Redirecting TGF-β signaling pathways: advancing targeted therapy for PAH and FOP

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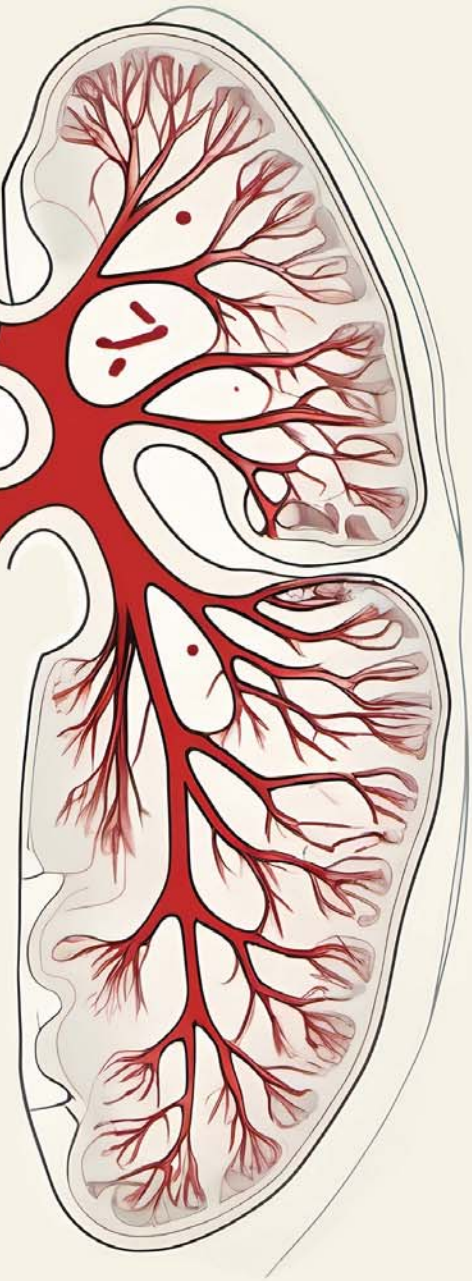
PART II

Unraveling new targets for treating PAH or FOP

Chapter 4



Sex-biased TGF-β signaling in Pulmonary arterial hypertension

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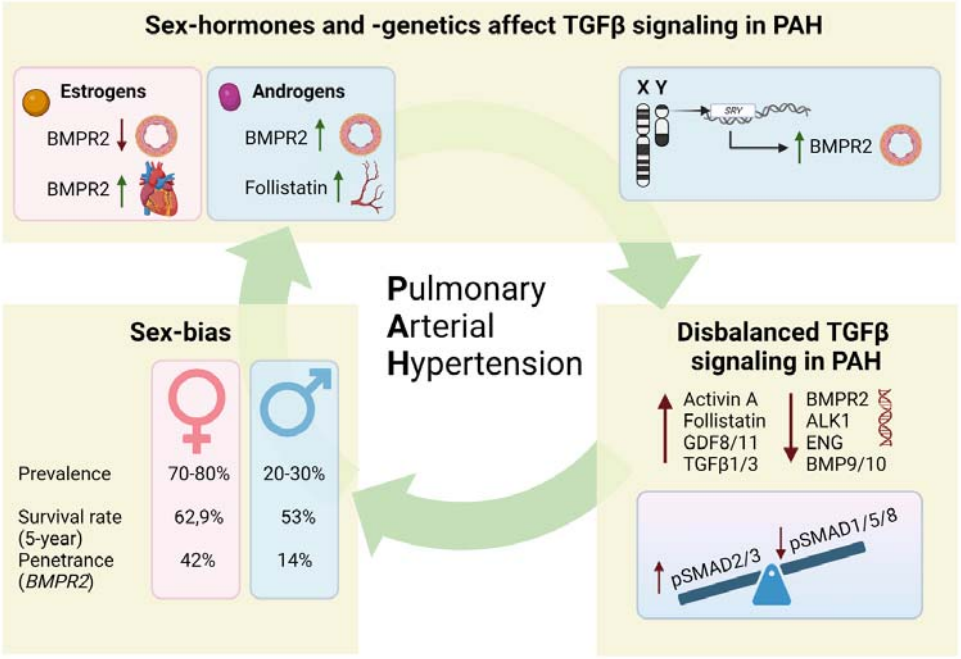
Cardiovascular Research (2023)

DOI: 10.1093/cvr/cvad129

Graphical abstract



Abstract

Pulmonary arterial hypertension (PAH) is a rare cardiovascular disorder leading to pulmonary hypertension and, often fatal, right heart failure. Sex-differences in PAH are evident, which primarily presents with a female predominance and increased male severity. Disturbed transforming growth factor-β (TGF-β) signaling and gene mutations in the Bone morphogenetic protein receptor 2 (*BMPR2*) are risk factors for PAH development, but how sex-specific cues affect TGFb signaling in PAH remains poorly understood. In this review we aim to gain deeper understanding of the sex-bias in PAH by exploring sex-differences in TGF-β signaling through mechanistical and translational evidence. Sex-hormones including estrogens, progestogens and androgens, can determine the expression of TGF-β receptors (including BMPR2), ligands and soluble antagonists in a tissue-specific manner. Furthermore, sex-related genetic processes, i.e. Y-chromosome expression and X-chromosome inactivation, can influence TGF-β signaling at multiple levels. Given the clinical and mechanistical similarities, we expect that the conclusions arising from this review may apply also to hereditary hemorrhagic telangiectasia (HHT), a rare vascular disorder affecting the TGF-β signaling pathway. In summary, we anticipate that investigating TGF-β signaling in a sex-specific manner will contribute to further understand the underlying processes leading to PAH and likely HHT.

Keywords: Activin, androgen, BMP, BMPR2, endothelial, estrogen, HHT, hypertension, PAH, TGF-β

Introduction

Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) belongs to group I in the total of five (I-V) groups of pulmonary hypertension. Group I is substratified in, among others, Idiopathic PAH (IPAH) and Heritable PAH (HPAH). HPAH has a known genetic origin, by either familial contribution or genetic correlation[1], while IPAH has an un-familial cause at the time of diagnosis. As established in the 2022 ESC/ERS Guidelines for the diagnosis and treatment of Pulmonary Hypertension, pre-capillary PH (including PAH) is defined by a mean Pulmonary Arterial Pressure (mPAP) of > 20 mmHg, pulmonary arterial wedge pressure (PAWP) of ≤ 15 mmHg, and pulmonary vascular resistance (PVR) of > 2 Wood Units (WU).[2] The increased workload on the right heart causes ventricular dilatation and hypertrophy, resulting in progressive right heart failure. Pulmonary vascular remodeling constitutes the main pathological event at the onset of PAH. Remodeling of the distal pulmonary arteries involves: abnormal proliferation of endothelial cells (ECs), smooth muscle cells (SMCs), and fibroblasts; apoptosis resistance of ECs; excessive EC migration that become dysfunctional, in part due to endothelial-to-mesenchymal transition (EndMT) (distal); migration of SMCs (proximal); inflammatory influx of macrophages and lymphocytes; and the formation of plexiform lesions.[3–5]

Although PAH is a disease caused by remodeling of the pulmonary vasculature, end-stage patients ultimately die from right heart failure.[6] To date there is no approved treatment curing or reversing disease progression. The current treatment of PAH mainly consists in the single or combined administration of pulmonary vasodilators acting on the guanylate cyclase, endothelin, or prostacyclin pathways[7], only postponing further progression and eventually requiring lung transplantation in severe cases.[6] Recently, the phase 3 clinical trial STELLAR has concluded excellent clinical outcomes in PAH patients using Sotatercept.[8]

Sex-related differences in disease prevalence and severity are known for PAH. The US REVEAL study showed that 80% of the PAH patients are women (4:1 ratio).[9,10] Comparably, multiple registries across Europe concluded a female bias in PAH of approximately 70% (2,3:1 ratio).[11–16] Interestingly, disease bias towards women declines by age when comparing age groups 18-65 with >65 years old in IPAH patients.[12] In addition, PAH disease penetrance is also defined by sex, with a 42% in females over 14% in male HPAH patients.[17] Remarkably, diagnosed PAH male patients are more severely burdened with nearly a 10% reduction in 5-year survival rate (53%) compared to females (62,9%).[9]

The underlying cellular and molecular causes of these sex-related differences in PAH have not yet been fully understood, although many hypotheses have been proposed. These often involve hormonal based alterations, although metabolism, genetics, and/or the immune system might also play a role.[18–20] In general, androgens are considered vasculo-protective and a contributor to pulmonary vasodilation[21], perhaps underlying the female predominance in PAH. However, estrogens have a cardioprotective effect on right ventricle adaptation in women[22], which might lead to a less severe phenotype in PAH compared to men.[23] Further, chromosomal differences also play a role, for instance, the Y-chromosome is thought to have vascular protective expression profiles in PAH.[24] In this review, we further assess if sex-

determinants, i.e. sex-hormones and -chromosomal effects, are a driver of PAH development by altering transforming growth factor-β (TGF-β) signaling.

Transforming growth factor-β signal transduction

Disturbances in TGF-β signaling contribute to PAH disease development and progression.[25–27] TGF-β signaling drives developmental processes and tissue homeostasis[28] within the cardiovascular system.[27,29] In mammals, the TGF-β family is comprised of 33 structurally related polypeptides, including TGF-β1-3, bone morphogenetic proteins (BMP1-15), nodal, growth differentiation factors (GDFs), activins, inhibins, and anti-Müllerian hormone (AMH).[30–36] TGFb ligands exert pleiotropic effects by controlling cell proliferation, migration and differentiation in a spatial and temporal manner.[28] Disturbed signaling can result in cancer[37], musculoskeletal disorders[38], fibrosis[39] and cardiovascular diseases[27,40–42].

Most TGF-β members, with BMPs being the exception[43], are secreted in an inactive form within a latent complex (reviewed in[44]). These large latent complexes include the mature TGF-β polypeptide shielded by latency-associated peptides and latent TGF-β binding proteins.[45] These additional factors also bind to the extracellular matrix (ECM) or the plasma membrane via receptors like glycoprotein-A repetitions predominant (GARP), creating an ECM storage of accumulated latent TGF-β. Mature TGF-β polypeptides are released via several mechanisms allowing a quick functional response on demand.[44]

Active TGF-β ligands signal via a heterotetrameric complex of type I and II serine-threonine kinase receptors (Figure 1).[46] In vertebrates, seven type I receptors (activin-like kinase (ALK)1-7) and five type II receptors (TGF-β receptor 2 (TGFβR2), activin receptor 2A (ACVR2A), ACVR2B, bone morphogenetic protein receptor 2 (BMPR2) and anti-Müllerian hormone receptor 2 (AMHR2)) exist. Since TGF-β ligands bind with different affinities to their receptor complexes, the relative expression level of the TGF-β receptors may determine sensitivity of a particular cell type or tissue to a TGF-β ligand.[47] Overall, TGF-βs and activins bind with a higher affinity to their type II receptors, whereas BMPs and GDFs exhibit a higher affinity for their type I receptors.[48] Co-receptors like TGFβR3 (betaglycan) or Endoglin (Figure 1 and 2) can enhance ligand binding to type I/II receptors when membrane bound, but can act as ligand trap when excreted in soluble form.[49] Next to these accessory proteins, soluble signaling modulators including Noggin, Gremlin, and Follistatin also exert regulatory effects on TGF-β signaling as ligand agonists or antagonists.[50]

Upon ligand-receptor interaction and receptor complex formation, the constitutively active type II receptor phosphorylates after which the activated type I receptor kinase initiates the signal transduction cascade by phosphorylating intracellular downstream proteins, i.e. receptor regulated-SMADs (R-SMADs) (Figure 1). Generally, TGF-β1-3 and Activins signal by SMAD2/3 phosphorylation whereas BMPs, GDFs and AMH signal via phosphorylation of SMAD1/5/8. In the vasculature for instance, BMP9 and -10 are important factors necessary for endothelial homeostasis, exhibiting a high affinity for BMPR2/ALK1 receptor complexes, mainly expressed in ECs.[51,52] Both ALK1/SMAD1/5/8 and ALK5/SMAD2/3 signaling are co-regulated by Endoglin in ECs.[53] Interestingly, the two splice variants short- and long-endoglin

favor different type I receptors, being S-endoglin pro-ALK5 and L-endoglin pro-ALK1 (Figure 2).[54]

Once phosphorylated, R-SMADs bind to the co-SMAD SMAD4 and form heterotrimeric complexes. Furthermore, Inhibitory SMADs (I-SMADs, SMAD6 and 7) are transcriptional targets of the TGF-β superfamily and create a classical negative feedback loop interacting with and promoting the degradation of TGF-β receptors by e.g. SMURF1/2.[55,56]

SMAD4-containing heterotrimeric complexes translocate to the nucleus, where they associate with cell type- and pathway-induced transcription factors to modulate target gene expression.[57] Different DNA motifs on the regulatory regions of genes have been described for the SMAD4, SMAD2/3 and SMAD1/5/8.[55,58–60] The binding affinity of SMADs for DNA is relatively low, and can be enhanced through association with other transcription factors, which may determine cell type-specific TGF-β responses.[55] Therefore, the transcriptional activity induced by TGF-β ligands can be 'fine-tuned' at multiple levels, including the relative expression levels of ligands, (co)receptors, (ant)agonists and nuclear transcription factors that are activated in a tissue and stimulus-dependent manner.[55,61] Much of the cell type-specific responses to TGF-β ligands is due to the so-called non-canonical pathways. The non-canonical signaling seems not to require the type I receptor kinase activity.[62] Furthermore, although the TGF-β type I and II receptors are known serine/threonine kinases they can also phosphorylate tyrosine residues and act as dual-specificity kinases. Therefore tyrosine phosphorylation may be an alternative route to mediate SMAD independent signaling.[63] TGF-β non-canonical signaling is often highly context dependent. For example in vascular settings, TGF-β-induced EndMT is also mediated through the activation of extracellular signal-regulated kinase (ERK)[64] and c-Jun N-terminal Kinase (JNK)[65]. Further, TGF-β-mediated inhibition of primary vascular smooth muscle cell proliferation has been demonstrated to be p38 dependent.[66] Unfortunately, much is still to be deciphered in the context of non-canonical TGF-β signaling and PAH. Accordingly, in this review we mainly focus on canonical TGF-β signaling.

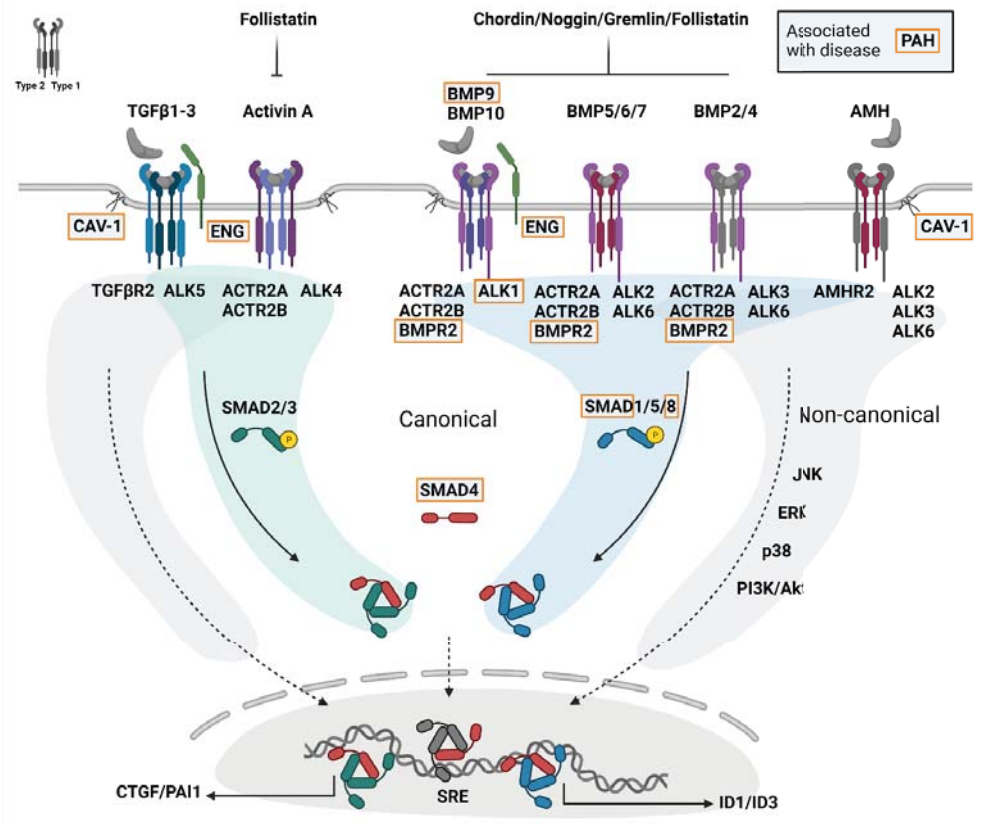


Figure 1. Schematic representation of TGF-β signaling. Ligands of the TGF-β family (TGF-β1-3, Activin A, BMP2/4/5/6/7/9/10, AMH) bind their type I (ALK1/2/3/4/5/6) and II (TGFβR2, ACTR2A/B, BMPR2, AMHR2) plasma membrane receptors. Soluble antagonists (Follistatin, Chordin, Noggin, Gremlin) can decrease ligand accessibility. Type III receptors (Endoglin) can further regulate ligand-receptor complex formation. Upon type I receptor activation the intracellular signaling molecules (R-SMADs) are phosphorylated and form a heterotrimeric complex with SMAD4. ALK4/5 (by TGF-β/Activin A ligands) signal via SMAD2/3 whereas ALK1/2/3/6 (by BMP/AMH ligands) signal via SMAD1/5/8. R-SMAD/SMAD4 complexes translocate to the nucleus where it functions as transcription factor. Also non-canonical signaling (JNK, ERK, p38, PI3K/Akt) can occur via TGF-β signaling. Mutations in genes encoding TGF-β factors have been linked to PAH development. Not all factors within the TGF-β signaling family have been incorporated in the figure for clarity purposes. Abbreviations; PAH: Pulmonary Arterial Hypertension, TGF-β: Transforming Growth Factor-β, BMP: Bone Morphogenetic Protein, AMH: Anti-Müllerian Hormone, CAV-1: Caveolin-1, ENG: Endoglin, ALK: Activin receptor-like Kinase, TGFβR2: TGF-β Receptor 2, ACTR2: Activin Receptor Type II, BMPR2: BMP Receptor Type II, SMAD: Small Mothers Against Decapentaplegic, JNK: c-Jun N-terminal Kinase, ERK: Extracellular signal-Regulated Kinase, PI3K: Phosphoinositide 3-Kinase, and SRE: SMAD Responsive Element.

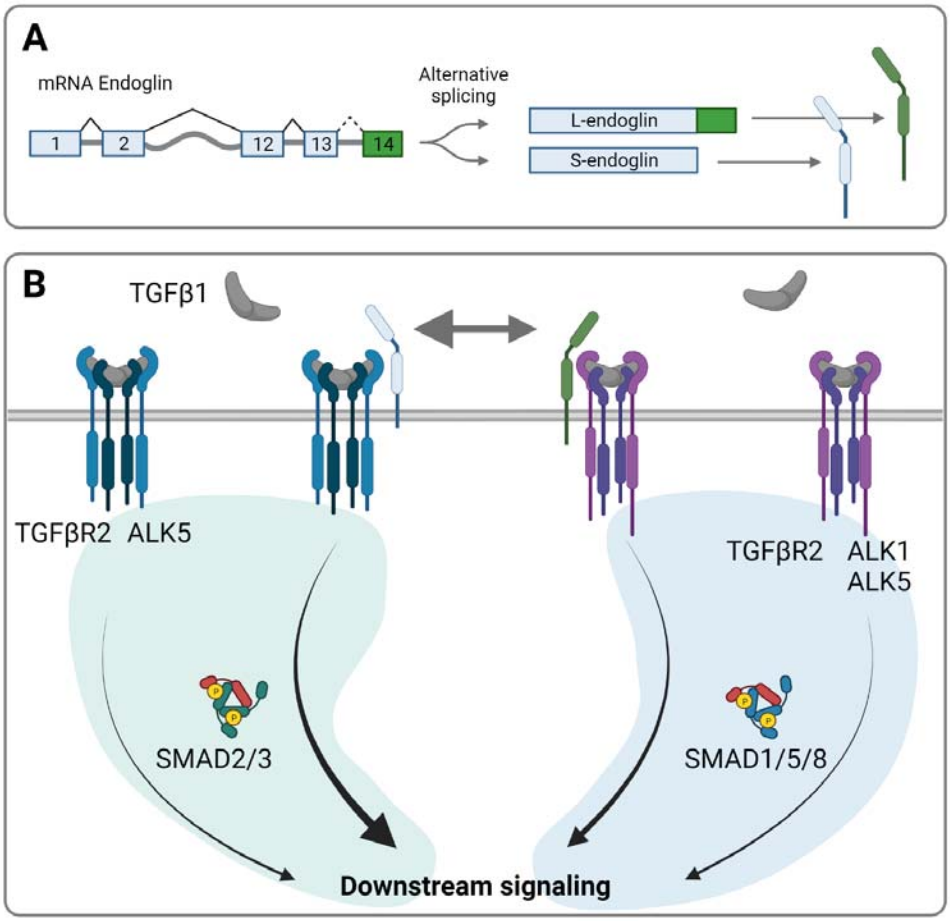


Figure 2. A schematic depiction of the splice variants (A) and signaling function (B) of Endoglin on TGF-β1 signaling. The short (S-) and long (L-)endoglin variants are alternatively spliced by excluding or including exon 14, respectively (a). Both S- and L-endoglin increases TGF-β1 signaling, however, S-endoglin favors ALK5 signaling where L-endoglin favors ALK1 dependent signaling (b). Therefore, as observed by[54,67], a balance shift towards S-endoglin increases TGF-β signaling by SMAD2/3 phosphorylation. Abbreviations; TGF: Transforming Growth Factor, ALK: Activin Like Kinase, SMAD: Small Mothers Against Decapentaplegic.

TGF-β signaling in PAH

PAH is linked to disturbances within the TGF-β signaling pathway. Mutations within the TGF-β signaling cascade have been identified, such as ACVRL1 (encoding ALK1), ENG (encoding endoglin), SMAD9 (encoding SMAD8)[68,69], SMAD1[68], SMAD4[68] and GDF2 (encoding BMP9)[70] (Figure 1). The most relevant gene mutation by far affects the BMPR2 gene, which is affected by loss of function mutations in 70-80% of the HPAH and in 10-20% of the IPAH patients.[71] Furthermore, genes not directly affecting TGF-β signaling have also been described (i.e., CAV1[72], TBX4[73], EIF2AK4[74], KCKN3[75]).

Currently more than 650 different BMPR2 mutations have been described.[76–78] These mutations can occur in non-coding regions but are mostly located in the coding regions containing the extracellular, transmembrane, kinase and cytoplasmic functional domains. Noteworthy, approximately 50% of total mutations are found in the kinase domain of BMPR2.[76,79] The different gene mutations consist of single nucleotide substitutions, leading to nonsense, missense or splice site mutations; and insertions or deletions causing small and partial insertions, deletions or duplications. A study looking at 144 different BMPR2 mutations from a broad international PAH patient cohort, predicted that around 70% of all the mutations result in non-mediated decay of the truncated transcripts.[79] Follow-up studies concluded similar findings.[76] The resulting haploinsufficiency is therefore the main cause of disrupted TGF-β signaling. Still, PAH penetrance is low in families with mutations causing haploinsufficiency. Comparing non-affected mutation carriers with PAH patients within the same family, Hamid and colleagues showed that the expression levels from the wild type BMPR2 allele impact disease progression, with lower BMPR2 expression levels observed in more affected individuals.[80] Therefore, next to loss of BMPR2 due to genetic mutations, additional triggers to reduce endogenous BMPR2 expression are needed to result in pathogenic disturbed TGF-β signaling.

In HPAH patients carrying a BMPR2 mutation, the BMPR2 and phosphorylated SMAD1/5/8 expression are decreased in lung tissues[41,81,82], consistent with a decreased expression of BMP transcriptional targets such as ID3.[83] Interestingly, BMPR2 expression is also decreased in idiopathic patients[81], which might be due to (post)transcriptional inhibition of BMPR2 expression in inflammatory environments.[65,84] Serum and lung expression of TGF-β1 and TGF-β3 ligands are increased in PAH patients[85,86], consistent with enhanced expression of a TGF-β target gene SERPINE1.[87] Additionally, Activin A and its natural antagonist Follistatin and Follistatin Like-3 are both increased in serum of HPAH and IPAH patients[88,89], of which Activin A is known to be secreted by macrophages, bronchial epithelial cells and lung microvascular ECs.[90] Given the widely acknowledged counterbalance between BMP and TGF-β signaling, it is well accepted that increased TGF-β and Activin A signaling in PAH results from inactivating mutations in BMP pathway components.[25,91] However, recent publications have unveiled novel mechanisms triggered upon loss of BMPR2. Hiepen and colleagues recently showed that loss of BMPR2 in ECs results in the formation of a mixed-tetrameric receptor complex TGF-β-TGF-βR2-ALK5 including a type I BMP receptor.[92] The inclusion of a type I BMP receptor allows the activation of pSMAD1/5/8 signaling, while this is prevented upon BMPR2 expression. Earlier work by other groups further strengthen this hypothesis of mixed-TGF-β/BMP receptor complexes and subsequent activation of pSMAD1/5/8 upon stimulation with TGF-β or Activins.[93–96] This can be a very relevant mechanism in PAH, as not only TGF-β1, but also Activin A levels are increased in serum of IPAH and HPAH patients.[88,89]

Loss of function mutations in ENG have been found in familial PAH patients.[97] IPAH patients display increased circulating and non-circulating ENG levels[85], measured in serum and EC isolation respectively. This increased soluble Endoglin is related with disturbed EC function. Moreover, alternative splice variants of Endoglin can shift the TGF-β/BMP signaling balance.[54] These variants differ in exon 14, transcribing a small intracellular domain, giving L-endoglin a longer intracellular part compared to S-endoglin.[98] This intracellular domain contains

phosphorylation sites for TGFβR2, ALK5, and ALK1.[99] As shown by Lee et al., increased short (S-)endoglin over long (L-)endoglin causes an increase in SMAD2/3 over SMAD1/5 phosphorylation in ECs (Figure 2).[67] Interestingly, this disbalance may also occurs in HPAH patients with mutations in exon 14 of the ENG gene, favoring the short splicing variant S-Endoglin and therefore increasing TGF-β signaling.

Taken together, alterations in BMP receptor complexes due to, for example, loss of function mutations in BMPR2 or ENG, can disbalance the cellular responses to the increased circulating levels of TGF-β/Activin ligands. Induction of BMP-driven pSMAD1/5/8 is often described as protective in PAH, however, irregular BMP signaling initiation by TGF-β or Activins may not be beneficial as well. One explanation might be that TGF-β- and Activin-induced mixed-tetrameric receptor complexes lead to a competition for canonical BMP-induced receptor complexes, resulting in less potent BMP-induced pSMAD1/5/8 and, perhaps, different non-canonical signaling activation. Further, it can lead to short-term signaling saturation (by e.g. SMAD4 competition). Therefore, comprehensive studies including not only BMPR2 downstream signaling, but also other TGF-β branches in the context of PAH are needed, as they all may contribute to vascular remodeling and subsequent PAH development.[92]

In line with a prominent role of aberrant TGF-β signaling as underlying cause of PAH, the ACTR2A-Fc fusion molecule Sotatercept aims to counter this imbalance by trapping soluble TGF-β ligands (Figure 3) and thereby restoring pathogenic tissue remodeling.[8,100] Indeed, in vitro evidence shows that ACTR2A-Fc treatment of pulmonary ECs reduces pSMAD2/3 while enhances pSMAD1/5/8 signaling. Further, pulmonary arterial thickening and cardiac hypertrophy were partially restored by only 2-4 weeks of Sotatercept treatment in PH rat models.[100] The type II receptor ACTR2A is able to bind many different TGF-β ligands (Figure 1) with different affinities. High affinity ligands of ACTR2A include Activin A, GDF8 and GDF11[48], which levels are all increased in PAH.[88,89,100] Due to the promiscuous role of ACTR2A in complex formation and binding capacity to many other ligands (also e.g. BMP10)[48], we stress that Sotatercept's success might rely on its unspecific targeting of TGF-β ligands. The balance of the combinatory levels of circulating TGF-β ligands in the patient and their differential affinities to Sotatercept therefore drives its pharmacological function. However, Sotatercept may also reduce BMP activity, which can underlie the undesirable side effects observed in PAH patients involved in a recent clinical trial (as reviewed in[101]). For instance, the inhibition of BMP10 by high doses of Sotatercept can interfere with BMP10 homeostatic function on the endothelium[52], possibly resulting in telangiectasias (Figure 3). Furthermore, thus far this drug has been tested in patients on background therapy. Whether a therapeutic approach based on solely targeting ACTR2A ligands is successful, remains to be investigated.

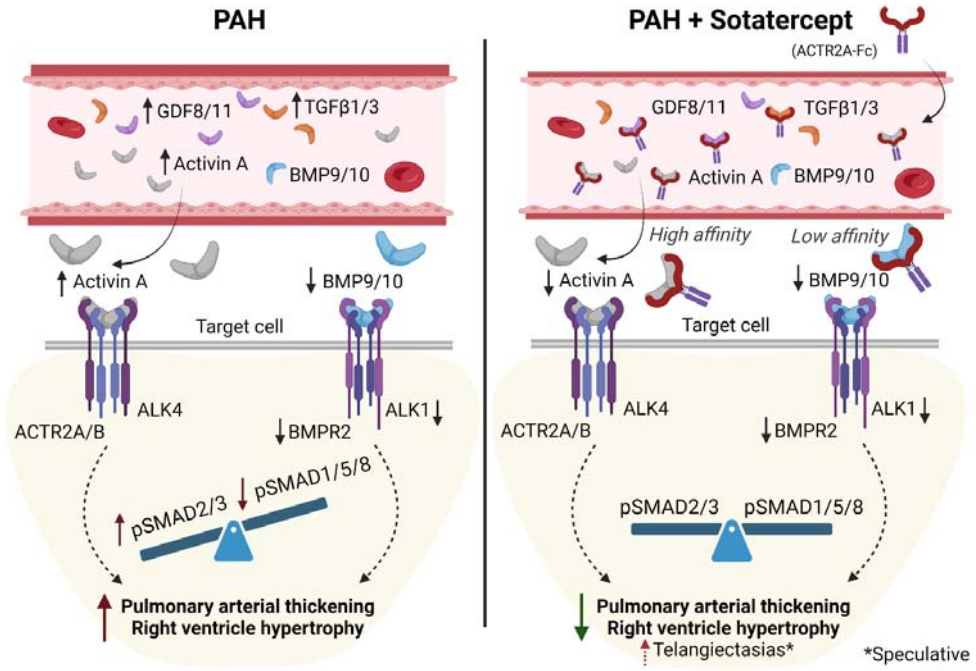


Figure 3. **Sotatercept (ACTR2A-Fc) traps TGF-β ligands to restore the disbalanced signaling in PAH.** The soluble ligands Activin A, GDF8/11 and TGF-β1/3 are increased in PAH causing increased SMAD2/3 phosphorylation over SMAD1/5/8 signaling. This disturbed TGF-β signaling causes increased pulmonary arterial thickening with a subsequent rise in pulmonary arterial pressure and right ventricle hypertrophy. Treatment with Sotatercept normalizes the signaling imbalance by trapping soluble TGF-β ligands, resulting in a decrease in pulmonary arterial thickening and right ventricle hypertrophy. *Possibly, low affinity inhibition of BMP10 by Sotatercept might disturb endothelial homeostasis and subsequently causing telangiectasias. Abbreviations; TGF: Transforming Growth Factor, GDF: Growth Differentiation Factor, BMP: Bone Morphogenetic Protein, ALK: Activin receptor-like Kinase, ACTR2: Activin Receptor type II, BMPR2: BMP receptor type II, SMAD: Small Mothers Against Decapentaplegic.

Sex-hormones and TGF-β signaling

As aforementioned, disturbed TGF-β signaling constitutes a hallmark in PAH development. Given the sex-bias observed in this disease, it becomes key to understand the mechanisms by which sex-specific cues may fine-tune TGF-β signaling. Sex hormones are derived from cholesterol. Female sex hormones are estrogens and progestogens, including estradiol and progesterone, respectively. Male hormones are Androgens, of which testosterone is the dominant effector. Sex steroids induce chemical signal transduction by binding to their soluble nuclear receptors; Estrogen Receptor (ER), Progesterone Receptor (PR), and Androgen Receptor (AR). These receptors act as signal transducer and transcription factors by binding to DNA responsive elements (RE; ERE, PRE, ARE).[102–104] In addition, membrane bound G-Protein-coupled receptors for all these sex hormones exist[105] which modulate non-canonical TGF-β signaling pathways.

Estrogens have strong implications in vascular diseases and promote cardiovascular protection.[106,107] Frump and colleagues showed that 17β-Estradiol substantially improves right ventricular function in the Sugen-Hypoxia (SuHx) PH rat model[108], and they further linked ERα signaling in the right ventricle to protective adaptation in PAH in a BMPR2-dependent manner.[109] Although less characterized than estrogens, progestogens and androgens are also cardiovascular active, and play a substantial role in vascular health and disease.[110–113] While the effect of sex hormones on the (pulmonary) vasculature is well appreciated[110,114,115], the molecular mechanisms underlying their functions remain elusive. Both sex hormones and TGF-β family members exert a tight control of the vasculature including pathogenic conditions like PAH.[25,115,116] For comprehensive understanding of the TGF-β and sex-hormone crosstalk we will summarize the molecular mechanisms described so far, mainly in vascular cells. Unfortunately, most mechanistic studies have been performed in non-vascular settings. Given that sex-hormones act on many non-cardiovascular tissues, influencing systemic levels of circulating TGF-β components and hence indirectly the cardiovascular system, we will learn from studies performed in non-vascular tissues, and discuss how the crosstalk between TGF-β signaling and sex hormones may be applicable to vascular biology and PAH.

Estrogens

Estrogen signaling involves several members of the TGF-β family signaling pathway in vascular context (Table 1). As such, transcriptome analysis of human umbilical vein endothelial cells (HUVECs) showed that the expression of ACVRL1 (encoding ALK1), and Latent-transforming growth factor beta-binding protein 3 (LTBP3) are increased in response to exogenous estradiol, while CAV2 (caveolin-2), a negative regulator of TGF-β1 induced ALK5/SMAD2/3 signaling in ECs[117], and SMURF2 are decreased, partially overlapping the transcriptome of TGF-β1-stimulated cells.[118] Additionally, administration of the selective estrogen receptor modulator (SERM) Raloxifene increased the protein expression of ALK1 and Endoglin in ECs[119], hence favoring SMAD1/5/8 over SMAD2/3 signaling. SERMs can have an agonistic and antagonistic effect, depending on the tissue type and availability of estrogen receptors.[120] These effects have been extensively studied in mammary and skeletal tissues but are underexplored in the cardiovascular system, which is evidently necessary in the context of PAH therapy.

The plasma membrane G-Protein-Coupled estrogen receptor (GPER, or GPR30) is an important mediator of estrogen-induced signaling in vascular etiologies.[121,122] Interestingly, GPER activation by Estradiol or the GPER agonist G1 increased SMAD1/5/8 phosphorylation and the downstream target ID1 in HUVECs.[123] These effects can be inhibited by a G-protein pathway inhibitor, indicating a specific role for canonical GPER signaling. This study suggests for the first time a cross-talk between GPER and canonical TGF-β signaling in ECs, and therefore more research is encouraged. Activation of GPER induces Src, MAPK, and PI3K/Akt signaling via transactivation of the epidermal growth factor receptor (EGFR) pathway.[124] GPER modulates hypoxia induced factor (HIF) and vascular endothelial growth factor (VEGF) signaling, which makes it an interesting receptor to target in the endothelium.[105] In addition, estrogen-GPER signaling enhances Notch-mediated epithelial-to-mesenchymal transition (EMT)[105,125], a process resembling EndMT (important in PAH as described above). Importantly, all these non-canonical TGF-β signaling routes (Figure 1) have shown relevance in PAH.[126–129]

Estrogens have been implicated to influence PAH disease development and are thought to be an important driver causing the sex-bias in PAH. As such, decreased expression of an important 2-hydroxyestrogen (2-OHE) catalyst, CYP1B1, may be a second-hit favoring PAH development in female HPAH patients.[130] In blood isolated lymphoblastoid cells, this enzyme showed a 10-fold decreased expression in affected compared to unaffected female BMPR2 mutation carriers.[130] As a follow-up, Austin and colleagues showed that female BMPR2 mutation carriers have a 4-fold decreased disease penetrance when expressing the N453S CYP1B1 variant compared to wild-type.[131] Further, they observed a decreased urinary 2-OHE/16α-OHE metabolite ratio in affected female BMPR2 mutation carriers. Unexpectedly, the enzymatic function of CYP1B1 was unrelated to 2-OHE levels but predominantly caused by increased levels of 16α-OHE (although highly variable).[131] This study therefore demonstrates the importance of estrogen metabolites in PAH disease penetrance in women.

Indeed, Mair and colleagues found that basal BMPR2 protein levels in female non-PAH hPASMCs are lower compared to male cells.[132] BMP4 induced pSMAD1/5/8 and ID1/3 expression was lower in female than male hPASMCs. Interestingly, administration of exogenous estradiol to male hPASMCs decreased ID1/3 expression to levels comparable to female cells.[132] Consistently, estrogen-ERα activation was reported to downregulate BMPR2 expression via an ERE in the promoter of BMPR2 in pulmonary microvascular ECs (MVECs).[133] Moreover, inhibition of estrogen synthesis by the aromatase inhibitor anastrozole alleviated experimental PAH in a SuHx rat model by restoring BMPR2 expression.[134] Conversely, in the right ventricles of multiple PH rat models and cultured rat right ventricle cardiomyocytes, E2-ERα signaling increased BMPR2 expression.[109] Further, basal BMPR2 levels were higher in female right ventricle samples compared to males. Interestingly, they showed a direct interaction between ERα and BMPR2, which improved cardiac function via Apelin upregulation. In this study, Frump and colleagues also showed a protective effect of E2, or an ERα agonist, by preventing PH disease development in multiple PH rat models, driven via this BMPR2/Apelin-axis. Compared to human control samples, IPAH patients showed decreased ERα levels in the right ventricle.[109] Taken together, estrogens can decrease BMPR2 expression in the vasculature, but promote BMPR2 levels in the right heart. This cell type-dependent effect can explain female predominance and increased male severity in PAH.

Mechanistically, estrogens have been shown to decrease BMPR2 levels in multiple vascular models[132–134] and increase BMPR2 levels in the right heart.[109] Further, DHEA treatment of PAH patient derived PASMCs increased BMPR2 mRNA expression[135], explaining an increased disease penetrance in individuals presenting with low DHEA-S levels.[136–138] Consequently, basal BMPR2 vascular levels are lower in females[132,133], which might explain the female predominance and male protection of PAH development. Further, estrogens increase right ventricular BMPR2 levels with subsequent cardiac protection[109], potentially justifying a more severe PAH phenotype in men. Additionally, the broad transcriptional effects of estrogen on TGF-β signaling components (Table 1), especially in BMPR2-mutated individuals could result in an increased disbalance in TGF-β and BMP signaling, thereby contributing to vascular remodeling and PAH development, particularly in women.

Circulating sex hormones may be also secreted by and affecting non-cardiovascular tissues, which in turn may impact the cardiovascular system indirectly. Through this angle, multiple studies have been performed using non-vascular cell models like MCF-7 and HEK293 that could help us to unveil the mechanistic crosstalk between TGF-β and sex hormones (summarized in Table 1). Researchers have shown that ERα/β can directly bind, inhibit and recruit protein degradation systems (by e.g. SMURF1) to SMAD2/3 in an estrogen dependent manner (Figure 4).[139–142] BMP stimulated SMAD1/5/8 phosphorylation was also reduced by estrogen treatment in the same non-vascular cell lines.[143] To add complexity to this estrogen-TGF-β crosstalk, SMADs can also be a cofactor for sex-hormone receptor-mediated transcription.[144,145] Evidently, as these studies made use of non-vascular cells, there is a need to confirm their findings towards vascular biology in the context of PAH.

In conclusion, accumulating evidence indicate that estrogens can regulate canonical TGF-β signaling by directly altering the expression of TGF-β receptors and signaling modulators, at the transcriptional and protein level. Moreover, estrogen signaling via GPER may indirectly modulate TGF-β non-canonical routes (Figure 4).

Progestogens

Progestogens may positively impact the cardiovascular system[146], by negatively regulating proliferation of ECs and SMCs.[111,147,148] Progesterone induces a strong vasodilatory response compared to estradiol and testosterone in male and female rat coronary and pulmonary arteries ex vivo.[113] Congruently, low progesterone levels correlate with increased risk of PAH in men.[149] To date, a direct link between progestogens and TGF-β signaling (including BMPR2 regulation) in cardiovascular cells is underexplored. In epithelial cells, progesterone dose dependently inhibits TGF-β1-induced SMAD3 phosphorylation[150], and antagonizes TGF-β1-mediated upregulation of the target genes CTGF, transgelin, and PAI-1. In human granulosa cells, BMP-15-induced signaling via BMPR2 and ALK6 was shown to suppress progesterone production[151], although the authors show indirect effects. In addition, Activin A repressed progesterone synthesis in the reproductive system[152,153], which might explain low progestogen levels in male PAH patients[149], as Activin A plasma levels are increased[88]. Similarly, BMP4 and BMP7 also suppressed progesterone synthesis in Granulosa-Lutein cells.[154] The crosstalk between progesterone and TGF-β signaling is most likely cell-type and context dependent.

In summary, although functional progesterone responses on vascular cells are well-described, data regarding cross-talk between progestogens and TGF-β signaling in this context is lacking, and more research is needed to further understand the sex-related differences in PAH.

Androgens

Androgens have been proposed as a therapeutic treatment for PH[115,155], because of its quick beneficial vasodilatory effect on the pulmonary vasculature[21] and its protective effect on right ventricle adaptation and function.[155,156] Androgens classical mode of action involves gene transcriptional responses through intracellular binding to AR[112,157,158], expressed in PASMCs and ECs. The androgen-induced vasodilation response occurs within 20 minutes after androgen administration.[21,113] As a direct effector, testosterone can antagonize calcium channels in SMCs, thereby triggering a fast cellular response, not mediated by classical AR-dependent gene transcription. The androgen metabolite DHEA is shown to restore cardiac

remodeling and increase right ventricular function in rat models for experimentally induced PAH.[135,155] As aforementioned, in PAH patient derived PASMCs, DHEA administration increased BMPR2 mRNA expression levels[135], and DHEA (or DHEA-sulphate, -S) treatment is currently investigated in a clinical setting.[156]

Beyond the vasculature, androgens are described to modulate TGF-β signaling at multiple levels (Figure 4 and Table 1). Also mechanistically, in prostate cancer cell lines such as LNCaP and PC3 cells, dihydrotestosterone (DHT)-induced AR transactivation can form a complex with SMAD3 and SMAD4, where SMAD3/AR complexes promote transcription via DNA binding to AREs while SMAD3/SMAD4/AR complexes inhibit androgen target gene expression.[144] Hayes and colleagues observed repression of androgen target gene expression by SMAD3/AR complexes[159], by direct binding of the MH2 domain of SMAD3 with the transcription activation domain of the AR. Interestingly, the androgen driven inhibitory effects on gene transcription is not specific for the TGF-β branch of the family, but also BMP signaling and its downstream targets are inhibited upon DHT treatment in e.g. intestinal stromal cells.[160] Furthermore, phosphorylated SMAD1 interacts with AR to suppress its transcriptional function[161], indicating that androgens may regulate both TGF-β and BMP signaling pathways and vice versa (Figure 4).

In conclusion, androgens and TGF-β cross-talk via direct AR and SMAD interactions and indirectly via transcriptional regulation through AREs (Figure 4). The vast majority of these data result from studies using prostate cancer or other non-vascular models, but may very well be applicable to PAH. For example, testosterone administration increased the expression of the circulating TGF-β regulators follistatin, chordin and noggin expression in muscle stellate cells[162] (Table 1), which may impact distant organs, including the heart and the pulmonary vasculature. PAH patients exhibit increased Activin A and Follistatin circulating levels[88], and Activin A levels correlate with increased mortality. Higher androgen-mediated Follistatin in males could potentially suppress high amounts of Activin A in PAH, and might contribute to the lower prevalence in men.[163] The decrease in androgens by age would lead to decreased follistatin levels with increased active Activin A levels and disturbed TGF-β and BMP signaling balance as consequence. In line, the sex-biased disease prevalence in PAH also decreases upon age.[12] The same mechanisms apply to chordin and noggin, suppressing the BMP branch with decreased effect upon age. Following this hypothesis, one might warrant the prescription of (Activin A) ligand traps like Sotatercept. Indeed, as described earlier, clinical trials have been performed treating Sotatercept to PAH patients with striking results.[8,164]

Taking into consideration the TGF-β/BMP balance and the effects sex hormones have on TGF-β signaling components, including BMPR2, one could hypothesize that BMPR2 expression levels are higher in men compared to women. Low androgen levels with a corresponding drop in BMPR2 expression could initiate PAH development, as low DHEA-S levels are correlated with worse disease outcome in male PAH patients.[138] Further, high androgen driven follistatin levels in men might protect from extensive signaling by e.g. Activin A in PAH. Taken together, this delineates a higher incidence in PAH development in predominantly younger women, but also a more severe disease outcome in men with low DHEA levels.[138]

Anti-Müllerian hormone

AMH is expressed in follicular sertoli and ovarian granulosa cells, and is known to be a circulating hormone throughout life, although declining by age. AMH is a TGF-β family member that binds its dedicated TGF-β type II receptor AMHR2[165], also expressed in the human heart.[166] Associated type I receptors include ALK2, -3 and -6, thereby involving BMP-like downstream signaling (Figure 1).[36,165] Although typically linked with sexual dimorphisms[167] and female fertility, other studies indicate AMH to have cardiovascular regulatory properties and it is long known to be a circulating hormone throughout life. From 2012 onward, higher levels of AMH have been correlated with cardiovascular protection[168], decreased plaque diameter in non-human primates[169], and decreased male aortic diameter (implications for aneurisms).[170] More recently, in the Doetinchem Cohort Study, they found that decreasing AMH trajectories are associated with a substantial elevated risk of CVD in women.[171]

A potential role of AMH in PAH was recently suggested as part of a case report study[172] describing a novel loss-of-function BMPR2 mutation in exon 2 associated with IPAH development. The resulting BMPR2 mutant protein is unable to translocate to the plasma membrane. Comprehensive analysis of the TGF-β/BMP signaling signature in peripheral blood mononuclear cells (BPMCs) of this patient confirmed low BMPR2 expression levels, and increased expression of AMHR2, ALK1, ALK3, and ALK6 protein levels, whereas TGF-β receptors remained unchanged.[172] Noteworthy, increased SMAD1/5 and SMAD2/3 phosphorylation was observed upon BMP2 and TGF-β stimulation. Furthermore, mRNA expression of the BMP target genes ID1, SMAD6 and STAT1 was increased, suggesting that BMP signaling was not compromised due to the BMPR2 mutation, at least in PBMCs. The expression of AMHR2 in PBMCs supports the hypothesis that AMH affects inflammation responses and therefore influences PAH. Indeed, higher circulating AMH levels has been correlated with reduced inflammation marker c-reactive protein in men.[173] Disturbed inflammation has been proposed as additional driver of PAH development[174], therefore, reducing inflammation via increased AMH signaling in BMPR2 mutation carriers might be beneficial in PAH. In this case-report however, increased AMHR2 not necessarily proves increased signaling as functional AMHR2 ligands activity was not quantified.

Studies using lung cancer epithelial cells reported cross-talk with AMHR2 and BMPR2 signaling causing increased SMAD2/3 phosphorylation upon loss of AMH or AMHR2[175], possibly via mixed-heteromeric receptor complexes driven by BMP ligands.[92] Correspondingly, in these cancerous epithelial cells, siRNA depletion of AMH or AMHR2 drives EMT[175], suggesting inhibitory functions of AMH in EMT. Early in life, males show higher AMH levels than females, but women have higher AMH levels throughout life.[170] To date, relevant data in relation to the pulmonary vasculature are lacking, but if the mechanisms described above for AMH are applicable to vascular cells too, unraveling the role of AMH in the vasculature might help understand PAH disease development.

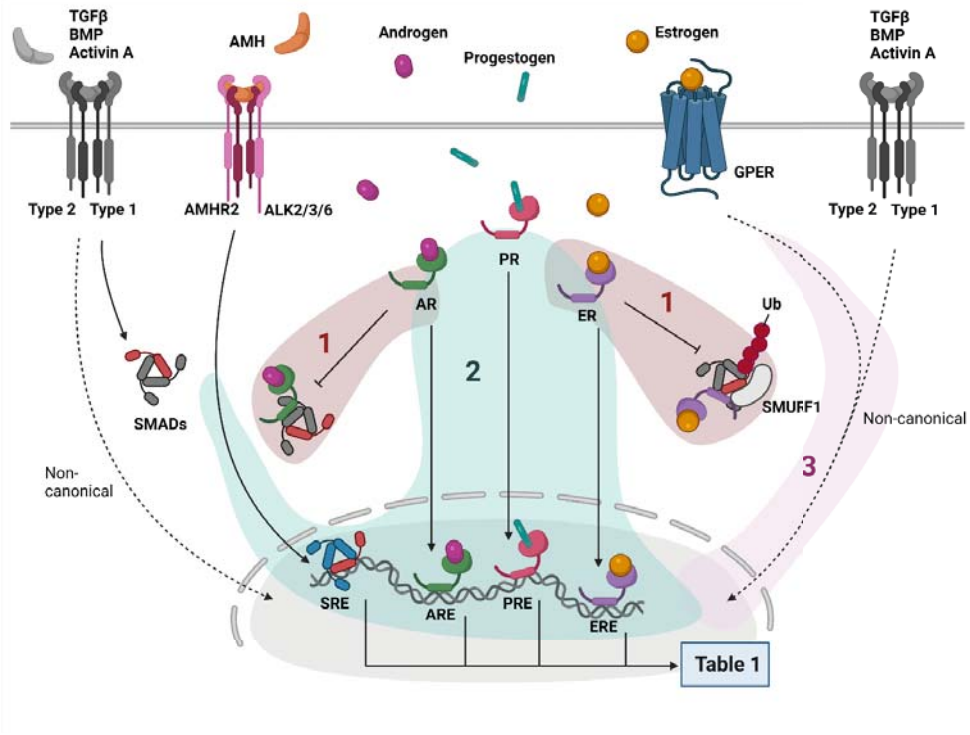


Figure 4. Signaling cross-talk of sex hormones and TGF-β signaling. The membrane permeable sex hormones androgens, progestogens and estrogens bind their nuclear receptors Androgen Receptor (AR), Progestogen Receptor (PR), and Estrogen Receptor (ER), respectively. Estrogens also bind the membrane receptor G Protein-coupled Estrogen Receptor (GPER). Sex-hormones cross-talk on three different levels with TGF-β signaling. 1) The activated nuclear receptors can directly interact with SMADs to inhibit downstream signaling. Estrogen-ER signaling has been associated with SMURF1-mediated proteasomal degradation of SMADs. 2) All sex-hormones have shown to regulate TGF-β target genes, via their corresponding responsive elements. 3) The Estrogen-GPER signaling cascade includes routes overlapping non-canonical TGF-β signaling routes. Abbreviations; TGF-β: Transforming Growth Factor-β, BMP: Bone Morphogenetic Protein, AMH: Anti-Müllerian Hormone, AR/PR/ER: Androgen/Progestogen/Estrogen Receptor, GPER: G Protein-coupled Estrogen Receptor, SRE/ARE/PRE/ERE: SMAD /Androgen /Progestogen /Estrogen Responsive Element, SMAD: Small Mothers Against Decapentaplegic, and SMURF: SMAD Specific Ubiquitin Ligase.

Hormone	*Expression ↑/↓*	*Level of expression*	*Model (tissue) / cell-type*	*Metabolite*	*Ref.*
Estrogens	↑ ALK1	mRNA & protein mRNA	HMEC-1 HUVECs	Raloxifene 17β-Estradiol	119 118
	↑ ALK5	Promoter Protein	Rat osteoblasts	Estradiol	176
	↑ BMP2	mRNA	Mouse MSCs	17β-Estradiol	177
	↑ BMP6	Promoter	Osteoblasts / MCF-7	17β-Estradiol	178
	↑ BMPR2	Protein	RV Su-Hx rat RVCM WT / Su-Hx rats	17β-Estradiol PPT	109
	↑ ENG	mRNA & protein	HMEC-1	Raloxifene	119
	↑ LTBP3	mRNA	HUVECs	17β-Estradiol	118
	↑ TGF-β3	Promoter & mRNA	Rat (bone)	17β-Estradiol Raloxifene	179
	↓ BMPR2	mRNA Protein Protein	Wild-type mice HPASMC Su-Hx rat	17β-Estradiol 17β-Estradiol Anastrozole	133 132 134
	↓ ID	Protein	HPASMC	17β-Estradiol	132
	↓ SMURF2	mRNA	HUVECs	17β-Estradiol	118
Progestogens	↓ CTGF (TGF-β1 induced)	Promoter mRNA Protein	A549 (lung epithelial cells)	Progesterone	150
	↓ PAI-1 (TGF-β1 induced)	Promoter	MLECs (mink lung epithelial cells)	Progesterone	150
	↓ TAGLN (TGF-β1 induced)	Promoter mRNA Protein	A549	Progesterone	150
Androgens	↑ BMPR2	mRNA	PAH HPASMC	DHEA	135
	↑ BMP7	mRNA	Stellate cells	Testosterone	162
	↑ Chordin	mRNA (array)	Stellate cells	Testosterone	162
	↑ FST	Protein	Stellate cells	Testosterone	162
	↑ Noggin	mRNA (array)	Stellate cells	Testosterone	162
	↑ SMAD7	mRNA	Stellate cells	Testosterone	162
	↓ ACVR2A	mRNA	Stellate cells	Testosterone	162
	↓ BMP2	mRNA (array)	Stellate cells	Testosterone	162
	↓ BMP4	mRNA (array)	Stellate cells	Testosterone	162
	↓ Nodal	mRNA (array)	Stellate cells	Testosterone	162
	↓ PAI-1	mRNA (array)	Stellate cells	Testosterone	162
	↓ SMAD2/3	Protein	Rat (kidney)	Testosterone Propionate	180
	↓ SMAD4	Protein	Rat (kidney)	Testosterone Propionate	180

	↓ SMURF1	mRNA (array)	Stellate cells	Testosterone	162
	↓ TGF-β1	mRNA	Stellate cells	Testosterone	162
		Protein	Rat (kidney)	Testosterone Propionate	180
	↓ TGFβR2	mRNA	Stellate cells	Testosterone	162
AMH	↓ ALK2	Protein	Lung epithelial cells	AMH (expressed)	175
	↓ ALK3	Protein	Lung epithelial cells	AMH (expressed)	175
	↓ BMPR2	Protein	Lung epithelial cells	AMH (expressed)	175

Table 1. An overview of studies investigating transcriptional effects of the different sex hormones on targets within the TGF-β signaling cascade. The table shows increased or decreased expression, at which level it has been investigated, in which model or cell-type and the specific metabolite used.

Sex hormonal therapy in the clinic

The cross-talk between estrogens and androgens and TGF-β signaling is relatively well described in the vascular system. The findings described in previous chapters indicated a protective effect of androgen, by increasing BMPR2 expression and circulatory Follistatin levels, and estrogens being an additional risk factor by decreasing BMPR2 levels in the vasculature but cardioprotective in the heart. Correspondingly, targeting sex-hormone signaling in PAH is a strategy applied within the clinic by multiple groups.

Baird et al. showed that lower levels of dehydroepiandrosterone-sulphate (DHEA-S, a prohormone for androgens and estrogens) and higher levels of E2 were associated with severe PAH in men[136] and in post-menopausal women[137]. This profile caused a worsened disease outcome, suggesting substantial roles of these sex-hormones in disease progression and response.[136] In a recent study analyzing a large Dutch PAH cohort we confirmed low DHEA-S levels in male and female PAH patients.[138] These studies validated a clinical trial to evaluate the effect of DHEA-S administration in PAH (NCT03648385).[156] Targeting high estrogen levels also seems a possible treatment option for PAH as estrogen inhibition by anastrozole (aratomase inhibitor) and fulvestrant (ER antagonist) treatment prevented and reversed PAH development in BMPR2 mutant mice.[181] There are two clinical studies conducted using anastrozole in PAH. The first small phase-2 clinical trial of anastrozole in PAH patients showed a 40% reduction of estrogen plasma levels, a good safety profile and a significant increased 6-minute walking distance, although other PAH clinical outcome measures were unchanged (NCT01545336).[182] A larger follow-up trial is performed (PHANTOM: NCT03229499), however, the outcomes are not published yet. Importantly, as estrogens show a protective effect on the right heart by increasing BMPR2 levels[109], anti-estrogen therapy might be detrimental and therefore tissue dependent effects of estrogen should be carefully considered. Correspondingly, pre-clinical data shows a protection of PH development when treating with E2.[109] Additionally, anti-estrogen therapy in reproductive aged women is far from ideal but treatment might be considered for post-menopausal women. Taken together, these studies underline the importance of sex-hormones in PAH disease initiation and progression and set

the stage for clinical (anti-)hormone therapies for PAH, although context dependent cellular and molecular mechanisms driving these effects are still incompletely understood.

Genetic-related sex-differences and TGF-β signaling

The X and Y sex-chromosomes contain specific genetic information which might differentially regulate TGF-β signaling in males and females. Although most of the genes expressed from the Y chromosome encode for proteins required during gonad development, some factors also have roles outside the reproductive system. In females, expression levels of genes located on the X chromosome are regulated by the inactivation of one of the two X chromosomes. As we will discuss below, in some occasions this process can be disturbed, leading to enhanced gene expression due to increased genetic load. In this section we elaborate on X and Y- linked genes in relation to TGF-β signaling in PAH.

Y Chromosomal expression

The Y chromosome is a relatively small chromosome containing a low number of genes in comparison with other mammalian chromosomes. There are 568 genes harbored on the Y chromosome, of which only 71 have protein encoding potential.[183] Multiple genes encode proteins of the same protein families, leaving only 27 non-related proteins encoded on the Y-chromosome. In a mouse model for PAH, Umar et al. found that the Y chromosome protects disease development, unrelated to gonadal sex (testes or ovaries)[24], suggesting an important role for Y-chromosomal expression in preventing PAH development. Of all Y-chromosomal genes, the sex-determining region Y (SRY) gene is the most studied.[184] SRY is a DNA binding transcription factor regulating gene expression at the early initiation of testes development, but SRY also functions outside the reproductive system.[185] As such, SRY directly binds the promoter of BMPR2 to upregulate BMPR2 expression in PAH fibroblasts.[186] As females lack SRY this BMPR2 transcriptional regulation does not occur. Correspondingly, BMPR2 mRNA levels in male PAH patient derived lymphocytes are higher compared to female equals.[133] Further, SRY may indirectly modulate TGF-β signaling by interacting with AR thereby dampening testosterone-induced transcription.[187]

Of all the genes found on the Y-chromosome in PAH patients, 8 genes showed decreased expression in diseased lung tissues.[24] One of these genes is USP9Y, a ubiquitin-associated hydrolase preventing ubiquitin-dependent degradation of proteins including SMAD4, thereby increases TGF-β signaling ([188] and ENSG00000114374). Another downregulated Y-linked gene in PAH lungs is the ATP-dependent RNA helicase DDX3Y [24]. Although DDX3Y interacts with SMAD2 and SMAD3[189], the functional consequences of this interaction is unknown. In summary, Y-specific expression profiles may alter TGF-β signaling (Figure 5B) and might prevent the initiation and progression of PAH. How these interactions with the TGF-β family results in changes of cellular behavior needs still to be deciphered.

X Chromosome inactivation

The X chromosome contains over 1,200 genes. In females, the expression of X-linked genes is tightly regulated by X-chromosomal inactivation. This process is necessary for genetic dosage, leading to similar gene expression levels of X-linked genes in female XX cells compared to XY male cells.[190] Silencing of the X chromosome is mediated by the long non-coding RNA (lncRNA)

antisense pair X-inactive specific transcript (XIST) and TSIX (XIST, opposite strand). While XIST shields (thereby silences) one of the X-chromosomes, TSIX impairs the inactivation of the active X-chromosome through complementary binding to XIST. Furthermore, epigenetic modifications of the XIST locus can cause XIST silencing.[191] In addition, the lncRNA X-active specific transcript (XACT) coats the active X-chromosome and also antagonizes XIST.[192] Most genes on the inactivated X-chromosome remain silenced, however, 15-25% of X-linked genes escape this silencing process (known as 'escapees').[193] These escapees have been linked to sex-differences in diseases like auto-immune diseases and cancers.[194]

Recently, in the EH_{itsn}-$KO^{ITSN+/-}$ PAH mouse model for plexiform arteriopathy, Xist expression levels were increased in female PAH mice compared to the male mice or female WT mice.[195] Noteworthy, female EH_{itsn}-$KO^{ITSN+/-}$ mice showed worsened vascular remodeling compared to their male equals. While no difference in Xist levels were observed in the SuHx PAH rat model, increased Xist expression was observed in human female PAH lungs compared to healthy subjects. Taken together, the upregulations of the lncRNA Xist/XIST may explain the sexual dimorphism in vascular remodeling, and therefore highlights the importance of X-chromosome inactivation in the sex-bias in PAH.

Several studies suggest an interplay with Xist and BMP/TGF-β signaling. Genetic knockdown of ACVR1B (ALK4), BMPR2, and SMAD2 inhibit the expression of Xist in mouse fibroblasts.[196] BMP signaling was found to induce and maintain the expression of XIST, while TGF-β signaling served as an antagonist. Furthermore TGF-β signaling induced TSIX expression in dermal fibroblasts.[197] Although specific XIST/TSIX expression levels are suggestive for X-chromosomal silencing, deeper comprehensive studies are needed for conclusive results. Nevertheless, dysregulation of TGF-β/BMP signaling could impact the chance of genes on the X-chromosome to escape gene silencing thereby contributing to sex-differences in PAH pathology.

Genetic impact on PAH development suggest a protective role for specific genes expressed from the Y-chromosome.[24] The Y-chromosomal expressed SRY transcription factor upregulates BMPR2 expression in PAH fibroblasts.[186] As discussed above, TGF-β signaling can influence X-chromosomal inactivation in females, further enhancing TGF-β signaling disbalance in PAH. These observations strengthen the link between sex-hormones, sex-related genetics, disturbed TGF-β signaling and PAH disease development.

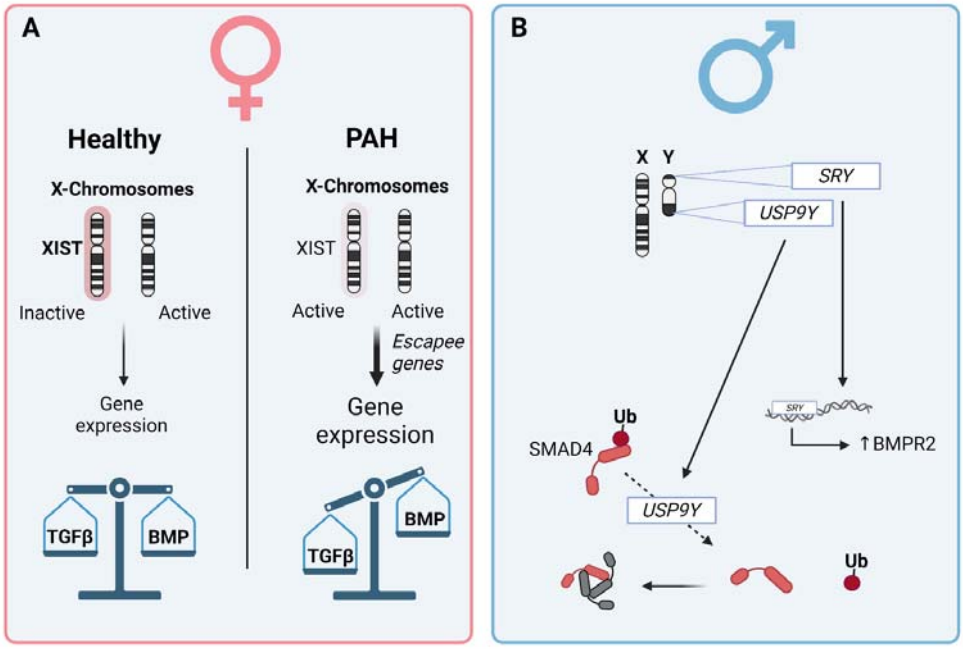


Figure 5. Genetics sex-related differences on TGF-β signaling in health and PAH. A) In females, proper X Chromosome inactivation results in healthy genetic output leading to a balanced TGF-β/BMP signaling. However, disturbances in X chromosome inactivation results in dysregulated genes (escapees) and increased genetic output which might cause a diseased disbalance in TGF-β/BMP signaling. B) In males, SRY has been linked to increased BMPR2 expression, while USP9Y is an ubiquitin-dependent hydrolase that targets SMAD4. Abbreviations; TGF-β: Transforming Growth Factor-β, BMP: Bone Morphogenetic Protein, SMAD: Small Mothers Against Decapentaplegic, SRY: Sex-determining Region of Y, USP9Y: Ubiquitin Specific Peptidase 9 Y-linked, BMPR2: BMP Receptor type 2.

Hereditary Hemorrhagic Telangiectasia

The genetic background and disease etiology in Hereditary Hemorrhagic Telangiectasia (HHT) (or Rendu-Osler-Weber syndrome) and HPAH patients sometimes overlap.[198] Interestingly, there is also a sex-bias observed in HHT although this is less pronounced compared to PAH. Therefore, many findings in this review are also relevant in a HHT context, which we shortly highlight in this chapter.

HHT is a vascular disorder presenting with malformed vessels leading to telangiectasia (spider veins), hemorrhages, and arteriovenous malformations (AVMs).[199] Similarly as HPAH, HHT originates in people harboring loss-of-function mutations in genes encoding BMP receptors, i.e. ACVRL1 (ALK1: HHT2) and ENG (Endoglin: HHT1).[97,200] It is thought that decreased BMP signaling causes endothelial dysfunction, leading to the malformed vasculature in HHT.[201,202] Sex differences in HHT present mainly by more severe symptoms in women compared to men (increased pulmonary and hepatic AVMs)[203,204], although some small registry studies describe a female predominance.[205–207]

In this review, we explored sex-differences in TGF-β signaling in PAH, but our findings can have implications for HHT too. For instance, administration of Raloxifene increases ALK1 and ENG expression in ECs[119] and is therefore proposed as treatment option for HHT (reviewed in[208]). Another SERM, Tamoxifen, showed promising effects in a clinical trial reducing severe epistaxis.[209] There is a marked influence of sex in pulmonary and hepatic vascular malformations in HHT, suggesting organ or tissue specific features in comparison with other organs.[210] It might be that expression levels of sex-hormone receptors in hepatic or pulmonary endothelial cells makes these cells more sensitive to circulating sex-hormones. This review highlights three levels on which sex-hormones can alter TGF-β signaling (Figure 4). Further research of these organ specific endothelial effects is warranted to delineate the sex-bias in HHT.

Discussion and concluding remarks

PAH is a cardiovascular disease with a clear sex-bias towards increased female predominance and more severe male phenotype. The molecular causes of this bias are incompletely understood. This review therefore explored sex differences in TGF-β signaling to understand sex-bias in PAH (and by extension in HHT).

We have emphasized that hormonal and genetic sex-differences may regulate TGF-β signaling in different ways to contribute to PAH. Noteworthy, many of the mechanistic findings described above originate from non-vascular cell models, hence translation into PAH should be done carefully. Future studies should be performed aiming to investigate sex-specific effects on TGF-β signaling in a cardiovascular setting. Often, sex-related genetics are not taken into account while investigating sex-hormonal effects on TGF-β signaling. For instance, researchers should include karyotypes of the cells or tissues studied. We further stress the importance of implementing sex-related genetics in sex-hormone based studies.

In the meantime, we can anticipate that personalized treatments will progressively become more relevant in clinical decision making, and therefore sex-related components need to be addressed accordingly. We highlight sex-specific features like hormones and genetic differences in relation to the TGF-β signaling pathway in pulmonary vascular diseases. These findings could implicate differential treatments based on sex, e.g. hormonal therapy like tamoxifen, raloxifene, anastrozole or DHEA-S, of which the latter two clinical trials are discussed in this review (chapter 4.5). These trials are eligible for all sexes although, dependent on the study outcomes, sex driven differential treatments should not be overlooked. Adverse effects of hormone therapies might be overcome by development and clinical testing of next generation SERMs like LY2066948.[120,211] Although anti-estrogen (anastrozole) has been studied in a clinical setting for PAH, pre-clinical evidence shows that estrogen administration also ameliorates PAH outcome in a tissue specific manner, by targeting the right heart.[109] Estrogen therapy targeting the heart, as an organ-specific treatment, might therefore be a promising treatment option, especially in men showing less right ventricular adaptation.

Furthermore, pregnancy might become a key determinant of PAH diagnosis and treatment. As such, pregnancy has been associated with increased risk of PAH development in BMPR2 mutation carriers, as patients have been diagnosed with PAH after pregnancy[212] and disease

outcomes are more severe peri- and post-partum.[213] These observations can easily be linked to drastic hemodynamic changes during pregnancy[213], but the long-term effects of hormonal changes are often not considered. As such, estrogens and progestogens rise dramatically during pregnancy. As we have described above, this affects the TGF-β signaling pathway by direct inhibition of SMADs, classical transcriptional effects (e.g. BMPR2 down-regulation in the vasculature and up-regulation in the right heart) and non-canonical cross-talk. Hence, sex-hormonal changes during pregnancy might enhance TGF-β signaling dysregulation (by an additional drop of BMPR2 levels in the vasculature) and subsequent PAH development.

Overall, sex-specific differences in TGF-β signaling potentially explains sex-differences in PAH. Many aspects of sex-related crosstalk with TGF-β signaling within the cardiovascular system are incompletely understood and more research is therefore warranted. Sex dependent determinants are getting increasingly important for biomarker identification, drug development and therefore curing PAH.

Author contributions

MW and CB wrote the initial draft of the manuscript and performed the literature search. GSD, FdM and MJG critically revised the work. GSD supervised and coordinated the writing. MW finalized the manuscript. MJG and GSD provided funding. All authors have approved the manuscript for publication.

Acknowledgements

Our research is supported by the Netherlands Cardiovascular Research Initiative (the Dutch Heart Foundation, Dutch Federation of University Medical Centres, the Netherlands Organization for Health Research and Development, and the Royal Netherlands Academy of Sciences), PHAEDRA-IMPACT (DCVA) and DOLPHIN-GENESIS (CVON). GSD is also sponsored by Fundacio La Marato de TV3 (#202038) and the Spanish Ministry of Science through the Ramón y Cajal grant RYC2021-030866-I. All figures were created with biorender.com.

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