Targeting soluble TGF-β factors: advances in precision therapy for pulmonary arterial hypertension

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STATE-OF-THE-ART REVIEW

Targeting Soluble TGF-β Factors

Advances in Precision Therapy for Pulmonary Arterial Hypertension

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HIGHLIGHTS

- **PAH is a disease characterized by significant heterogeneity and high variability in response to treatment and clinical outcomes. Precision medicine, using thorough clinical phenotyping, has the potential to enhance disease classification and treatment selection for improved clinical outcomes.**
- **Dysfunctional bone morphogenic protein receptor type 2-mediated signaling and an overactive TGF-β pathway are hallmarks of PAH and the focus of a multitude of treatment strategies. This review explores how alterations in TGF-β signaling may serve as both biomarkers and therapeutic targets for disease management.**
- **Precision medicine in PAH may involve substratification of patients through a deep characterization of altered TGF-β signaling. Identifying and focusing on unique patient profiles aims to optimize treatment strategies and improve patient outcomes.**

SUMMARY

Pulmonary arterial hypertension (PAH) is a rare progressive disease characterized by pulmonary artery vascular remodeling, increased vascular resistance, and subsequent right ventricular hypertrophy and right heart failure. It is triggered by disrupted transforming growth factor (TGF)-β signaling, including loss-of-function mutations in the bone morphogenetic protein (BMP) receptor 2. Emerging treatments aim to inhibit elevated TGF-β levels or enhance diminished endothelial BMP signaling. This review aims to summarize the role of the TGF-β superfamily in the pathobiology of PAH and recent discoveries highlighting altered expression of TGF-β–related soluble factors in PAH patients that can serve as potential biomarkers and drug targets. The discussion focuses on how these altered factors can guide treatment decisions and monitor therapeutic responses, facilitating personalized patient care through the integration of diagnostics and therapy, that is, precision medicine. This approach tailors treatment strategies to individual patients based on their unique disease characteristics. (JACC Basic Transl Sci. 2024;9:1360–1374) © 2024 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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PULMONARY ARTERIAL HYPERTENSION

Defined by the most recent 2022 European Society of Cardiology/European Respiratory Society guidelines for the diagnosis and treatment of pulmonary hypertension, precapillary pulmonary hypertension (PH) is defined by mean pulmonary arterial pressure >20 mm Hg, pulmonary vascular resistance >2 WU, and pulmonary arterial wedge pressure ≤15 mm Hg.[1] Pulmonary arterial hypertension (PAH) is embedded within group 1 of a total of 5 groups as PH in the absence of other causes. PAH is a severe and progressive disease, characterized by the remodeling of distal pulmonary arteries, subsequently leading to an increase in pulmonary vascular resistance, alteration of the pulmonary vasculature, and eventually right ventricular (RV) dysfunction. Inflammation, apoptosis, thrombosis, and hyperproliferation, along with dysfunction of pulmonary microvascular endothelial cells and pulmonary artery endothelial cells (PAECs), as well as pulmonary artery smooth muscle cells (PASMCs), are recognized as contributing factors to the distinctive vascular remodeling seen in PAH.[2] As a consequence, there is an increase in pulmonary artery pressure and RV overload, leading to hypertrophy and ultimately RV failure. This is recognized as one of the most crucial factors influencing the clinical outcome and survival of PAH patients.[3] Current treatment options consist of monotherapy or combination therapy targeting 3 major pathways (eg, nitric oxide, prostacyclin, and endothelin-1) to normalize the pulmonary vasomotor tone, with an estimated 5-year survival rate of 57%.[4]

As discussed in the following paragraph, genetic predisposition determines the incidence of PAH, for example, through gene mutations in several members of the transforming growth factor (TGF)-β superfamily. In addition, sex is a well known risk factor, with disease incidence significantly higher in women than in men,[5] although male patients exhibit poorer survival.[6] Importantly, sex cues may affect the activity of TGF-β family members,[7] underscoring the relevance of this signaling pathway in PAH. Alterations in the TGF-β superfamily are reflected in the circulation of PAH patients and may contribute to precision disease management (PDM) (**Central Illustration**). Through PAH PDM, decision making, treatments, and interventions may be tailored to the patient's unique genetic, molecular, and clinical characteristics, aiming to optimize therapeutic outcomes and provide personalized care. In this review, we discuss how alterations in soluble TGF-β factors can guide PDM in PAH.

TGF-β SIGNAL TRANSDUCTION

In mammals, the TGF-β superfamily comprises 33 structurally related soluble polypeptides, which exert pleiotropic effects by regulating essential cell processes such as proliferation, migration, differentiation, and tissue homeostasis.[8] Members of this family include the TGF-β$s_{(1-3)}$,[9] bone morphogenetic proteins (BMPs 1-20),[8,10] nodal ligands,[11] growth/differentiation factors (GDFs),[12] activins, inhibins, and anti-müllerian hormone.[13,14] TGF-β family ligands are synthesized in an inactive form consisting of an N-terminal pro-domain and a C-terminal receptor-binding domain.[15] Active TGF-β ligands are released upon mechanical deformation or proteolytic cleavage. Unlike TGF-βs, BMPs do not form complexes with latency-related proteins and in some cases (eg, BMP9, BMP10) circulating pro-domain-bound ligands are active.[16] The intracellular TGF-β signaling cascade is initiated through the formation of specific TGF-β receptor (TGFBR) complexes at the membrane, containing type I and II TGFBRs endowed with intrinsic serine/threonine kinase activity. Upon ligand-receptor binding, the type I receptor is transphosphorylated by the type II receptor. The now active type I receptor phosphorylates specific subsets of receptor-regulated small mothers against decapentaplegic (R-SMAD) factors, which form a complex with the co-SMAD (SMAD4) and translocate to the nucleus to regulate gene expression. TGF-β superfamily members play key pleiotropic roles in cell biology. Therefore, their activity must be tightly and precisely controlled, both temporally and spatially. Natural soluble antagonists (such as gremlin, noggin, follistatin, and chordin) are able to bind to circulating ligands, thereby shielding their receptor interaction domain and preventing the activation of TGFBRs.[17]

In addition, bioavailable ligands and receptors have different interaction affinities, and the relative expression levels of TGFBRs ultimately influence the sensitivity of specific cell types or tissues to a given TGF-β ligand. For example, TGF-β isoforms$_{(1-3)}$ and activins show enhanced binding affinity for the TGFBR type 2 (TGFBRII) and activin receptor type IIA/B (ACVRIIA/B), respectively.[18] Following TGF-β ligand/type II receptor interaction, a type I receptor is recruited, forming a heterotetrameric complex. Seven activin-like kinase (ALK, 1-7) type I receptors are

ABBREVIATIONS AND ACRONYMS

ACVR = activin A receptor

ALK = activin-like kinase

BMP = bone morphogenic protein

BMPRII = bone morphogenic protein receptor type 2

EC = endothelial cell

GDF = growth differentiation factor

lncRNA = long noncoding RNA

miR = microRNA

MCT = monocrotaline

PAEC = pulmonary arterial endothelial cell

PAH = pulmonary arterial hypertension

PASMC = pulmonary arterial smooth muscle cell

PH = pulmonary hypertension

RV = right ventricular

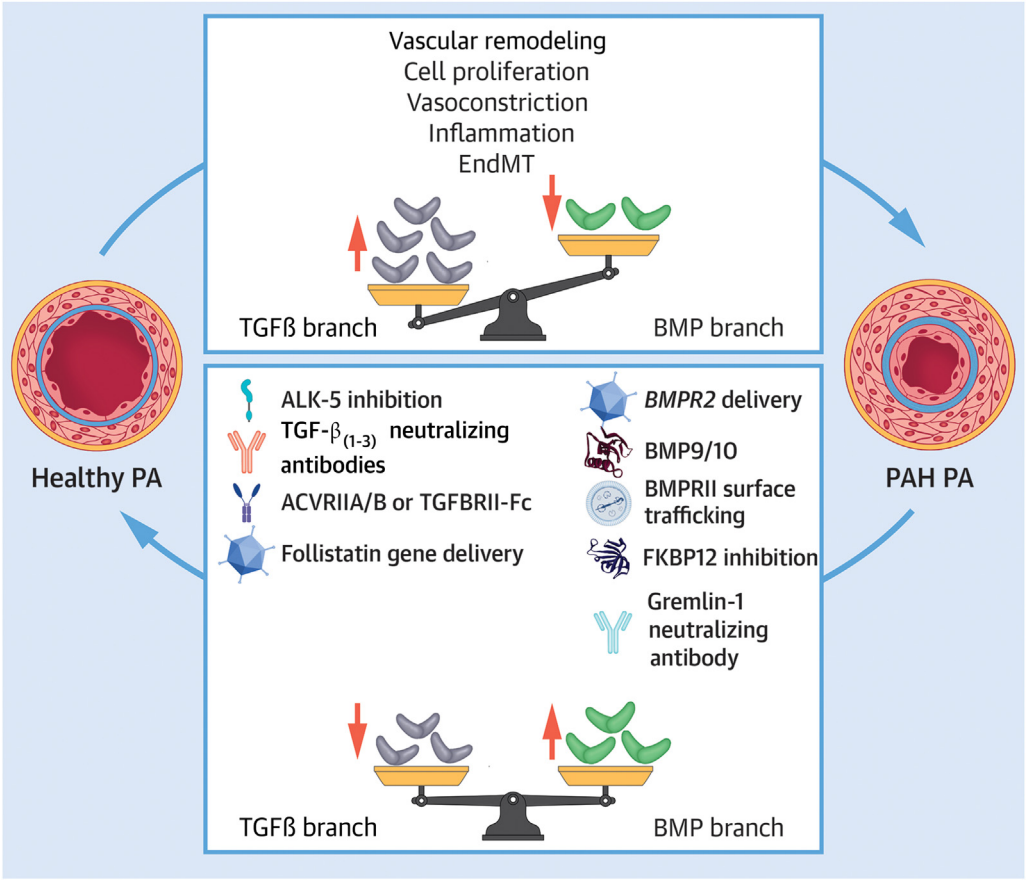
sEV = small extracellular vesicle

SMAD = small mothers against decapentaplegic

TGF = transforming growth factor

TGFBR = transforming growth factor-β receptor

CENTRAL ILLUSTRATION **Biomarker-Based Therapeutics in PAH Targeting TGF-β Signaling**



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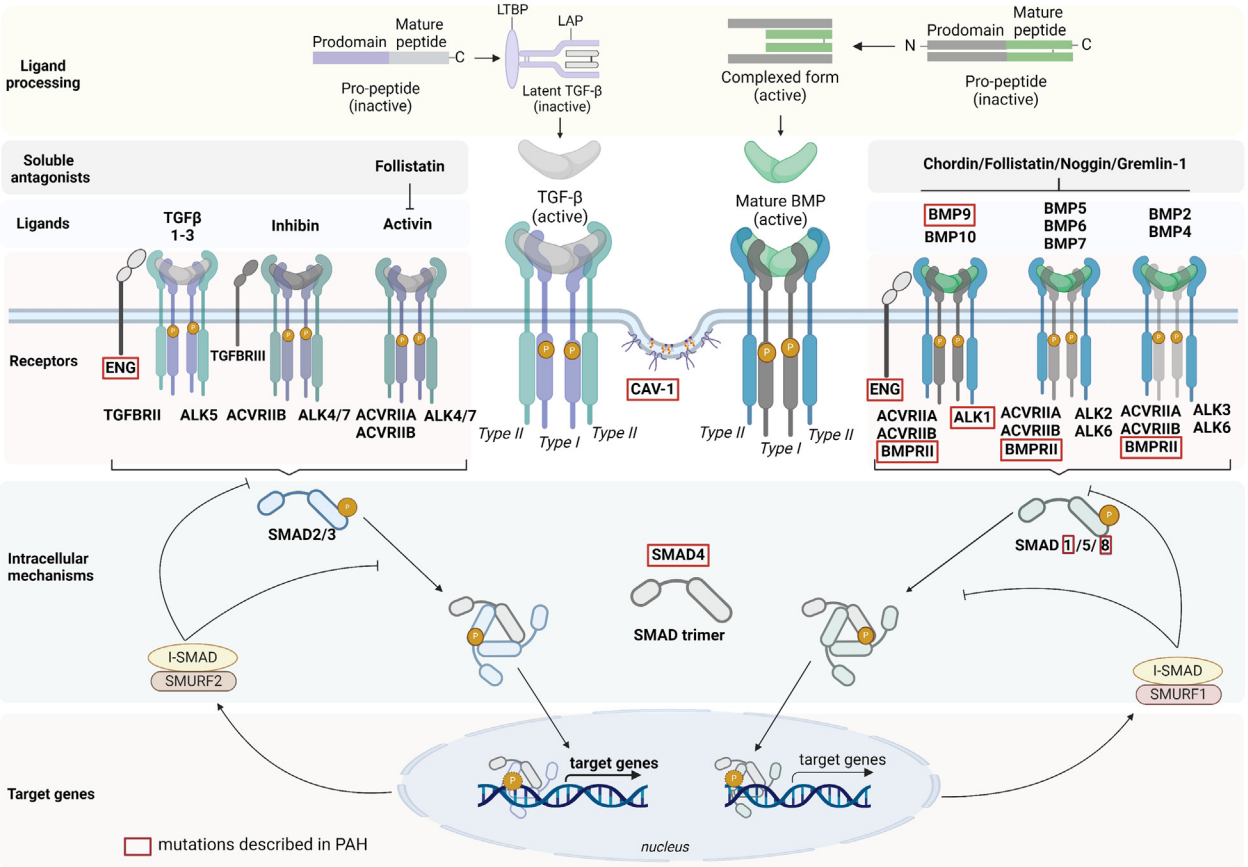
Pulmonary arterial hypertension (PAH) is characterized by vascular remodeling and a dysfunctional BMPR-II-mediated signaling as well as an overactive TGF-β pathway. Altered factors related to the TGF-β superfamily are used as biomarkers and have the potential to be utilized for precision medicine to guide therapeutic approaches and treatment decisions for PAH patients.

known in vertebrates.[19] Unlike TGF-β and activin ligands, BMPs display a higher affinity for type I receptors and display different specificity.[20] The recruitment of a constitutively active type II receptor follows the BMP ligand-type I receptor interaction. BMP receptor heterotetrameric complexes may include 3 different type II receptors, including BMPRII and ACVRIIA/B, which phosphorylate the type I receptors on their glycine/serine-rich domain, thereby activating the receptor and transducing the signal intracellularly[19] (**Figure 1**).

Ligand-receptor affinity can be modulated owing to the tissue-specific expression of TGF-β membrane receptors type III (co-receptors). These membrane-bound proteins lack a catalytic kinase domain but can either favor or impede the interaction between ligands and TGFBRs.[21] Known co-receptors are betaglycan (TGFBRIII), cripto (TDGF1) and endoglin (ENG). Endoglin is highly expressed by endothelial cells and can modulate both ALK1 and ALK5 canonical pathways.[21] Upon receptor oligotetrameric complex formation and internalization, the phosphorylation of the type I receptors activates intracellular SMADs. In general, activation of ALK4/5/7 leads to SMAD2/3 phosphorylation, whereas the BMP receptors ALK1/2/3/6 induce SMAD1/5/8 activation. This pathway is also known as the canonical SMAD-dependent pathway. These sets of R-SMADs are phosphorylated and recruit the co-SMAD into a heterotrimeric SMAD complex to regulate gene transcription (**Figure 1**).[22]

FIGURE 1 **TGF-β Superfamily Signal Transduction**



TGF-β superfamily signal transduction. Schematic overview of the TGF-β signaling pathway. TGF-β family proteins are synthesized as precursor peptides. Through proteolytic cleavage, the latent TGF-β complex is formed consisting of the latency-associated peptide (LAP) and the mature TGF-β peptide which can form disulfide bonds with the latent TGF-β binding protein (LTBP). Binding of the active TGF-β ligands to specific TGF-β type I and II receptors in the cell membrane activates the intracellular signaling cascade. (Left) TGF-β ligands (TGF-β_{1-3}, inhibins and activins) bind to receptor complexes consisting of a type I (ALK4/5/7) and type II (TGFBRII, ACVRIIA/B) receptor. The binding of the ligands to its receptor complexes leads to phosphorylation and activation of SMAD2/3. (Right) BMP ligands (BMP2/4/5/6/7/9/10) generally bind to receptor complexes of ALK1/ALK2/ALK3/ALK6 (type I receptor) and ACVRIIA/ACVRIIB/BMPRII (type II receptor) resulting in phosphorylation of SMAD1/5/8. These SMADs interact with SMAD4 and induce the transcription of specific target genes. Co-receptors (endoglin/TGFBRIII) can fine tune receptor-ligand affinity. Soluble antagonists (Follistatin/Chordin/Noggin/Gremlin-1) can function as ligand traps to decrease their availability. Several genes encoding TGF-β factors have been described to be mutated in PAH (encircled in red) leading generally to a decreased BMP and (in)direct increased TGF-β signaling. Inhibitory SMADs (I-SMADs 6 and 7) interact with Smurf 1 and 2 and regulate TGF-β signaling by blocking activation of R-SMADs and induction of receptor degradation. ACVRII = activin receptor type II; ALK = activin receptor like kinase; BMP = bone morphogenic protein; BMPRII = bone morphogenic protein receptor type 2; CAV-1 = caveolin-1; ENG = endoglin; SMAD = small mothers against decapentaplegic; TGF-β = transforming growth factor beta; TGFBRII/III = transforming growth factor beta receptor type II/III.

Furthermore, inhibitory SMADs (I-SMADs; SMAD6 and SMAD7) function as transcriptional targets of the TGF-β superfamily and generate a conventional negative feedback loop by facilitating the degradation of TGF-β receptors, through, for example, SMAD-specific E3 ubiquitin protein ligase (SMURF) 1/2.[23]

In addition to R-SMADs activation, soluble TGF-β ligands induce the activation of non canonical pathways, including mitogen-activated protein kinase (MAPK) branches (such as extracellular regulated kinase [ERK], c-Jun N-terminal kinase, p38), rho-like guanosine triphosphatase, or the phosphatidylinositol 3 kinase (PI3K)/Akt axis. Activation of these pathways may be independent from the type I receptor kinase activity and are often influenced by specific cellular contexts.[24] For example, in vascular environments, TGF-β-induced endothelial-to-mesenchymal transition (EndMT) involves the activation of ERK

and JNK.[25] Endothelial cell migration is modulated by JNK and ERK upon activation of ALK1.[26] Furthermore, the inhibition of primary vascular smooth muscle cell proliferation by TGF-β has been linked to p38-dependent pathways.[27] JNK and p38 MAPK pathways are critical for TGF-β-induced apoptosis and epithelial-mesenchymal transition (EMT). Inhibition of the p38 MAPK pathway has been shown to alleviate renal interstitial fibrosis and reduce lipid accumulation, as well as to attenuate TGF-β-mediated EMT and cell migration, highlighting its importance in these processes.[24] To date, many aspects of non canonical TGF-β signaling and its relevance to PAH remain poorly understood.

DISRUPTED TGF-β SIGNALING IN PAH ENDOTHELIAL CELLS

A dysfunctional endothelium is one of the first triggers of PAH development. Therefore, soluble TGF-β family molecules derived from the pulmonary endothelium might be specifically relevant for disease early detection. Accordingly, we will focus on them in this review. Genetic studies in PAH patient cohorts have identified loss-of-function mutations in several genes belonging to the TGF-β superfamily. Heterozygous germline mutations in the BMPRII (*BMPR2*) gene have been most frequently reported (80% of all heritable PAH patients) and correlated with worsened disease progression compared with non-mutation carriers.[28] Furthermore, reduced BMPRII expression is a hallmark of the disease, even in non-genetic patients.[29] Other mutations within the TGF-β pathway associated with PAH are: activin A receptor-like type 1 (*ACVRL1*),[30] endoglin (*ENG*),[31] caveolin 1 (CAV1),[32] SMAD family members (SMAD9 and *SMAD1*),[33] and *GDF2*, which encodes BMP9[34] (**Figure 1**).

The expression of most of these genes is enriched in vascular endothelial cells (ECs), directly impairing EC function because of those mutations. The endothelium can be activated in response to systemic or tissue-specific challenges (such as hypoxia, shear stress, inflammation, epigenetic factors, and genetic predisposition). This activation triggers the secretion of growth factors and cytokines, initiating a secondary phase involving inflammation, apoptosis, abnormal proliferation, and coagulation favoring chronic activation of the endothelium, EndMT, and vascular dysfunction.[35] EndMT is a process by which ECs undergo a shift toward a mesenchymal-like phenotype, leading to, for example, medial migration and thence arterial thickening. All 3 TGF-β isoforms can induce EndMT via SMAD-dependent as well as SMAD-independent pathways.[36] In addition, several studies show imbalanced TGF-β signaling in PAH ECs as a consequence of genetic mutations and feedback mechanisms in both BMP and TGF-β branches of the pathway.[37] For example, *BMPR2*-deficient ECs can form receptor complexes of TGFBRII/ALK5/ALK1 that may lead to TGF-β-induced SMAD1/5/8 activation.[38] Our group demonstrated that ACVRIIA can partially compensate for the loss of BMPRII protein expression in BMP9-induced receptor complexes in ECs. Although SMAD1/5/8 signaling remained unaltered in response to BMP9, non canonical pathways were differentially regulated in *BMPR2*-deficient EC, thereby contributing to EndMT.[39] In summary, balanced TGF-β/BMP signaling is crucial to maintain EC homeostasis, and disruption of the quiescent endothelium contributes to the development of PAH.

IMBALANCE OF TGF-β ACTIVITY IN THE PATHOLOGY OF PAH

Considering the pathogenic impact of disrupted TGF-β signaling in PAH, which is frequently linked to the abnormal expression of specific TGF-β superfamily pathway components, we hypothesize that novel approaches in precision medicine may arise through quantification of soluble factors within the TGF-β superfamily. How such quantification can provide insights into the underlying pathophysiology of the disease, guiding treatment decisions and facilitating the development of innovative therapies, will be explored. **Table 1** lists how soluble factors modulating TGF-β signaling in PAH may be valuable as therapeutic targets or disease biomarkers, summarizing expression level, tissue type, and how they modulate signaling. The imbalance between the different branches of the pathway, mainly TGF-β and BMP ligands and receptors, has been exploited therapeutically not just in PAH, but also in other conditions characterized by overactive or hampered activity of TGF-β family members. **Table 2** lists several (pre) clinical agents investigated, including specific targets, mechanisms of action (if known), and the experimental models or diseased populations in which the agents were studied.

TARGETING BMP LIGANDS AND RECEPTORS. BMPRII protein levels are reduced in lung tissues of heritable PAH patients.[29] It was also found that reduced *BMPR2* mRNA levels in blood samples of PAH patients carrying a *BMPR2* mutation correlated with diseased hemodynamic parameters, such as pulmonary vascular resistance, RV wall thickness, and pulmonary arterial pressure.[40] Targeting the BMP signaling

cascade, for example, to enhance BMP activity or recover BMPRII expression on the cell surface, are well studied strategies for the treatment of PAH.[41-43] In summary, the most explored strategies include: 1) exogenous adenoviral delivery of *BMPR2*;[44] 2) small molecule-mediated inhibition of the interaction of the BMP type-1 receptor with the receptor inhibitor FK-binding protein 12 (FKBP12) (ie, tacrolimus, also known as FK506);[45,46] 3) restoration of BMPRII trafficking to the cell surface (ie, sodium 4-phenylbutyrate, hydroxychloroquine);[47,48] and 4) recombinant BMP9-based treatment[49] (**Table 2**). The importance of the last approach is further supported by the reduced BMP9/BMP10 levels seen in some PAH patients, in both the absence and the presence of *GDF2* mutations, although *GDF2* mutations seem to further reduce BMP activity in plasma. BMP10 plasma levels might be influenced by sex and disease severity: Hodgson et al found reduced circulating BMP10 in female (but not male) PAH patients.[50] Recombinant human BMP (rhBMP) administration has been approved by the U.S. Food and Drug Administration (FDA) only for BMP2 and BMP7 to treat orthopedic diseases such as open fracture healing and maxillofacial bone enhancement.[51] However, owing to safety concerns (osteolysis, inflammation, soft tissue swelling, heterotopic ossification, and calcification),[52] rhBMP7 was withdrawn from the market, and limitations were placed on the clinical use of rhBMP2. Currently, the use of rhBMP6 is tested in clinical trials using an osteogenic device (OSTEGROW) to promote bone healing and regeneration in patients undergoing various orthopedic procedures, including distal radial fracture, high tibial osteotomy and posterior lumbar interbody fusion (EudraCT 2017-000860-14).[53,54]

Another drug, already FDA approved because of its beneficial effects in vivo, is rapamycin (also known as sirolimus). Rapamycin treatment in a PAH rodent model reduced pulmonary arterial remodeling, RV systolic pressure, and RV hypertrophy and overall attenuated PAH.[55,56] Clinically it is used as an immunosuppressive drug and is getting more attention as a rejuvenating agent.[57] Furthermore, LAM-001 (inhaled sirolimus) is being currently tested in a clinical trial phase IIa as an add-on therapy for PH (NCT05798923). In the context of PAH, its crosstalk with BMP signaling is particularly intriguing: In human embryonic stem cells[58] and mesenchymal stem cells,[59] rapamycin-induced mammalian target of rapamycin (mTOR) inhibition and enhanced BMP/SMAD signaling, which was also seen in prostate cancer cells.[60] The underlying mechanism of rapamycin points to a direct inhibition of FKBP12 binding to the BMP type I receptor, thereby enhancing BMPR downstream signaling.[61] These results suggest that FKBP12 monitoring or targeting may become a promising agent for PAH therapy.

In addition, the BMPRII-B-cell lymphoma (Bcl) xL axis has been described to control the dysregulated apoptosis seen in PAH.[62] *BMPR2* haploinsufficiency was shown to modulate the ratio of anti-apoptotic Bcl-xL over proapoptotic Bcl-xS transcripts in PAH patients and PH animal models. Determining their ratio might be promising as novel biomarker. BMPRII deficiency promoted the expression of Bcl-xL transcripts in PASMCs, leading to apoptosis resistance. In PAECs, BMPRII deficiency reduced Bcl-xL expression, promoting a proapoptotic phenotype, and selective inhibition of Bcl-xL increased apoptosis in wild-type and BMPRII-deficient PASMCs, suggesting that

TABLE 1 Soluble Molecules Modulating TGF-β Signaling in Pulmonary Arterial Hypertension

Marker	Tissue/Cell Type	Expression	Function/Target	Ref. #
Activin A	PAH patient blood	↑	Ligand	66
Bcl-xL	PAH patient blood and leukocytes, Hypoxia-induced PAH rat lungs	↑	Antiapoptotic protein	62
BMP9/10	PAH patient blood	↓	Ligand	50
BMPRII	PAH patient blood	↓	Receptor	40
Follistatin	PAH patient blood	↑	Antagonist	66
Gremlin-1	PAH patient blood	↑	Antagonist	90,91
	CHD-associated PH patient blood	↑		
TGF-β_1	IPAH and HPAH patient blood	↑	Ligand	67
miR-125a	PAs of hypoxia-induced rats	↓	BMPRII	101
	Lungs of hypoxia-induced mice	↑		100
	Precapillary PH patient blood	↓		
	Hypoxic mouse blood	↓		
miR-125-5p	PAs of MCT-PAH rats	↓	PASMC	99
miR-145	Lung tissue from PAH patients	↑	BMPRII	119
	SMCs of remodeled vessels	↑		119
	PASMCs from *BMPR2* mutation carrier	↑		99
miR-17-92 sEVs	PASMCs from PAH patients, IPAH patient blood	↑	BMPRII	117
miR-19b, miR-20a, miR-20b	IPAH patient blood	↑	–	117
	MCT mouse model	↑		
miR-21	Hypoxia-induced PH patient blood	↑	BMPRII, BMP9	106,107
	MCT rat model for PAH	↓		108
	IPAH patient blood	↓		
miR-424(322)	PAH patient blood	↑	BMPRII	96
Zeb1- and TGF-β-enriched sEVs	hPASMCs from PAH patients	↑	PAEC	125

BMP = bone morphogenic protein; BMPRII = bone morphogenic protein receptor type 2; sEV = small extracellular vesicle; IPAH = idiopathic pulmonary arterial hypertension; miR = microRNA; MCT = monocrotaline; PAEC = pulmonary arterial endothelial cell; PA = pulmonary artery; PAH = pulmonary arterial hypertension; PASMC = pulmonary arterial smooth muscle cell; PH = pulmonary hypertension; SMC = smooth muscle cell; TGF = transforming growth factor.

TABLE 2 Investigational Therapeutic Agents Targeting the TGF-β Superfamily

Agent	Target	Mechanism of Action	Study/Disease	Ref. #
Exogenous BMPRII	BMPRII ↑	Adenoviral *BMPR2* delivery	Preclinical PH model	44
BMP9	BMP9 ↑	Recombinant protein	Preclinical PH model	49
Tacrolimus/FK506	BMP ↑	Inhibition of FKBP12 signaling suppression	Preclinical PH model PAH patients	45,46 NCT01647945
Sodium 4-phenylbutyrate hydroxychloroquine	BMPRII ↑	Restoration of BMPRII trafficking to the cell surface	Preclinical PH model	47,48
Sirolimus (rapamycin/LAM-001)	BMP ↑	Inhibition of FKBP12 signaling suppression	Preclinical PH model PH patients	55,56 61 NCT05798923
IN-1233, SB-525335	ALK5 ↓	Small molecule inhibition	Preclinical PH model	68,69
TGFBRII-Fc fusion proteins	TGF-β ligands ↓	TGF-β ligand traps	Preclinical PH model	70
TGF-β–neutralizing antibodies (fresolimumab)	TGF-β ligands ↓	TGF-β neutralization	Preclinical PH model Renal cell carcinoma Malignant melanoma Breast cancer Glioma Mesothelioma	72 NCT00356460 NCT00923169 NCT01401062 NCT01472731 NCT01112293
ACVRIIA-Fc fusion protein (sotatercept)	Activin ↓ GDF ↓	Activin and GDF ligand trap	PAH patients	71 NCT04576988
Trabedersen (AP12009)	TGF-β_2 ↓	Antisense oligonucleotides	Different cancer types	NCT00431561 NCT00761280
Activin A–blocking antibodies (garetosmab or REGN2477)	Activin A ↓	Inhibition of activin A	FOP patients	79-81 NCT0318866
ACTR2A/B-Fc fusion protein (RKER-012/RAP-011)	Activin ↓	Activin ligand trap	Preclinical PH models	82,83
Follistatin gene therapy	Activin ↓	Activin A inhibition	Inflammatory myopathy Muscular dystrophy	85 86
Gremlin-1–neutralizing antibodies (UCB6114)	Gremlin-1 ↓	BMP activity restoration through gremlin-1 inhibition	Preclinical PH model Patients with advanced solid tumors	92 93 NCT04393298
miR-424(322) mimic	miR-424(322) ↑	Inhibition of SMAD2/3/RUNX2 expression	Cancer therapy CVD (AAA)	97 98
miR-125a-5p mimic/agon-miR	miR-125-5p ↑	Inhibition of TGF-β_1 and IL-6 Inhibition of macrophage infiltration Inhibition of proliferation and migration	Preclinical PH model Preclinical retinal model to study vascular defects Preclinical model of head and neck carcinoma	99 103 104
miR-21 antag-miR/anti-miR Genzyme (RG-012)	miR-21 ↓	miR-21 binding and inhibition	Preclinical PH model Alport nephropathy	109,110 NCT03373786
miR-30a	miR-30a ↓	Modulation of miR-30a/P53 pathway	Preclinical PH model	113
Anti-miR-145	miR-145 ↓		Preclinical PH model	119

AAA = abdominal aortic aneurism; BMP = bone morphogenic protein; BMPRII = bone morphogenic protein receptor type 2; CVD = cardiovascular disease; EV = extracellular vesicle; FOP = fibrodysplasia ossificans progressiva; miR = microRNA; PAH = pulmonary arterial hypertension; RUNX = runt-related transcription factor; SMAD = small mothers against decapentaplegic; TGF-β = transforming growth factor beta; TGFBRII = TGF-β receptor 2.

interfering with the BMPRII-Bcl-xL axis might offer a novel therapeutic approach. In a clinical setting, different Bcl-2 inhibitors, such as navitoclax (ABT-263) and venetoclax (ABT-199), are being explored, mainly to promote apoptosis in cancer.[63] Venetoclax shows a much higher affinity for Bcl-2 and was approved by the FDA in 2016 for the treatment of hematologic malignancies.[64] In the context of PAH, administration of navitoclax reversed pulmonary vascular remodeling in a PH rat model through the inhibition of Bcl-2 and Bcl-xL, indicating its potential as a target for therapeutic intervention in PAH.[65]

TARGETING TGF-β LIGANDS AND RECEPTORS. The most antagonistic interaction between the TGF-β and BMP pathways in PAH is mirrored in the systemic circulation of PAH patients. Circulating activin A and TGF-β_1 levels are elevated in the serum of PAH patients, and high activin A expression correlates with increased mortality.[66,67] Inhibiting the overactive TGF-β signaling branch in PAH has been pursued to normalize the balance between TGF-β and BMP

signaling. Different approaches have been evaluated (**Table 2**), including pharmacologic inhibition of ALK5 kinase activity (IN-1233,[68] SB-525335[69]), the sequestration of active TGF-β family ligands with the use of TGFBRII-Fc (a fusion protein against $TGF\beta_1$ and $TGF\beta_3$),[70] sotatercept (an ACVRIIA-Fc ligand trap against activins and GDFs),[71] and TGF-β/activin monoclonal antibodies,[72,73] and interference with latent TGF-β maturation through integrin inhibition (Cdp22, an integrin-linked kinase inhibitor[74]) (**Table 2**). Strategies to rebalance TGF-β signaling in PAH have been reviewed in more detail elsewhere.[43,75]

Mechanistic similarities in TGF-β signaling underlying different forms of cardiovascular disease, cancer, fibrotic diseases of the lung, liver, and kidney, and musculoskeletal disorders have been described. Therapeutic agents studied within a disease-specific context may also find potential use in addressing different pathologies driven by similar abnormalities in TGF-β signaling. For example, fresolimumab (also known as GC1008), a well characterized monoclonal antibody inhibiting all TGF-β isoforms, has been described in renal cell carcinoma (phase I, NCT00356460), malignant melanoma (phases I and II, NCT00923169), breast cancer (phase II, NCT01401062), glioma (phase II, NCT01472731), and mesothelioma (phase II, NCT01112293). In PAH, however, fresolimumab has not yet been tested, perhaps owing to the limited effects seen in the previous clinical trials. Another strategy to reduce TGF-β expression involves antisense oligonucleotides (ASOs) that prevent the translation of TGF-β mRNA. ASOs have been shown to reduce tumor growth in vivo. Owing to their promising and specific actions in preclinical models, trabedersen (also known as AP12009), which blocks the expression of $TGF\text{-}\beta_2$, has progressed into phases II and III clinical trials to treat different cancer types (NCT00431561, NCT00761280). Unfortunately, trabedersen did not show increased antitumor responses in patients compared with standard chemotherapy in the phase II clinical trial, and phase III had to be terminated due to insufficient patient recruitment. We are not aware of reported studies making use of ASOs to inhibit TGFβ signaling in PAH. However, the potential application of ASOs to target epigenetic marks in PAH is currently being considered.[76]

As previously mentioned, the levels of GDFs and activins are increased in pulmonary PAH lesions and plasma from patients.[77] Recently, sotatercept, a GDF and activin ligand trap (ACVRIIA-Fc) has successfully completed a phase III clinical trial (STELLAR, NCT04576988), with exciting clinical outcomes in patients with PAH,[71] resulting in the approval by the FDA as first-in-class treatment for adults with PAH.[78] This recent discovery of therapy for PAH patients underscores the potential of soluble TGF-β factors to advance precision medicine approaches in PAH. Disturbed activin A signaling not only is associated with PAH, but also plays a crucial role in other diseases, for example, fibrodysplasia ossificans progressiva (FOP), an ultrarare autosomal-dominant musculoskeletal disorder in which activins trigger the formation of ectopic bone through mutations in the *ACVR1* gene encoding for ALK2. In FOP, the monoclonal antibody garetosmab (also known as REGN2477) shields exclusively activins A, AB, and AC, which contain the inhibin βα subunit, and does not bind other TGF-β family members, including activin B and inhibin A. In vivo, specific inhibition of activin A in combination with GDF8 inhibition led to increased muscle mass and force production in mice.[79] After the successful completion of a phase I clinical trial to evaluate safety, immunogenicity, pharmacokinetics, and tolerability,[80] garetosmab reported a successful reduction of ectopic bone volume in adults with FOP in the phase II LUMINA-1 trial (NCT03188666).[81] In PAH, garetosmab has not been tested yet, but other ACTRIIA/B ligand traps (RKER-012 and RAP-011, a sotatercept analogue) are being investigated in preclinical models, and preliminary results look promising.[82,83] These new emerging classes of ligand trap-based therapeutics show once more the importance of targeting this pathway in PAH pathogenesis.

TARGETING SOLUBLE TGF-β SUPERFAMILY ANTAGONISTS. Not only TGF-β superfamily ligands, but also extracellular antagonists are altered in PAH. Follistatin is a natural soluble trap for activin A, that prevents ligand-receptor interaction by binding to activin A with high affinity.[84] Follistatin and follistatin-like 3 are increased in patients with PH.[66] Because activin A induces follistatin expression, it may play a counter-regulatory and protective role in PAH. Follistatin-based gene therapy using adeno-associated viruses has been described to reduce activin/myostatin-triggered fibrosis in inflammatory myopathy and muscular dystrophy.[85,86] Overexpressed follistatin leads to the inactivation of the TGF-β signaling pathway through binding to activin A, thereby diminishing SMAD2/3 phosphorylation. In PAH, follistatin-like 1 has been described to protect against hypoxia-induced PAH in mice and might be of interest as a potential target for future therapies.[87]

Gremlin-1 is a BMP antagonist with a high affinity for BMP2 and BMP4,[88] which is increased in PAECs under hypoxic conditions.[89] Elevated plasma levels of gremlin-1 were quantified in a small cohort of PAH

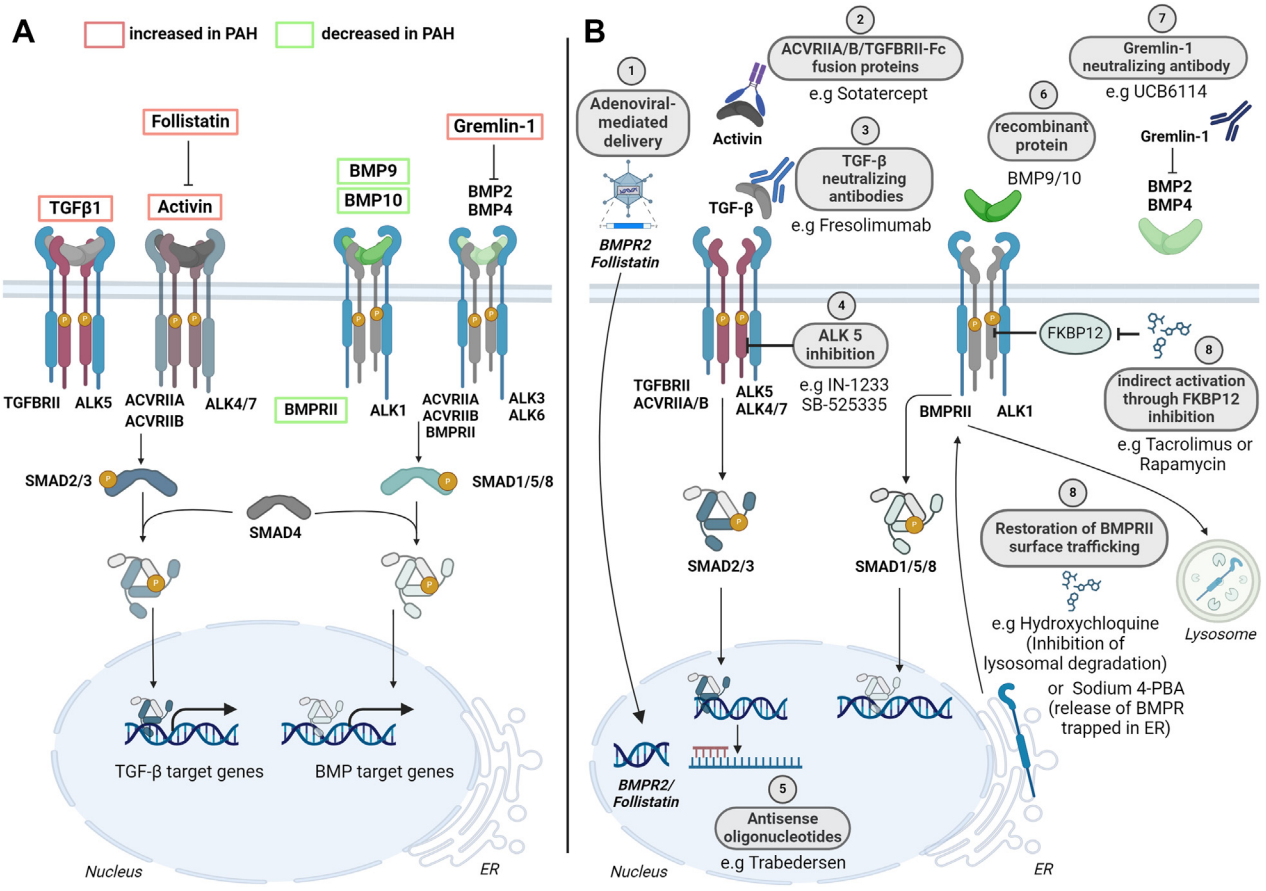
patients compared to healthy controls, correlating with higher N-terminal pro-B-type natriuretic peptide levels.[90] In addition, gremlin-1 was increased in patients with congenital heart disease-associated PAH and in lungs after systemic-to-pulmonary shunt model in rats.[91] In those lungs, gremlin-1 was predominantly expressed in PAECs and PASMCs of remodeled pulmonary arteries, where it promoted proliferation and inhibited apoptosis in PASMCs. Gremlin-1 is also increased in small pulmonary vessels of PAH patients' lungs, and hypoxia-induced increase in gremlin-1 was reported to have a role in vascular remodeling and increased vascular resistance in mice.[89] Experimental PH in mice could be partially reversed by gremlin-1-neutralizing antibodies.[92] In addition, a humanized IgG4P anti-gremlin-1 monoclonal antibody (UCB6114) was tested in a phase I/II study in patients with advanced solid tumors to neutralize the activity of gremlin-1 and restore BMP signaling (NCT04393298). That study showed preliminary evidence of clinical activity and that UCB6114 was well tolerated.[93] Although, anti-gremlin-1 therapy has shown promise in the preclinical models of PAH, it has not been tested in patients yet. Altered factors in PAH within the TGF-β pathway and general therapeutic approaches to target the TGF-β superfamily signaling are depicted in **Figure 2**.

REGULATION OF TGF-β SIGNALING IN PAH THROUGH NON-CODING RNAs. MicroRNAs (miRs) and long non-coding RNAs (lncRNAs) play a crucial role in gene regulation. miRs are small RNA molecules of 19 to 22 nucleotides, often highly conserved among species. Numerous non-coding RNAs modulate TGF-β signaling at different levels, including receptors, SMADs (R-, co-, and I-SMADs), and ligands.[94,95] miR-424(322) levels were higher in PAH patients than in healthy subjects and correlated with disease severity. Therefore, it was proposed to serve as a diagnostic and prognostic marker in PAH. miR-424(322) expression is induced by hypoxia in PAECs, leading to sustained *BMPR2* signaling by inhibiting SMURF1, mainly in cardiomyocytes.[96] This might suggest that miR-424(322) integrates signals between the diseased pulmonary endothelium and the right heart. Incongruently, miR-424(322) did not play a protective role in PAH, as miR-424(322)-mediated down-regulation of SMURF1 in cardiomyocytes contributed to maladaptive RV hypertrophy and heart failure. No clinical trials on miR-424(322) have been reported, but in vivo studies have investigated the role of miR-424(322) in cancer therapy[97] as well as cardiovascular diseases.[98] Importantly, miR-424(322) mimics exhibit a protective effect against abdominal aortic aneurysm (AAA) by directly inhibiting the expression of the Smad2/3/Runt-related transcription factor 2 (RUNX2), thereby suppressing Smad2/3 signaling and the expression of inflammatory mediators. Another study revealed that miR-125a-5p expression is reduced in the pulmonary arteries of a rat monocrotaline (MCT) PAH model, and increasing its expression ameliorated the disease by reducing systolic pulmonary arterial pressure and pulmonary vascular remodeling. The authors of that study suggested a negative feedback regulation loop with TGF-β_1 and interleukin (IL)-6 and its downstream target STAT3 in PASMC, and showed that up-regulation or down-regulation of miR-125a-5p resulted in a reduced or increased expression of TGF-β_1 or IL-6, respectively. Notably, overexpressing miR-125a-5p inhibited the proliferation of PASMCs and promoted apoptosis in those cells, whereas down-regulation showed the opposite effect.[99]

The role of miR-125a in PAECs remains controversial. Huber et al showed a hypoxia-induced up-regulation of miR-125a that correlated with a decrease in *BMPR2* expression in human PAECs. Levels of miR-125a were up-regulated in lung tissues of hypoxia-induced PH mice, however, circulating miR-125a was found to be decreased in plasma samples of PH mice as well as in patients with precapillary PH.[100] In contrast, Ma et al reported a lower expression of miR-125a in the pulmonary arteries of rats upon hypoxia.[101] In none of these studies a distinction was made between miR-125a-5p and miR-125a-3p. The miR-125 family acts in a highly context-dependent manner, which might explain contradicting results as both groups used different animal models.[102] In addition, different tissues were analyzed, and PASMCs expressing low levels of miR-125 are more abundant in the pulmonary arteries of rats, whereas whole-lung lysates have a relatively high weight of ECs, which generally express high levels of miR-125. Although miR-125-5p-based therapy has also been described in other diseases,[103,104] the lack of efficacy and safety in clinical trials remains a major challenge for the clinical application of miR therapeutics.

miR-21 has anti-proliferative effects in PAECs and PASMCs,[105] and elevated levels of circulating miR-21 are reported in patients with hypoxia-induced PH and are correlated with RV dysfunction.[106] Furthermore, it has been described to be up-regulated in pulmonary tissue from rodent models and PH patients. Hypoxia, BMPRII signaling, and IL-6 have been shown to independently up-regulate miR-21 expression in PAECs and PASMCs,[107,108] pathways that all play a crucial role in PAH disease progression. Furthermore, miR-21 was up-regulated by BMP9 in

FIGURE 2 Therapeutic Approaches to Target Altered TGFβ Signaling



Altered TGFβ related factors in PAH and therapeutic approaches to target the TGFβ superfamily. (A) Members of the TGFβ superfamily altered in PAH comprise ligands (BMP9, BMP10, TGFβ, activin (A) Soluble antagonists (Follistatin, Gremlin-1) and membrane-bound receptors (BMPRII). Most of those factors are increased (encircled in red) in the context of PAH, others are reported to be decreased (encircled in green) in PAH patients. (B) Therapeutic approaches to target the TGFβ superfamily comprise: 1) adenoviral-mediated delivery of BMPRII or Follistatin, inhibition of TGFβ/Activin signaling; with 2) ACVRIIA/B and TGFBRII-Fc fusion proteins; 3) neutralizing antibodies; 4) ALK-5 inhibition with small molecules; 5) antisense oligonucleotides blocking the production of $TGFβ_2$, or enhancement of BMP signaling; by 6) recombinant BMP9; 7) inhibition of soluble antagonists with Gremlin-1 neutralizing antibodies; 8) FKBP12 inhibition; and 9) restoration of BMPRII surface trafficking by e.g., inhibiting lysosomal degradation (Hydroxychloroquine) or the release of trapped BMPRII in the endoplasmic reticulum (ER) (Sodium 4-PBA). Created with BioRender.com. ACVRII = activin receptor type II; ALK = activin receptor like kinase; BMP = bone morphogenic protein; BMPRII = bone morphogenic protein receptor type 2; FKBP-12 = FK-binding protein 12; SMAD = small mothers against decapentaplegic; sodium 4- PBA = sodium 4-phenylbutyrate; TGFβ = transforming growth factor beta; TGFBRII/III = transforming growth factor beta receptor type II/III.

PAECs, which was abrogated by a knockdown of *BMPR2*.[105] Pharmacologic inhibition of miR-21 by an antagomir or modified anti-miR, ameliorated hypoxia-induced pulmonary vascular remodeling and reduced PASMC proliferation.[109,110] In contrast to these findings, in vivo studies have shown that miR-21-null mice develop an aggravated PH phenotype when exposed to chronic hypoxia and Su5416 through the activation of the programmed cell death 4 (PDCD4)/caspase-3 axis in pulmonary tissues.[107,111] Conversely, miR-21-overexpressing mice displayed reduced PDCD4 expression and seemed to be protected from the onset of PH in response to chronic hypoxia/Su5416.[111] Others also reported decreased miR-21 levels in an MCT PAH rat model, which was further confirmed in serum and human lung tissue from idiopathic PAH patients.[112] The controversial results obtained with miR-21 are likely due to highly context-dependent mechanisms, such as different tissues and animal models. There are very few clinical

trials aiming to suppress the activity of miR-21. For example, Genzyme is testing a chemically modified oligonucleotide (RG-012) that can bind to miR-21 to prevent Alport nephropathy (NCT03373786).

Concentrations of miRNA-30a are increased in the serum of PAH patients as well as in the pulmonary arterioles of the Su5416/hypoxia-induced mouse model. In addition, inhibition of miR-30a could improve experimental PAH by modulating the miR30a/P53 signaling pathway.[113] Crosstalk with the TGF-β superfamily was described during osteogenic differentiation, in which members of the miR-30 family inhibited osteoblast differentiation targeting *Smad1* and *RUNX2*, leading to decreased p-SMAD1/5 expression.[114] In addition, activin A significantly increases miR-30a expression in human prostate epithelial cells.[115] However, no clinical trials are currently ongoing to test miR-30a therapy in PAH or other diseases. Although there is no FDA-approved miRNA therapy available yet, candidates are being tested in phase I and II clinical trials, and miRNA-based therapies are gaining more attention as new treatment options.[116] Challenges in miRNA delivery, including stability, specific targeting, and effectiveness, are partly addressed by extracellular vesicle-based therapies. Small extracellular vesicles (sEVs) are nanosized membrane-bound particles that encapsulate cytoplasmic material (nucleic acids, metabolite, lipids, proteins) of the donor cell. sEVs protect their cargo from degradation, and alterations in sEV content may mirror cellular processes. They are involved in cell-to-cell communication via paracrine or endocrine signaling, such that altered sEV content can influence surrounding cells as well as have systemic effects in PAH, thereby potentially contributing to disease progression. sEVs are implicated in the pathogenesis of PAH and have been proposed as biomarkers, therefore they represent an interesting target. For example, Aliotta et al detected 19 miRNAs to be differentially expressed in exosomes in MCT mice plasma compared with healthy control mice, and 11 were also found to be up-regulated in plasma from idiopathic PAH patients: miR-17/92 cluster and miR-19b, -20a, -20b, and -145.[117] *BMPR2* has been described to be a direct target of various members of the family, including miR-17 and -20a, and a STAT-mediated down-regulation of BMPRII expression through the miR-17/92 cluster was proposed.[118] Furthermore, expression of miR-145 was described to be up-regulated in lung tissues of PAH patients as well as in smooth muscle cells of remodeled vessels from those patients.[119] Those authors showed increased miR-145 levels in primary PASMCs from patients that carry the *BMPR2* mutation, as well as in lungs from *Bmpr2*-deficient mice. Lack of miR-145 in mice protected against the development of PAH, indicating a potential target for PAH therapy.

lncRNAs are longer than 200 nucleotides and regulate gene expression on transcriptional and posttranscriptional levels. Some lncRNAs are implicated in the pathology of PAH and are involved in different cellular processes, including EC proliferation, EndMT, and angiogenesis.[120] lncRNA H19 has been linked to a variety of diseases and was proposed as a new biomarker and therapeutic target in PAH.[121] The study showed that H19 expression is increased in the RV of preclinical models of PAH, with a positive correlation with RV hypertrophy and fibrosis, and H19 genetic inhibition is protective against PAH. Crosstalk with the TGF-β pathway was not discussed in that study, but H19 was reported elsewhere to promote osteoblast differentiation in human mesenchymal stem cells by activating TGF-β_1/SMAD3 signaling,[122] which makes it an interesting target to explore in PAH. In conclusion, various non-coding RNAs have substantial implications in PAH, underscoring the need for more research to facilitate novel therapeutic approaches targeting these molecules.

DISCUSSION

Increased TGF-β ligand activity at the expense of reduced BMP activity has been documented in association with the initiation and progression of PAH, prompting a growing focus on the role of activin A in this context.[123] Understanding the molecular mechanisms underlying the pathology of PAH is imperative, because these alterations frequently manifest as distinctive indicators in a patient's blood and may serve as valuable biomarkers and therapeutic targets. For example, circulating levels of sEVs from various cell types are altered in PAH patients. These sEVs contribute to pulmonary vascular remodeling, vasoconstriction, and inflammation, thereby contributing to PAH progression.[124] In the context of the TGF-β pathway, it was reported that PASMC-derived sEVs are enriched in Zeb1 and TGF-β superfamily ligands (TGF-β_3 and GDF11) and are efficiently incorporated into PAECs, resulting in vascular remodeling and EndMT.[125] In addition, lung- and plasma-derived sEVs from MCT-induced PAH mouse models induce PH in healthy mice.[117] By examining altered molecular markers in the blood, we can gain insights into the pathologic processes at the molecular level and determine the state of the disease. This, in turn, guides us toward more precise and effective therapies.

Novel circulating biomarkers, also explored as therapeutic approaches, seem encouraging. A combination of different biomarkers is vital to gaining deeper insights about the disease status. Furthermore, substratifying patients based on different categories, including age, sex, medical interventions, and comorbidities, before determining soluble markers is crucial to enhance biomarker selectivity. For example, it is widely accepted that the incidence of PAH is higher in women,[6] suggesting that biomarkers for men and women may give different formation. For example, BMP10 elevation was seen in exclusively female PAH patients.[50]

Moreover, PAH develops through the combination of multiple factors (eg, biomechanical conditionings, inflammation, female sex, genetic predisposition). Integrating this wide input into a common PAH signature might be an approach for early biomarker detection with the use of machine learning and network-based bioinformatics.[107,126] In addition, the technologic advances in genomics, (phospho)proteomics, and metabolomics are further facilitating high-throughput analysis (eg, of plasma from PAH patients). Multiomics approaches may also be useful to identify differentially expressed factors in mutation carriers that develop the disease and carriers that have not yet developed PAH. The PVDOMICS initiative (NCT02980887) and the PVRI GoDeep Global Deep Phenotyping Meta-Registry for Pulmonary Hypertension (NCT05329714) are examples of deep clinical phenotyping and integrated multiomics approaches. The use of machine learning techniques has the potential to analyze big data to decipher the molecular mechanisms that underpin pulmonary vascular remodeling and the identification of new therapeutic targets for PAH.

Following the exciting outcome from the clinical studies evaluating sotatercept,[71,127] we propose to consider factors related to the TGF-β superfamily to improve precision medicine in PAH, because they are known to play an important role in vascular remodeling, and drugs that target members of the superfamily are under investigation as therapies in PAH. We emphasize the use of biomarkers in PAH not only for their diagnostic and prognostic role, but also as a guide for treatment decisions and indications for appropriate treatment strategies.

CONCLUSIONS

In summary, the goal of precision medicine in PAH is to bridge the gap between diagnosis and treatment by using blood-based clues for better disease management and opening a new era in personalized medicine, essentially improving the quality of life for individuals with PAH.

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