances the integrity of ECs in PAECs and blood outgrowth ECs from PAH patients. Furthermore, therapeutic BMP9 delivery prevents and reverses PAH in several mouse models [70]. BMP10 is the least studied BMP ligand; however, it may present a better treatment than BMP9 since it binds to ALK1 and BMPRII with higher affinity and because of its lack of osteogenic activity in vitro [151]. Further studies have to be performed to evaluate the delivery strategies, efficiency, and potential side effects of BMP9 and BMP10 in vivo. Finally, the development of a small peptide mimetics of BMP9 or BMP10, with an increased affinity for the receptor, is a theoretical alternative for efficiently activating BMP signaling and thereby reversing PAH [150].

**Enhance downstream SMAD signaling**

An additional approach to reverse the effect of mutant BMPRII is use small molecules to enhance signaling of the wild-type functional proteins. Sildenafil is a phosphodiesterase type-5 (PDE5) inhibitor currently used in the clinic for PAH treatment [152–154]. Its mode of action is to block the degradation of cyclic guanosine monophosphate (cGMP) resulting in corrective vasodilatory and anti-proliferative effects in the arterial wall [155]. Furthermore, it has been described that protein kinase G (PKG) activated by cGMP, is a modulator of BMP signaling [156] and that PASMCs expressing a BMPRII mutant, showed an increase in BMP signaling after Sildenafil treatment via a cGMP/PKG-dependent mechanism. In addition, in vivo studies confirmed that Sildenafil treatment enhanced BMP signaling and partially reversed PAH development in the MCT rat model [157, 158]. Although Sildenafil therapy during 12 weeks improves multiple clinical symptoms in PAH patients, it appears to have no effect on reducing either mortality or serious adverse events [159]. Furthermore, the long-term efficiency and safety of Sildenafil therapy in PAH requires further studies based on large and well-designed clinical trials [159].

Another promising strategy is to identify compounds in drug libraries that activate BMP/SMAD signaling. FK506 (Tacrolimus) was identified as the best BMP coactivator among 3756 FDA-approved drugs and bioactive compounds (using a high-throughput BMP/SMAD-driven transcriptional reporter assay) [160]. FK506 promotes BMP
signaling and endothelial-specific gene regulation of genes such as APELIN. This occurs even in the absence of exogenous ligand via a dual mechanism of action: acting as an inhibitor of phosphatase CALCINEURIN and binding FK-binding protein-12 (FKBP12), a repressor of BMP signaling. FK506 promotes the release of FKBP12 from the type I receptor which leads to activation of SMAD1/5 down-stream of BMP as well as MAPK signaling and ID1 gene regulation [161]. Furthermore, FK506 treatment increases ALK1 and ENDOGLIN expression in ECs [162]. Recently, a randomized, double-blind, placebo-controlled phase Ila trial was performed to investigate the efficacy of FK506 treatment in three patients with end-stage PAH. The results suggest a potential clinical benefit of low-dose FK506 (the evidence being that patients demonstrated cardiac function stabilization and required less intensive hospital care for RV failure despite the severity of the illness). It was also found that changes in serologic biomarkers indicated that BMPRII had been successfully targeted [163]. However, these results are based on a limited group of patients and the efficacy of this therapy must be validated in appropriate, well-designed clinical trials. FK506 (also known as Tacrolimus) is an immunosuppressive drug with a known pharmacokinetic and toxicity profile. It is widely used in solid organ transplantations to lower the risk of organ rejection [164]. High doses of FK506 caused systemic hypertension and transplant vasculopathy in animal models [165]. Also, organ transplant patients treated with FK506, have an increased risk of renal injury, which might occur due to the inhibition of calcineurin expression in the kidney [166–168]. In contrast, low doses of FK506 did not induce systemic hypertension in animal models, even after 3 weeks of treatment. FK506 has shown significant clinical benefits, nonetheless long-term use of this agent for treating PAH still needs to be rigorously monitored for toxicity effects.

Conclusions and perspectives

Exogenous BMPRII delivery to ECs has been shown to be an effective means to restore BMPRII expression and function [77, 78]. An interesting approach, which is yielding promising results in mice, is to deliver BMPRII specifically to ECs using BMPRII adeno viral vectors carrying a bi-specific conjugate antibody that targets the virus to ACE, a membrane-bound protease highly expressed on pulmonary endothelial cells [77, 78]. One of the drawbacks of this strategy is the use of two components namely, adenovirus and antibody. Additional restrictions related to the use of viral transduction such as safety, specificity, and delivery of sufficient protein to revert the phenotype must also be taken into consideration. The utilization of CRISPR/Cas9 may overcome some of these limitations, for instance by minimizing the risk that the foreign gene will be integrated in the wrong place in the genome. Furthermore, it will place the gene under the control of its natural promoter. However, the delivery of CRISPR/Cas9 into the patient is still challenging and the Cas9 enzyme could cleave at unwanted locations. Similarly, the use of miRNAs targeting BMPRII has to be evaluated for off-target effects and an effective delivery system has to be found in order to consider this approach as a promising treatment. A solution for both plasmid DNA and miRNA delivery might be using the liposomes [169] or iTOP (induced transduction by osmocytosis and propanebetaine), which is an active uptake mechanism in which NaCl-mediated hyperosmolarity together with propanebetaine triggers the uptake of macromolecules [170]. Another therapeutic strategy is the use of FDA-approved drugs that have been found to be beneficial in PAH mice models or similar diseases. Ataluren, for example, allows the cellular machinery to read-through premature stop codons [115]. Although most of the BMPR2 mutations (~70%) are non-sense mutations, not all patients will benefit from this approach. Nevertheless, further in vivo studies are worth pursuing in the context of PAH. Likewise, clinical trials using chloroquine have to be performed to test its effectiveness in PAH patients. The use of chloroquine has to be carefully evaluated because blocking lysosomal degradation might trigger non-specific off-target effects when used as a long-term treatment. An alternative drug showing significant clinical benefits for PAH is FK506/Tacrolimus. However, it still needs to be monitored for side effects since it is an immunosuppressive drug (currently utilized after allogeneic organ transplant). Moreover, the effectiveness of FK506 at low doses has to be rigorously tested.

It is important to highlight that although several drugs showed beneficial outcomes in animal models, most of the drugs have failed in the clinic. In light of this, we should focus on a more personalized approach which takes into account the co-existence of modifier genes, infections, toxic exposure, inflammation, or alterations in estrogen metabolism. Combining treatments which target not only BMPRII signaling but also inflammation and hypoxia should improve outcomes. Lastly, the use of human ex vivo models such as lung or vessel on a chip [171] could be beneficial for drug discovery and efficacy testing in the context of PAH. We anticipate that such models may improve the relevance of pre-clinical results by using patient derived cells, especially since animal models of PAH are frequently difficult to translate into clinical practice.

Taken together, previously discussed data suggest that modulation of BMPRII signaling in PAH is a promising alternative that could prevent and reverse pulmonary vascular remodeling. However, different therapeutic approaches
aimed at to increasing the levels of BMPRII signaling are needed, and these approaches will depend on the particular genetic background of each patient. In addition, for more efficient treatments, targeting other genetic and environmental factors that contribute to the disease must be taken into consideration. In this regard, modulators of the inflammatory response and estrogen metabolism could be used to help restore BMPRII signaling.

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