New mutations and Pathogenesis of Pulmonary Hypertension:

Progress and puzzles in disease pathogenesis

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Abstract

Pulmonary arterial hypertension (PAH) is a complex multifactorial disease with poor prognosis characterized by functional and structural alterations of the pulmonary circulation causing marked increase in pulmonary vascular resistance (PVR), ultimately leading to right heart failure and death. Mutations in the gene encoding *Bone Morphogenetic Protein Receptor type 2* (*BMPR2*), a receptor for the transforming growth factor-beta (TGF-β) superfamily, account for over 70% of families with PAH, and approximately 20% of sporadic cases. In recent years, however, less common or rare mutations in other genes have been identified. This review will consider how these newly discovered PAH genes could help to provide a better understanding of the molecular and cellular bases of the maintenance of the pulmonary vascular integrity, as well as their role in the PAH pathogenesis underlying occlusion of arterioles in the lung. We will also discuss how insights into the genetic contributions of these new PAH-related genes may open up new therapeutic targets for this, currently incurable, cardiopulmonary disorder.

Keywords

pulmonary hypertension; pulmonary vascular remodeling; functional genetic; gene mutation; familial

1. Introduction

The term pulmonary arterial hypertension (PAH) encompasses a heterogeneous group of devastating and incurable cardiopulmonary vascular disorders that are characterized by

vasoconstriction and remodeling in the distal pulmonary arteries [1, 2]. PAH may occur in a number of different clinical contexts (Table 1): sporadically, without an identifiable underlying cause, called idiopathic PAH, or in association with exposure to certain weight reducing drugs and certain toxins, or other diseases (aPAH) like congenital heart disease, connective tissue disease, human immunodeficiency virus (HIV), schistosomiasis, portal hypertension or as a pulmonary veno-occlusive disease (PVOD). When PAH occurs in a familial context (called heritable PAH), heterozygous germline mutations in the *Bone Morphogenetic Protein Receptor type 2* (*BMPR2*) gene are detected in at least 70 to 80% of cases [3, 4]. Recently, mutations located in other genes of different members of the transforming growth factor (TGF)-β superfamily have been reported (Table 2 and Figure 1, 2, and 3), including genes encoding general control nonderepressible (GCN)-2 (*EIF2AK4*), activin receptor-like kinase (ALK)-1 (*ACVRL1*), transcription factor T-box 4 (*TBX4*), BMP9 (*GDF2*), SRY-related HMG-box transcription factor (*SOX17*), endoglin (*ENG*), a potassium two pore domain channel called TWIK-related acid-sensitive potassium channel (TASK)-1 (*KCNK3*), sulfonylurea receptor (SUR)-1 (*ABCC8*), ATPase 13A3 (*ATP13A3*), SMAD family member 8 (*SMAD9*), BMP10 (*BMP10*), aquaporin-1 (*AQP1*), SMAD1 (*SMAD1*), SMAD4 (*SMAD4*), and caveolin-1 *(CAV1)*. Currently, it remains unclear how mutations in these newly discovered genes in certain familial PAH cases affect the maintenance of the pulmonary vascular integrity and lead to occlusion of arterioles in the lung. We review here the current understanding of the connections between these newly discovered PAH-predisposing gene mutations, disease pathology and clinical manifestation. We also discuss how dissecting the connections between these newly identified PAH-related genes could increase our current understanding of the PAH pathogenesis, and offer new therapeutic targets.

2. Genes implicated in PAH

Heritable PAH (HPAH) includes not only cases of familial PAH defined by the presence of two or more members of the family with PAH with or without identified germline variant, but also idiopathic PAH cases when a pathogenic variant in one of the known PAH-related genes has been identified. Usually, HPAH is inherited by heterozygous mutations in an autosomal dominant manner with an incomplete penetrance (approximately 10-30%) [5, 6], which means that not all mutation carriers develop PAH during their life. Notably, the penetrance in males is estimated to be about three times lower than in females [5]. Therefore, HPAH may require a second hit or a further stress for it to manifest clinically such as chronic exposure to hypoxia, oxidative stress, inflammatory signals, or to certain drugs, toxins or hormones, or even general changes in expression of genes that occur with aging of the pulmonary circulation. Additional genetic or epigenetic factors, diet, lifestyle, or a combination of all these factors may also contribute to the onset and progression of the pulmonary vascular remodeling in HPAH patients. Some pedigrees may appear to show genetic anticipation, where the disease presents at a younger age in successive generations, but this likely represents an artefact of incomplete time of observation [5]. The notable exception to the dominant inheritance pattern is pulmonary veno-occlusive disease (PVOD) or pulmonary capillary hemangiomatosis (PCH), which are inherited as an autosomal

recessive trait. Other, more complex genetic patterns are now starting to emerge, and will be discussed in more detail below.

2.1. Bone morphogenetic protein pathway genes in Heritable PAH

2.1.1. BMPR2—*BMPR2* was the first gene identified in families with PAH [7, 8], and accounts for around 70-80% of heritable cases. It encodes the bone morphogenetic protein (BMP) receptor type-II (Figure 2). The majority of pathogenic variants in *BMPR2* are nonsense, frameshift or splice-site mutations that are predicted to lead to degradation of the mutant transcript by nonsense-mediated mRNA decay. Deletions or duplications involving one or more exons, which would significantly disrupt protein structure, are also common. This indicates that haploinsufficiency - a 50% reduction in functional protein - is the primary molecular mechanism [9]. The remaining mutations are amino acid substitutions, often occurring in functionally-important domains, such as the kinase domain. Some missense mutations may act in a dominant negative manner, downregulating the function of the remaining wildtype allele, although evidence for this remains contradictory and may be mutation-specific [10, 11].

2.1.2. ACVRL1 and Endoglin—*ACVRL1* encodes activin A receptor-like kinase I, known as ALK1, which is the predominant type-I receptor in endothelial cells (Figure 2). ALK1 complexes with BMPRII to activate signaling in response to BMP9 and BMP10 [12]. Endoglin (gene symbol *ENG*) is an accessory protein, also known as CD105, that is highly expressed in endothelial cells and interacts with ALK1 to promote BMP signaling. Heterozygous loss-of-function mutations in *ACVRL1* or *ENG* primarily cause hemorrhagic telangiectasia (HHT), a multi-system vascular disorder characterized by epistaxis, multiple telangiectases on the skin and mucosal surfaces, and arteriovenous malformations (AVMs) in major organs such as the lung, liver or brain [13, 14]. PAH has been reported in a small proportion of individuals with HHT, predominantly in association with *ACVRL1* mutations [15, 16]. *ENG* mutations are less commonly associated with PAH, but have been reported in several large studies [15-18]. The penetrance of HHT is considerably higher than for PAH, and thus almost all of these PAH cases also show signs of HHT. However, pulmonary hypertension may in some cases pre-date the onset of HHT, and is therefore not solely attributable to high cardiac output associated with liver AVMs [19].

2.1.3. Smads—Smad proteins operate downstream of the receptor complex, transmitting the signal to the nucleus, where they bind Smad-responsive elements to regulate gene expression (Figure 2). In the BMP pathway, Smad-1, −5 and −8 (encoded by *SMAD1*, *SMAD5* and *SMAD9* genes, respectively) are the receptor-Smads, while Smad-4 (*SMAD4*) is common to both the BMP and TGF-β pathways. Of these genes, *SMAD9* mutations have been reported most frequently in PAH patients, although the overall prevalence is low. Interestingly, loss of function mutations in *SMAD4* primarily lead to a syndrome of juvenile polyposis of the colon and HHT, with few reports of PAH [20]. This may suggest that non-canonical signaling pathways downstream of BMPRII activation may predominate over canonical BMP signaling in the pathogenesis of PAH.

2.1.4 BMP9 and BMP10—BMP9 (encoded by *GDF2*) and BMP10 are the two major ligands of the ALK1-BMPRII receptor complex [12]. Mutations in *GDF2* account for up to 3-5% of HPAH cases [17, 21], although incomplete penetrance and variable expressivity have been recently reported [6], as discussed in more detail below. While some of these variants are clear loss of function that lead to premature truncation of the protein, many are missense substitutions that change a single amino acid. In vitro overexpression studies of these mutations have demonstrated that they interfere with normal protein processing. BMP9 and BMP10 are both synthesized as a pro-protein, which then dimerizes and undergoes furin cleavage to generate the mature protein that is secreted from the cell [22]. Modeling of *GDF2* mutations found in HPAH patients showed a significant reduction in mature BMP9, with most of the protein remaining uncleaved [17, 21, 23]. This has been confirmed in vivo, with significantly lower levels of BMP9 in the plasma of PAH patients carrying pathogenic *GDF2* variants compared to controls [21, 23]. Recently it has been shown that BMP9 and BMP10 can form heterodimers that are biologically active in plasma [24]. Interestingly, those patients with a *GDF2* pathogenic variant and reduced plasma BMP9 levels also showed reduced levels of prodomain-bound BMP10 (pBMP10). Indeed, plasma BMP9 and pBMP10 levels were highly correlated, both in controls and PAH patients, with significantly lower levels of both cytokines in males than females [23]. Overall, however, there were no differences in PAH versus controls, except for a slight reduction in pBMP10 in female patients, although some patients without *GDF2* mutations also exhibited very low levels of BMP9 [23]. In contrast, plasma BMP9 levels were markedly reduced in patients with portopulmonary hypertension, even in the absence of genetic mutation, and predicted transplant-free survival [25]. *BMP10* mutations have also been reported in PAH patients, but less frequently than *GDF2* [23, 26] (Figure 1).

2.2. Other PAH risk genes

Caveolin-1 (*CAV1*) and *KCNK3*, encoding a potassium channel known as TASK1, were the first HPAH genes identified by whole exome sequencing in families that were negative for mutations in *BMPR2* [27, 28]. More recently, whole exome or genome sequencing has identified several additional novel genes, including aquaporin-1 (*AQP1*), ATPase 13A3 (*ATP13A3*), SRY-box transcription factor 17 (*SOX17*), ATP binding cassette subfamily C member 8 (*ABCC8*), gamma-glutamyl carboxylase (*GGCX*), kallikrein 1 (*KLK1*), and vascular endothelial growth factor receptor 2 (*KDR*) [17, 18, 29, 30]. SOX17 is especially interesting, with rare loss of function variants identified in HPAH patients, but also more common variants in an enhancer region were associated with a modest increase in PAH risk in a large meta-analysis of genome wide association studies [31] (Figure 1).

Recently, *TBX4* mutations have also been described in HPAH. *TBX4* encodes the T-Box Transcription Factor 4, one of a family of transcription factors that share a common DNA-binding domain known as the T-box. T-box genes are important in developmental processes, including bone development and lung branching morphogenesis. Loss-of-function mutations in *TBX4* were originally found to cause small patella syndrome (SPS), a bone developmental abnormality characterized by small or absent knee-caps, and in some cases abnormalities of the pelvic bones and/or feet. The first potential link with PAH came with the identification of a microdeletion at 17q22q23.2 in a child with multiple

congenital anomalies and PAH [32]. The deletion encompassed *TBX2* and *TBX4*, as well as multiple other genes. A subsequent study of patients with PAH and developmental delays or dysmorphic features narrowed down the common deletion region to suggest *TBX2* and *TBX4* as candidate genes [33]. Sequencing of these two genes in non-syndromic PAH cases then identified intragenic loss-of-function mutations in *TBX4* but not *TBX2*. Skeletal surveys revealed unrecognized SPS in all of the patients, and in two parents who carried the *TBX4* mutation but were unaffected with PAH [33]. Since then, multiple studies have validated *TBX4* as a significant cause of PAH, with or without SPS, and it is now recognized as the major locus in pediatric cases [17, 18, 34].

2.3. *EIF2AK4* mutations in PVOD/PCH

Familial PVOD and PCH, classified as group 1.6 at the 6th World Symposium of PH [2] (Table 1), shows autosomal recessive inheritance, and therefore differs from HPAH. *EIF2AK4*, encoding eukaryotic translation initiation factor 2 alpha kinase 4 also known as general control nonderepressible 2 (GCN2), was identified as the causative gene for heritable PVOD/PCH, with biallelic mutations reported in familial cases, and up to 25% of sporadic cases [35, 36]. Interestingly, biallelic *EIF2AK4* mutations were also identified in approximately 1% of idiopathic PAH patients in a large whole genome sequencing study [37]. These patients could not be distinguished by chest CT, but showed significantly reduced diffusing capacity for carbon monoxide (DLCO) and earlier age at diagnosis. As discussed below, correct classification is important for clinical management, and therefore genetic testing for *EIF2AK4* is valuable in making a differential diagnosis between PVOD/PCH and idiopathic PAH.

2.4. Mutations in Associated forms of PAH

Early reports suggested that *BMPR2* mutations were found not only in familial or idiopathic PAH, but also in PAH associated with congenital heart defects or anorexigen exposure [38, 39]. However, the only large systematic study of associated PAH has been from the US-based PAH Biobank, which included 1239 associated PAH cases analyzed by whole exome sequencing [18]. Mutations were identified in 8 of 110 cases (7.2%) of drugs and toxins-associated PAH, in the genes *BMPR2, ACVRL1, TBX4* and *SOX17*. For connective tissue disease-associated PAH, the frequency was 33 of 722 cases (4.6%), involving all genes except *SOX17. ABCC8* was particularly frequent, representing 9 (27%) of these variants. And in CHD-associated PAH, mutations were identified in 22 of 268 cases (8.2%) with *BMPR2, ACVRL1, SMAD9, TBX4, SOX17*, and *ABCC8* accounting for 2 or more variants each. A subset of these cases was also included in a prior publication that concluded SOX17 may account for up to 3% of CHD-PAH cases [40].

2.5. Genetic complexities: possible semi-dominant genes and digenic inheritance

Recent large-scale next generation sequencing efforts have started to uncover more complex modes of inheritance. One childhood-onset PAH case was reported with a homozygous *GDF2* nonsense mutation [6], and a second case was homozygous for the Arg110Trp substitution [41], previously determined to be functionally pathogenic [23]. Two other homozygous cases with protein truncating variants have been reported with pulmonary arteriovenous malformations (AVMs) but not PAH [42, 43], while a sibling of one was

homozygous for the same mutation, yet unaffected. The four affected individuals showed spider-like telangiectases on the skin. All heterozygous parents were unaffected. This underlines that the incomplete penetrance of PAH extends beyond *BMPR2* to other PAH genes, and also illustrates considerable variable expressivity of *GDF2* mutations. This is further illustrated by several cases with heterozygous *GDF2* variants and an HHT-like vascular anomaly syndrome [44], and a family with typical HHT, including childhood-onset pulmonary AVMs in the proband [45].

The occurrence of more severe disease in homozygous cases compared with heterozygotes suggests that the inheritance pattern may be semi-dominant, rather than a true dominant where homozygous cases are not more severe. However, since PAH varies considerably in the age of onset and clinical course, it can be difficult to distinguish these two scenarios. With respect to *GDF2*, only two of five homozygous individuals developed PAH, albeit in childhood, suggesting that the presentation is not necessarily more severe than the many documented heterozygous cases. However, it remains unclear how mutations that appear functionally equivalent can give rise to different phenotypes, either PAH and/or a spectrum of vascular anomalies that overlap with HHT.

ATP13A3 provides more convincing evidence of a dose-dependent effect, with biallelic mutations reported in three families [46]. All homozygous or compound heterozygous individuals presented with PAH in early childhood and had a severe clinical course with high mortality. Their heterozygous parents and siblings were unaffected. A homozygous mutation in *KCNK3* has also been reported in a consanguineous PAH family [47]. The proband, who was homozygous for the variant, presented at two months of age and required lung transplantation at five years-old, whereas the heterozygous mother presented at age 19, a year after the child's birth. The heterozygous father was unaffected. Although only a single family, this suggests that *KCNK3* may also show semi-dominance.

In the reverse scenario, whereas heterozygous carriers of recessive *EIF2AK4* mutations are expected to be healthy, Hadinnapola *et al* found a significantly higher frequency of heterozygous rare, likely deleterious, *EIF2AK4* variants in patients with idiopathic PAH compared with controls, suggesting that the heterozygous state may contribute to PAH risk [37]. Two of these cases also had mutations in *BMPR2*. Similarly, co-occurrence of a *BMPR2* mutation and heterozygous *EIF2AK4* variant was documented in two members of a family affected with PAH [48]. Two other individuals carried only the *BMPR2* mutation and were unaffected. This raises the possibility that heterozygous *EIF2AK4* mutations might act as modifiers of disease penetrance, although in this family, the two unaffected *BMPR2* carriers were both male, where the penetrance is known to be lower. At least five other cases have been reported with likely deleterious variants in *BMPR2* plus one other gene (*TBX4* in one case, *ACVRL1* in two, and *SMAD9* in two) [17, 18]. Further studies are required to understand these examples of potential digenic inheritance, and whether the second mutation increases the overall likelihood of developing PAH.

3. What does this mean for the patient or clinician?

Currently, PAH-specific therapies should only be used in patients diagnosed with PAH and not in unaffected mutation carriers. The search for the presence of a mutation in one of the known predisposing genes provides information for both the patient and clinician.

First, the presence and the type of mutation can give information about the clinical phenotype and outcomes and thus is recommended. It is indeed known that PAH patients carrying a *BMPR2*[3], *ACVRL1*[49], and *EIF2AK4*[50] are younger at diagnosis than non-carriers. PAH patients with *BMPR2* mutations are also known to have a more severe hemodynamic at diagnosis and a worse prognosis[3]. They are also less likely to show an acute vasodilator response and their diffusing capacity of the lung for carbon monoxide (DLCO) values are relatively preserved[3, 51]. In addition, even with a similar afterload, right-ventricular (RV) function is more severely affected in *BMPR2* mutation carriers than in noncarriers[52]. In explanted lungs from *BMPR2* patients with PAH, a more pronounced bronchial vascular changes (including bronchial arterial hypertrophy/ dilatation and bronchial microvessel density) have been reported, along with formation of large fibrous vascular structures that appeared to connect the systemic vasculature to pulmonary veins[53]. The resultant increase in communication between systemic and pulmonary circulations could explain why higher amounts of hemoptysis are seen in PAH patients with *BMPR2* mutations. Despite less severe initial hemodynamics and similar management, *ACVRL1* patients with PAH had also worse prognosis compared with other patients with PAH, suggesting more rapid disease progression[49]. Pulmonary veno-occlusive disease and/or pulmonary capillary haemangiomatosis (PVOD/PCH) very often share an overlapping disease phenotype with PAH, but a genetic test identifying a bi-allelic *EIF2AK4* mutation could serve as a tool for the early diagnosis of PVOD[50]. Heritable PVOD/PCH due to bi-allelic *EIF2AK4* mutations display similar disease severity compared with mutation non-carriers, but the response to therapy approved for PAH drugs in PVOD/PCH is rare[50]. The identification of a bi-allelic *EIF2AK4* mutation can therefore allow an appropriate and early referral for lung transplantation.

Second, the presence of a mutation can also help to detect PAH in an early phase and to identify predictors of outcomes in asymptomatic mutation carriers. A systematic screening for PAH in adults carrying a *BMPR2* mutation demonstrates that asymptomatic *BMPR2* mutation carriers have a significant risk of developing incident PAH ranging from 0.99% per year in males to 3.5% per year in females[54]. Due to the severity of this devastating disease and the potential impact of abortion on parents, screening program might also lead to the consideration of pre-natal diagnosis or pre-implantation diagnosis[55, 56].

The development of genetic counselling and the analysis of the phenotypic profile of patients according to their genotype have already helped to develop preventive medicine and a specific monitoring for *BMPR2* mutation carriers. This explains why the 2015 European Society of Cardiology (ESC)/ European Respiratory Society (ERS) guidelines currently recommend that "individuals who test positive for PAH-causing mutations and first-degree relatives of heritable PAH cases may be considered to have an annual screening echocardiogram (IIb-C)". The modality for genetic testing varies between countries, but

often the first step is to perform testing of a panel of the known HPAH genes. This should ideally be performed in an accredited clinical testing laboratory to ensure the highest quality assurance and correct interpretation of the results. If there is a strong suspicion of a genetic cause, a negative test may be followed up by whole exome or whole genome sequencing, either in a clinical lab or on a research basis. Practices vary as to whether the patient may be informed of the results from a research test. Due to the complexities of incomplete penetrance and variable expressivity discussed above, as well as the implications of a positive test result, it is important that patients and their families are fully informed prior to genetic testing. Such education should be provided by a genetics professional, or a PAH physician with in-depth genetic knowledge. It is important to note that a negative test result in a patient with PAH does not exclude the possibility of heritable PAH, since there are still families in whom the causative gene has not been identified. When a pathogenic variant is identified, asymptomatic family members may choose to be tested for the familial mutation, and here, a negative test result returns their PAH risk to population level (i.e., very low). Genetic counseling from a specialist clinical genetics provider is essential for asymptomatic family members who are considering a genetic test.

Pediatric PAH differs from the adult PAH in several important aspects, including sex bias, clinical presentation, etiology, response to therapy, but the genetic basis in children is also very different from adult. Indeed, children have a greater predominance of rare pathogenic inherited variants including in the *TBX4* and *SOX17* genes [57]. Thus, there is a strong case for offering genetic counseling and testing in children with PAH.

4.   Rare genetic mutations shed light on the pathogenesis of PAH: one or more paths to pathogenesis?

4.1.   Experimental evidence supporting a role of *BMPR2* mutations on PAH precipitation and progression

To date there is compelling in vitro and in vivo evidence for a contribution of the loss and/or alterations of the BMP pathway in the characteristic pathogenic pulmonary vascular remodeling in PAH. Defects in the BMP pathway have been indeed associated with modulation of cell proliferation, survival, and differentiation, but also with glycolytic metabolism, persistent inflammation and genome instability. Despite enormous progress, however, our understanding of the detailed mechanisms by which reduction or alteration of the BMP pathway contribute to the pulmonary vascular remodeling in PAH is still far from complete. BMP activation is indeed highly context- and dose-dependent, and the cellular response depends on which ligands are present and what receptors are expressed by the signal-receiving cell. For example, a recent study has shown that BMP9 acts as an angiogenic switch that can either promote or prevent endothelial proliferation, depending on the level of BMPRII at the endothelial cell surface [58]. The activities of BMPs are dynamically and precisely regulated at different molecular levels through for example the action of highly regulated diffusible and cell surface-bound agonists and antagonists or via caveolin-1, a cell membrane protein involved in the formation of caveolin-rich microdomains in the plasma membrane essential not only for endocytosis, transcytosis, mechanotransduction, but also for the transmission of the BMP signalling

and other signaling pathways. Recently, it was also shown that pulmonary artery smooth muscle cells (PA-SMCs) exposed to tumour necrosis factor (TNF)-α exhibit a reduction of BMPRII receptor presence on their cell surface, leading to BMP6-mediated PA-SMC proliferation via preferential activation of an ALK2/ACTRIIA signalling axis [59]. These experimental evidences highlight how slight changes to the cellular microenvironment can result in dramatic changes to the response of pulmonary vascular cells. Therefore, a better understanding of these precise molecular mechanisms behind the subversion of the BMP signaling in PAH is essential and might help to understand the low penetrance of the *BMPR2* mutations.

Transgenic animals, especially mice, have been extensively used to study the susceptibility of various knockout (KO), knockin (KI) animals to remodel pulmonary vessels in response to different known PH inducers such as chronic hypoxia, monocrotaline or SU5416 combined with chronic hypoxia (Table 3). Even if they do not reproduce all aspects of human PAH pathology, they have enriched our understanding of biology behind the BMP signaling and their role in the homeostasis and remodeling of the pulmonary circulation. For example, the demonstration that mice deficient in ALK1, BMPRII, endoglin, Smad1, Smad8 or in KLF develop spontaneous PH or were more prone to develop experimental PH relative to their wild type littermates underlines their importance in the PAH physiopathology. However, some of these results are still unclear, and much remains to be learned. Many important questions regarding the contributions of these different PAH-related genes in the pathophysiology indeed remain unanswered, not only in relation to how they regulate pulmonary vascular cell behaviors involved in vascular function, but also why they predominantly affect the pulmonary vasculature. It is also important to know to what extent the regulation of these pathways controlled by these PAH-related genes is the same as in humans.

Because the recruitment or modulation of the BMP signalling is critical for proper function of the cardiovascular system with partial redundancy among members and complex interrelationship with other pathways, many unexpected and unexplained phenotypes can be observed in transgenic animals. For example, a recent study has highlighted redundant roles for BMP9 and BMP10 in cardiovascular homeostasis under normoxic conditions, but has also underlined specific roles under chronic hypoxic conditions [60]. These authors have shown that adult *Bmp9* and *Bmp10* KO mice do not develop spontaneous PH under unstressed conditions, but that the combined deficiency in *Bmp9* and *Bmp10* lead to vascular defects resulting in a decrease in peripheral vascular resistance and blood pressure and the progressive development of high-output heart failure (HOHF) and pulmonary hemosiderosis [60]. Therefore, the use of genetically engineered animals and preclinical PAH animal models, combined with functional analyses of human pulmonary vascular cell responses to exogenous BMPs in different environment not only greatly improved our understanding of the regulatory mechanisms and signaling of the different BMPs but also provided a powerful strategy to decipher their critical roles in the maintenance of the pulmonary vascular integrity and remodeling.

4.2. Other PAH-related genes offering avenues or new therapies

Although the discovery of these novel PAH related genes plays a crucial part in furthering our understanding of the basis of disease, the challenge remains whether these pathways can be targeted by therapeutic interventions. Such interventions might target a related part of the pathway or specifically correct the signaling defect brought about by a mutation. Of the recently reported PAH genes, receptors, transporters and ion channels in particular represent druggable targets, whereas transcription factors are more challenging. This section will focus on those pathways and genes that suggest potential druggable targets.

ATP13A3.—Recently, whole genome sequencing identified rare variants in *ATP13A3* in PAH patients, accounting for 1.1% of a large I/HPAH cohort [17]. Missense mutations in *ATP13A3* are enriched within the catalytic domains, suggesting a potential disruption of normal ATP13A3 function. The finding of protein truncating variants in PAH patients suggests that loss-of-function of ATP13A3 could contribute to the pathogenesis of PAH. ATP13A3 is a member of the P-type ATPase family of transporters [61]. Although the function of ATP13A3 was largely unknown, it has recently been shown to be a polyamine transporter [62]. Polyamines, such as putrescine, spermidine, and spermine, are physiologically important polycations that have previously been shown to play a role in the pathobiology of PAH [63]. The mechanisms by which mutations in *ATP13A3* perturb polyamine metabolism remain to be determined but offer the possibility that modulation of polyamine metabolism might offer new therapeutic avenues in PAH.

AQP1.—Aquaporin 1 (AQP1) is a widely expressed water channel and is predominantly recognised for maintaining water homeostasis in the kidney. In the UK/EU PAH Cohort 9 patients were found to harbour heterozygous mutations in *AQP1*. Of note, 5 of these unrelated individuals had the recurrent mutation p.Arg195Trp (R195W). Structural analysis of the mutations based on the high-resolution bovine AQP1 structure predicts that the majority of identified human *AQP1* rare variants are situated within the critical water channel. In particular, the recurrent variant R195W locates to the hydrophilic face of the pore. The arginine at position 195 helps define the constriction region of the AQP1 pore structure and is conserved across the water-specific aquaporins. Recently, knockout of *AQP1* was shown to alleviate hypoxia-induced PAH in mice [64]. Reduced angiogenesis was also observed in *Aqp1* knockout mice [65]. Conversely, aquaporin 1 overexpression induced proliferation and migration of pulmonary artery smooth muscle cells [66]. Collectively, these findings suggest an essential role of AQP1 in vascular homeostasis and suggest that inhibitors of AQP1 are worthy of further investigation in PAH [67].

KCNK3.—Impaired potassium channel function had become a well-recognized feature of PAH pulmonary artery smooth muscle cells even prior to the identification of mutations in the *KCNK3* gene, which encodes 2-pore domain potassium channel, TASK-1. The role of potassium channels in PAH has been reviewed recently [68, 69]. Activators of TASK-1 have been shown to restore function and warrant further study in PAH [28]. Moreover, dysfunction of BMPRII has been associated with reduced KCNK3 expression and function in rats with targeted disruption of BMPRII [70].

4.3 Possible developmental origins for PAH: *TBX4, SOX17* and *KDR*

TBX4 is known to play a crucial role in embryonic lung development. In mouse, *Tbx4* and the closely related gene *Tbx5* are strongly expressed in the trachea and lung mesenchyme, and are important in lung branching morphogenesis [71]. Homozygous knockout *Tbx4* embryos die around day E10.5 due to failure of allantois development and placental insufficiency [72]. Conditional alleles are therefore needed to study the effects of loss of *Tbx4* expression. Loss of *Tbx4/Tbx5* expression leads to reduced expression of *Fgf10*, which in turn reduces expression of *Bmp4*, a target of *Fgf10* [71]. As noted earlier, *TBX4* mutations are more frequent in childhood-onset PAH than adults, suggesting that developmental lung defects may directly contribute to PAH susceptibility. Galambos *et al.* [73] published a retrospective series of 19 neonates and children with pulmonary hypertension and either an intragenic *TBX4* mutation or a large heterozygous deletion that encompassed the entire *TBX4* locus and a variable number of neighboring genes. Ten of these children presented with persistent pulmonary hypertension of the newborn (PPHN), a condition that is distinct from PAH and reflects a failure to transition from the fetal to postnatal circulatory pattern. PPHN initially improved or resolved in eight cases, but the other two died before the age of one year. Subsequently, all 17 remaining children developed PH, with a variable clinical course that ranged from death or lung transplantation to resolution of their disease. Histological samples obtained via biopsy or at transplant/death showed significant defects in lung development, elements of interstitial lung disease and vascular remodeling that in some cases extended to the bronchial circulation. In one case, where a biopsy was performed at age 2 and transplant at 18, longitudinal histology showed marked progression of alveolar growth defects, as well as vascular remodeling, over time. These studies suggest that although the neonatal period may be unremarkable, abnormal lung development resulting from loss of *TBX4* function can predispose to subsequent development of PH.

At the other end of the age spectrum, *KDR* mutations were associated with an older age of onset than other HPAH cases, many with low DLCO and evidence of interstitial lung disease [30, 74]. *KDR* encodes the receptor for vascular endothelial growth factor (VEGF-A). It is essential for embryonic blood vessel development, as evidenced by the embryonic lethal phenotype in *Kdr* loss-of-function mice [75]. In the neonatal period, inhibition of VEGF signaling in rats leads to reduced alveolarization and vascular density [76], suggesting that PH due to *KDR* mutation may have its roots in a subtle developmental abnormality, and with diminished ability to maintain a healthy vasculature during adult life [74]. Extreme KDR blockade with SU5416, a VEGF-receptor antagonist, is used to generate one of the well-studied rodent models of PH [77], and endothelial-specific deletion also leads to pulmonary vascular remodeling and PH (Table 3) [78]. Further studies of animals with more subtle defects in VEGF signaling are needed to understand the origins of PAH resulting from heterozygous loss of KDR function, and the stressors that may precipitate development of the disease.

SOX17 is one of a family of SRY-box transcription factors containing a DNA-binding domain with homology to that of the sex-determining region Y (*SRY*) gene. SOX genes are involved in various developmental processes, and SOX17 plays specific roles in cardiac development and pulmonary vascular morphogenesis (among others). Thus, SOX17 variants

- both common and rare - may give further insight into the early origins of PAH. Indeed, rare SOX17 variants are a significant contributor to CHD-associated pediatric PAH [40]. We refer the reader to a recent review for a comprehensive summary of the current state of knowledge regarding *SOX17* and PAH [79]. Lastly, preterm birth may itself be a risk factor for pulmonary vascular disease in adult life [80, 81].

5. Current therapeutic developments related to the genetic pathways

5.1 Targeting the BMPRII pathway

The fact that *BMPR2* mutation is the most common genetic finding in patients with PAH, coupled with the finding that loss of expression or function of BMPRII is a feature common to many forms of PAH in animals and patients, has led to attempts to restore function in this pathway as a potential therapeutic approach. There are various approaches that have been proposed to achieve restoration of BMPRII signalling based on insights into the transcription, translation, cell surface trafficking, and degradation of BMPRII. These approaches have been reviewed extensively recently [82, 83]. One promising approach is developing a version of human BMP9 to agonize the deficient BMPRII/ALK1 pathway in PAH, but this is yet to be tested in the clinic. Here, we will focus on approaches that are already being actively explored in the clinic.

Sotatercept.—Early studies of BMP and TGF-β signalling in PAH showed that loss of BMP signalling was a common finding in patients with PAH as well as animal models of disease. In addition, these changes were often accompanied by an increase in signalling mediated by TGF-β [84]. Classically, TGF-β signals via Smads 2 and 3, whereas BMPs signal via Smads 1,5 and 8, although both pathways utilise Smad4. Activins are members of the TGF-β superfamily and also signal predominantly via Smad2/3. Sotatercept is a recombinant fusion protein consisting of the extracellular domain of the human activin receptor type IIA (ACTRIIA) linked to the Fc portion of human IgG1 [85]. A number of TGF-β family ligands bind with high affinity to ACTRIIA including among others activin A, activin B, GDF8 and GDF11. Thus, sotatercept acts as a soluble ligand trap for these factors and blocks their engagement with cell surface receptor complexes, thereby inhibiting their signalling. The application of sotatercept analogues in preclinical rodent models of PAH reverses aspects of disease pathology and is associated with a reduction in Smad2/3 activation in the pulmonary vasculature [86]. The precise mechanism of action of sotatercept in PAH remains unclear, although the observed efficacy suggests that activins play a more significant role in PAH than previously appreciated. Previous preclinical studies have targeted Smad2/3 activation by TGF-β in PAH using, for example, small molecule inhibitors of ALK5 [84, 87] or Fc-TGFβRII [88], but it appears that activin-induced Smad2/3 activation is also playing an important role in PAH pathobiology. The available data from animals with experimental PAH (e.g., Sugen-hypoxia model) treated with a murine sotatercept analogue does not suggest any rescue of the suppressed BMP signalling pathway. No matter what the precise mechanism of action is, sotatercept has shown very promising results in the Phase 2 (PULSAR) trial [89]. The trial met its primary endpoint of a reduction in pulmonary vascular resistance, despite many patients already being on double or triple therapy with existing vasodilator agents. On the basis of these promising data, Phase 3 trials of sotatercept

are now underway in PAH: STELLAR [NCT04576988], HYPERION, [NCT04811092] and ZENITH [NCT04896008].

Tacrolimus.—Since the discovery of loss of function mutations in *BMPR2* in 2000, efforts to identify approaches to restore BMPRII signalling have gained momentum. Most of these approaches remain in the preclinical stages of development, and have been extensively reviewed recently, but at least two approaches have been tested in the clinic. The first potential BMPRII modulator to be tested in the clinic was low dose tacrolimus (FK506). This small molecule was identified from an in vitro screen for BMP signalling activity from a panel of FDA approved drugs [90, 91]. The mechanism of action involves the displacement of FKBP12 from between the type I and type II BMP receptors, leading to phosphorylation of the type I receptor and subsequent downstream Smad phosphorylation. This effect is thought to be independent of calcineurin inhibition and the immunosuppressive effects of tacrolimus, and may occur at a lower dose than that required for immunosuppression. In rodent models of experimental PAH, low dose tacrolimus was shown to reverse aspects of the pathology and physiology of PAH. Building on these findings, a small open label Phase 2 (n=23 subjects, 16 weeks) study was conducted with tacrolimus in patients with PAH [91]. Although primarily a safety and tolerability study, the trial was negative for efficacy endpoints overall, but this was a small-scale single centre study which included patients with diverse forms of PAH. The investigators developed biomarkers of tacrolimus target engagement to determine whether the drug was actually increasing BMP signalling in individual patients. These assays comprised transcriptional readouts of known BMP targets in peripheral mononuclear cells. There was some suggestion that patients in whom target engagement could be demonstrated had a better response to the drug. This approach is undergoing further clinical development for PAH (https://vivus.com).

Genetically-targeted therapies.—Small molecule approaches to correct specific types of mutation, such as ribosomal readthrough of nonsense mutations or molecular chaperones that correct abnormal protein trafficking, are already in clinical use for some genetic diseases, including Duchenne muscular dystrophy and cystic fibrosis (CF). They have also shown promise for HPAH, although they remain in the early pre-clinical phases [92-94]. One of the challenges is that, unlike CF, no mutation in *BMPR2* or other HPAH gene occurs at a high frequency, and thus these approaches need to be tested on tens or even hundreds of different mutations. *Bmpr2* gene replacement therapy has been successfully tested in animal models, but presents challenges for human translation [95]. Likewise, the potential promise of gene editing approaches such as Crispr-Cas9 is offset by the challenges of delivering these therapies to the lung. In contrast, gene editing of autologous hematopoietic stem cells is showing great promise in early clinical trials for sickle cell disease and beta-thalassemia [96]. Multiple groups have shown that bone marrow-derived cells play a role in the pathogenesis of PAH [97-100], raising the intriguing possibility that perhaps gene editing of hematopoietic cells might be an alternative route for genetically-targeted PAH therapy.

6. Conclusion

Recent whole-exome and whole-genome sequencing efforts in large patient cohorts have transformed our understanding of the genetic basis of Group 1 pulmonary hypertension.

Despite these advances, many questions still remain. Some of these new genes have a clear functional relevance to what we already know about vascular remodeling and PAH, whereas others are obscure. One of the biggest puzzles is why many PAH genes have such low penetrance and variable expressivity, and what are the additional factors that contribute to disease initiation and progression? How do variants in HPAH genes interact with co-morbidities in APAH? For example, as we identify pathogenic variants in known HPAH genes in cases of CTD-PAH, is it coincidence that the patient has connective tissue disease (i.e., they would have developed PAH anyway, and been classified as idiopathic PAH), or did the co-occurrence of CTD hasten the onset of PAH in someone who is genetically predisposed? In CHD-PAH, to what extent does the HPAH gene play a role in cardiac development? At the individual case level, it is impossible to know; careful genetic epidemiological studies will be required in even larger cohorts to unravel these complexities.

The identification of *BMPR2* mutations, and our subsequent understanding of the pivotal role of BMP signaling, is translating into novel therapies for PAH that are relevant beyond just those patients with a heritable cause. To some, progress may seem slow, but this reflects the detailed level of study that is needed to understand the pathways and the effects of individual mutations, followed by drug development and rigorous clinical trials. The timeline from the identification of *CFTR* to the new modulator therapies now transforming the lives of patients with cystic fibrosis is similar. The concerted effort of the field to further improve our understanding behind these newly discovered genes in PAH will continue to enhance our understanding of this disease and drive the development of disease-modifying therapies.

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Nonstandard Abbreviations and Acronyms:

ACTRIIA	activin receptor type IIA
ALK	activin receptor-like kinase
AQP1	aquaporin-1
BMP	bone morphogenetic protein
BMPRII	bone morphogenetic protein receptor type-II
GCN2	general control nonderepressible-2
HHT	hemorrhagic telangiectasia
HPAH	heritable pulmonary arterial hypertension
KI	knockin
KO	knockout

PAH	pulmonary arterial hypertension
pBMP10	prodomain-bound bone morphogenetic protein 10
PCH	pulmonary capillary hemangiomatosis
PVOD	pulmonary veno-occlusive disease
SUR1	sulfonylurea receptor-1
TASK-1	TWIK-related acid-sensitive potassium channel-1
TGF-β	transforming growth factor-beta
TNF-α	tumor necrosis factor-alpha
VEGF	vascular endothelial growth factor

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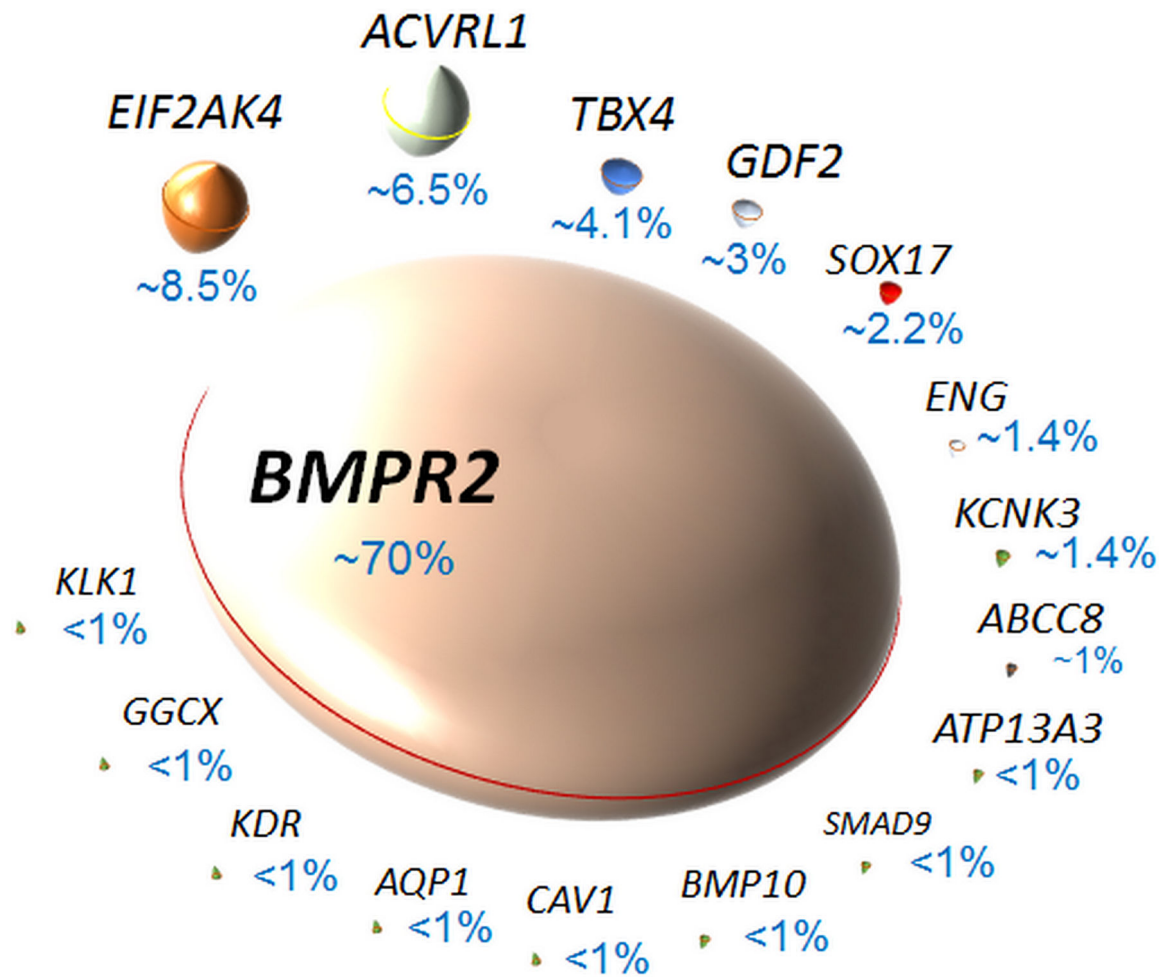
Figure 1: Genetic mutations associated with familial pulmonary arterial hypertension (PAH): (adapted from [4]).
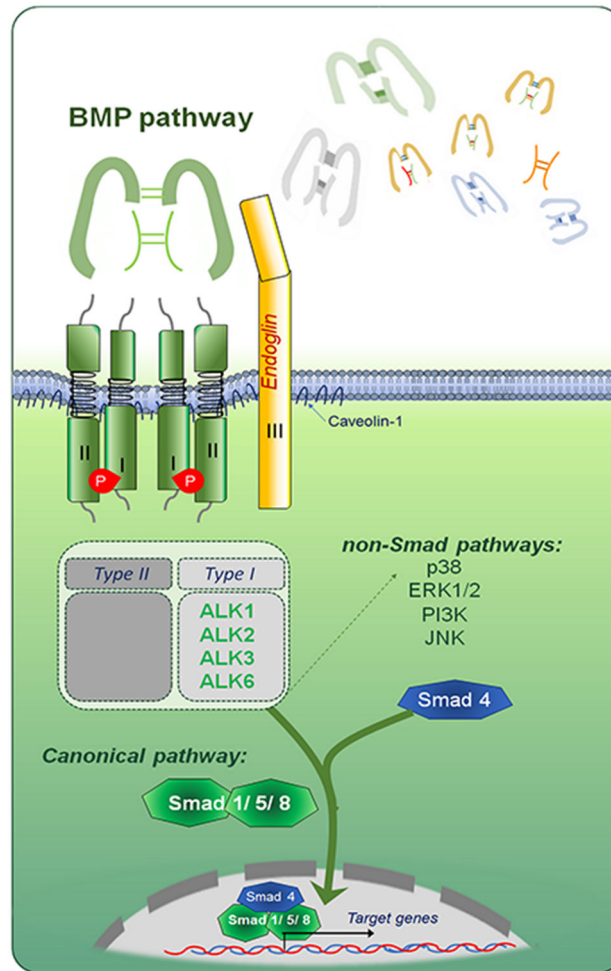
Figure 2: The BMP signalling pathway.
BMP ligands can form homo- or heterodimers which signal through a tetrameric set of receptors, comprising two type I and two type II receptors. Binding of BMP dimers to the receptors triggers a phosphorylation cascade that result in phosphorylation of Smad 1/5/8 and players of various non-SMAD pathways to initiate SMAD and non-SMAD signaling cascades. The non-SMAD pathways include signaling by mitogen-activated proteins kinases (MAPKs p38, ERK, and JNK), PI3K/AKT, and small Rho-like GTPases. Phosphorylated Smads are able to form complexes with Smad4 and translocate to the nucleus. (Illustration credit: Ben Smith)
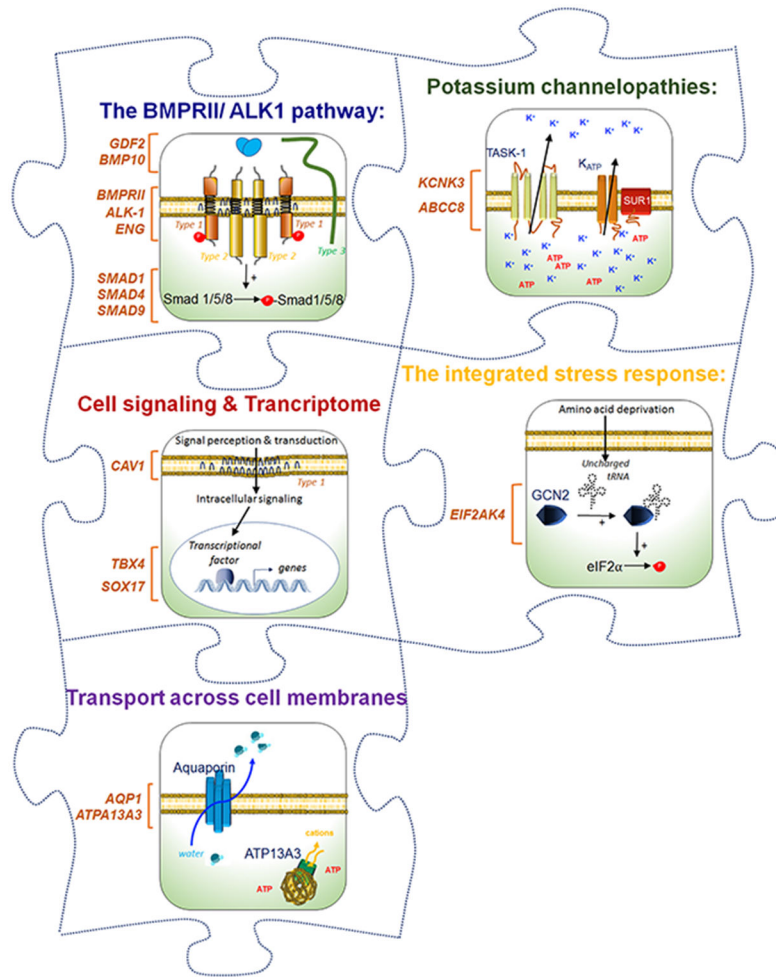
Figure 3: PAH physiopathology: a puzzle with many missing pieces.
(Illustration credit: Ben Smith)

Table 1:

Classification of Pulmonary Hypertension (From [2])

1. Pulmonary Arterial Hypertension (PAH)	1.1 Idiopathic PAH
	1.2 Heritable PAH
	1.3 Drug- and toxin-induced PAH
	1.4 PAH associated with:
	1.4.1 Connective tissue disease
	1.4.2 HIV infection
	1.4.3 Portal hypertension
	1.4.4 Congenital heart disease
	1.4.5 Schistosomiasis
	1.5 PAH long-term responders to calcium blockers
	1.6 PAH with overt features of venous/capillaries involvement
	1.7 Persistent pulmonary hypertension (PH) of the newborn syndrome
2. PH due to left heart disease	
3. PH due to lung diseases and/or hypoxia	
4. PH due to pulmonary artery obstructions	
5. PH with unclear and/or multifactorial mechanisms	

Table 2:

Gene name, functions of 16 genes related to pulmonary arterial hypertension (PAH)

Gene Symbol	Location	Product name	Function
ABCC8	11p15.1 (38 exons)	sulfonylurea receptor 1 (SUR1)	This gene encodes SUR1 that is a member of the ATP-binding cassette (ABC) family of transporter proteins which function as transporters, ion channels and channel regulators.
ACVRL1	12q13.13 (11 exons)	Activin receptor-like kinase-1 (ALK1)	This gene encodes a transmembrane type I receptor that is abundantly expressed by endothelial cells and that leads to activation of SMAD1/5/8.
AQP1	7p14.3 (4 exons)	Aquaporin-1	This gene encodes a protein that is a membrane channel that allows rapid water movement driven by a transmembrane osmotic gradient.
ATP13A3	3q29 (36 exons)	ATPase 13A3	This gene encodes a P-type ATPase that transport a variety of cations across membranes.
BMP10	2p13.3 (2 exons)	Bone morphogenetic protein 10 (BMP-10)	This gene encodes BMP-10 that is a secreted protein that is predominantly expressed by the right atria. Among BMPs, BMP9 and BMP10 are two high affinity ligands for ALK1 and BMPRII present in a heterotetrameric complex on pulmonary endothelial cells.
BMPR2	2q33.1-q33.2 (13 exons)	Bone morphogenetic protein receptor, type II (BMPRII)	This gene encodes a transmembrane type II receptor with an intrinsic serine/threonine kinase domain that is expressed on pulmonary vascular endothelial and smooth muscle cells and different inflammatory cell types. On ligand binding, BMPRII phosphorylates and activates type I receptors which then activates SMAD1/5/8.
CAV1	7q31.2 (4 exons)	Caveolin-1	This gene encodes a small, oligomeric scaffolding protein, typically required to generate membrane curvature in structures, such as caveolae. In addition, caveolin-1 has been reported to bind to multiple other proteins, to control cholesterol homeostasis, and to modulate different cell functions, such as endocytosis, cholesterol accumulation, receptor internalization, and cell signaling, proliferation, and cell apoptosis.
EIF2AK4	15q15.1 (39 exons)	General control nonderepressible 2 (GCN2)	This gene encodes the protein kinase GCN2 that predominantly responds to amino acid deprivation by binding uncharged transfer RNAs. When activated, GCN2 phosphorylates the alpha subunit of eukaryotic translation initiation factor-2 (EIF2), resulting in the downregulaton of protein synthesis.
ENG	9q34.11 (15 exons)	Endoglin	This gene encodes the transmembrane type III receptor endoglin that is predominantly expressed on proliferating endothelial cells *in vitro* and on angiogenic blood vessels *in vivo*.
GDF2	10q11.22 (2 exons)	Bone morphogenetic protein 9 (BMP-9)	This gene encodes BMP-9 that is a secreted protein that is predominantly expressed by the liver. Among BMPs, BMP9 and BMP10 are two high affinity ligands for ALK1 and BMPRII present in a heterotetrameric complex on pulmonary endothelial cells.
GGCX	2p11.2 (15 exons)	Gamma-glutamyl carboxylase (GGCX)	This gene encodes an integral membrane protein of the rough endoplasmic reticulum that carboxylates glutamate residues of vitamin K-dependent proteins to gamma carboxyl glutamate.
KCNK3	2p23.3 (3 exons)	TWIK-related acid-sensitive potassium channel (TASK-1)	This gene encodes TASK-1, which is an acid-sensitive potassium (K^+) channel containing two pore-forming P domains and contributing to resting membrane potentials.
KDR	4q12 (30 exons)	Vascular endothelial growth factor receptor 2 (VEGFR2)	This gene encodes the vascular endothelial growth factor receptor 2 (VEGFR2) that is considered to be one of the main receptors involved in endothelial cell proliferation, migration and survival
KLK1	19q13.33 (6 exons)	Kallikrein 1	This gene encodes a member of the kallikrein subfamily of serine proteases that are involved in diverse physiological functions.
SMAD1	4q31.21 (12 exons)	SMAD family member 1 (SMAD1)	This gene encodes SMAD1. Once phosphorylated, SMAD1 binds with Smad4 and migrates to the nucleus.
SMAD4	18q21.2 (12 exons)	SMAD family member 4 (SMAD4)	This gene encodes SMAD4 that plays a pivotal role in mediating the downstream effects of the BMP/TGF-β superfamily signaling.
SMAD9	13q13.3 (10 exons)	SMAD family member 8 (SMAD8)	This gene encodes SMAD8. Once phosphorylated, SMAD8 binds with Smad4 and migrates to the nucleus.

Gene Symbol	Location	Product name	Function
SOX17	8q11.23 (2 exons)	SRY-related HMG-box transcription factor (SOX17)	This gene encodes a member of the Sry-related high mobility group domain family F (SoxF) transcription factors that is a key developmental regulator of endothelial and hematopoietic lineage.
TBX4	17q23.2 (11 exons)	T-box transcription factor 4 (TBX4)	This gene encodes the T-domain transcription factors TBX4 that is expressed in the lung mesenchyme in which it plays a central role in the transcriptional regulation of genes required for mesoderm differentiation.

Table 3:

Susceptibility of various transgenic animals to remodel pulmonary vessels

Gene Symbol	Animal	Spontaneous PH	Susceptibility to chronic-hypoxia-induced PH	References
ABCC8	---	---	---	---
ACVRL1	• *Alk1*$^{+/-}$ mice	↑RVSP ↑Fulton index ↑Vessel wall area	---	101
AQP1	• *Aqp1*$^{-/-}$ mice	= RVSP = Fulton index = % Wall area = % Muscularized PAs	↓RVSP ↓Fulton index ↓% Wall area ↓% Muscularized PAs	64
ATP13A3	---	---	---	---
BMP10	• *Bmp10*$^{-/-}$ mice (tamoxifen inducible)	= RVSP = Fulton index = % Wall thickness = % Muscularized PAs	↓RVSP = Fulton index = % Wall thickness = % Muscularized PAs	60
BMPR2	• *Bmpr2*$^{+/-}$ mice	↑mPAP ↑% Wall thickness	= mPAP ↓% Wall thickness	102
	• *Bmpr2*$^{+/R899X}$ mice	↑RVSP ↑Fulton index ↑% Muscularized PAs	---	103
	• *Bmpr2* $^{Ex2/+}$ mice	= RVSP = Fulton index = % Muscularized PAs	↑RVSP ↑Fulton index = % Muscularized PAs	104
	• *Bmpr2*$^{+/-}$ mice	= RVSP = Fulton index = % Muscularized PAs	---	105
	• *Bmpr2*$^{+/\ 527bp}$ rat • *Bmpr2*$^{+/\ 16bp}$ rat	= RVSP = Fulton index = % Muscularized PAs	= RVSP = Fulton index = % Muscularized PAs	106
	• *Bmpr2*$^{+/\ 71}$ rat • *Bmpr2*$^{+/\ 140}$ rat	↑mPAP ↑Fulton index ↑% Muscularized PAs	↑mPAP ↑Fulton index ↑% Muscularized PAs	70
	• *L1cre$^+$; Bmpr2$^{f/f}$ and L1cre$^+$; Bmpr2 $^{f/+}$* mice	*in a small proportion of mice:* ↑RVSP ↑Fulton index ↑Muscularized vessels ↑% Wall thickness	---	107
	• *SM22-rtTA x TetO^7-BMPR2^{R899X}* mice	↑RVSP ↑Fulton index ↑Muscularized vessels	---	108
CAV1	• *Cav-1*$^{-/-}$ mice	↑RVSP ↑Fulton index	↓RVSP ↑Fulton index	109
	• *EC-Cav-1*$^{-/-}$ mice	= RVSP = Fulton index = Vessel area = Vessel thickness	↑RVSP ↑Fulton index ↑Vessel area ↑Vessel thickness	110
EIF2AK4	• *Eif2ak4*$^{-/-}$ rat	= RVSP = Fulton index = % Muscularized PAs	---	111
ENG	• *Eng*$^{+/-}$ mice	↑RVSP ↑Fulton index ↑Vessel wall area	= RVSP = Fulton index = Vessel wall area	112
	• *Eng*$^{+/-}$ mice	= RVSP = Fulton index	↓RVSP ↓Fulton index	113

Gene Symbol	Animal	Spontaneous PH	Susceptibility to chronic-hypoxia-induced PH	References
		= % Wall thickness = % Muscularized PAs	↓ % Wall thickness ↓ % Muscularized PAs	
GDF2	• *Bmp9* $^{+/-}$ mice	= RVSP = Fulton index = % Wall thickness = % Muscularized PAs	↓ RVSP ↓ Fulton index ↓ % Wall thickness ↓ % Muscularized PAs	60, 114
GGCX	---	---	---	---
KCNK3	• *Kcnk3* mutated rat	↑ RVSP = Fulton index ↑ % Muscularized PAs	↑ RVSP	115
KDR	• *Kdr end* mice	↑ RVSP ↑ Fulton index ↑ % Wall thickness	↑ RVSP ↑ Fulton index ↑ % Wall thickness ↑ % smoth muscle + area	78
KLK1	---	---	---	---
SMAD1	• *L1Cre(+); Smad1* mice • *Tagln-Cre(+);Smad1* mice	*in a small proportion of mice:* ↑ RVSP in a small proportion of mice ↑ Fulton index ↑ Muscularized vessels ↑ % Wall thickness	---	116
SMAD4	---	---	---	---
SMAD9	• *Smad8* ^{ex4,5} mice	Presence of remodeled vessels in lungs	---	117
SOX17	---	---	---	---
TBX4	---	---	---	---