Sex affects BMPR-II signalling in pulmonary artery smooth muscle cells.

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**At a Glance Commentary:** Females develop pulmonary arterial hypertension (PAH) more frequently than males and decreased BMPR-II signalling is associated with the development of PAH. Our research shows that control pulmonary artery smooth muscle cells from non-PAH women demonstrate depressed BMPR-II signalling and these effects may predispose to proliferation and be mediated by estrogens. Our research suggests that differential BMPR-II signalling may account for the increased frequency of PAH observed in women.

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This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org
Abstract

Rationale: Major PAH Registries report a greater incidence of PAH in females; mutations in the BMP type II receptor (BMPR-II) occur in approximately 80% of patients with heritable pulmonary arterial hypertension (hPAH).

Objectives: We addressed the hypothesis that females may be predisposed to PAH due to normally reduced basal BMPR-II signalling in control pulmonary artery smooth muscle cells (hPASMCs).

Methods: We examined the BMPR-II signalling pathway in hPASMCs derived from men and women with no underlying cardiovascular disease ('non-PAH hPASMCs'). We also determined the development of PH in male and female mice deficient in Smad1.

Measurements and main results: PDGF, estrogen and serotonin induced proliferation only in non-PAH female hPASMCs. Female non-PAH hPASMCs exhibited reduced mRNA and protein expression of BMPR-II, the signalling intermediary Smad1, and the down-stream genes, inhibitors of DNA binding proteins, Id1 and Id3. Induction of phospho-Smad1/5/8 and Id protein by BMP4 was also reduced in female hPASMCs. BMP4 induced proliferation in female, but not male, hPASMCs. However, siRNA silencing of Smad1 invoked proliferative responses to BMP4 in male hPASMCs. In male hPASMCs, estrogen decreased mRNA and protein expression of Id genes. The estrogen metabolite 4-hydroxyestradiol decreased phospho-Smad1/5/8 and Id expression in female hPASMCs whilst increasing these in males commensurate with a decreased proliferative effect in male hPASMCs. Female Smad1+/- mice developed PH (reversed by ovariectomy).
**Conclusions:** We conclude that estrogen-driven suppression of BMPR-II signalling in non-PAH hPASMCs derived from women contributes to a pro-proliferative phenotype in hPASMCs that may predispose females to PAH. **247 words**
Introduction

Pulmonary arterial hypertension (PAH) is a devastating disease characterised by severe pulmonary arterial remodelling and occlusive pulmonary vascular lesions, leading to right ventricular failure. PASMCs normally exhibit low rates of proliferation, migration, and apoptosis to maintain a low resistance pulmonary circulation. However, alterations in key signalling pathways, including the BMPR-II pathway, can lead to abnormal cell growth and remodelling of the pulmonary vasculature. Heterozygous germ-line mutations in the gene (BMPR2) encoding the bone morphogenetic protein type II receptor (BMPR-II) occur in up to 80% of families with PAH, and are found in approximately 20% of patients with sporadic PAH (1). The majority of BMPR2 mutations cause haploinsufficiency and thus, reduced cell surface levels of BMPR-II (2).

BMPR-II belongs to the transforming growth factor \(^2\) (TGF\(^2\)) receptor superfamily (3). Signalling by BMPs requires the formation of heterodimeric complexes of the constitutively active type II receptor, BMPR-II and a corresponding type 1 receptor. After BMP ligand-induced heteromeric complex formation, the type II receptor kinase phosphorylates the type I receptor which in turn phosphorylates Smad1/5/8 (R-Smads) to propagate the signal into the cell. Phosphorylated Smad1/5/8 forms heteromeric complexes with Smad4 (Co-Smad) which then translocate to the nucleus and associate with other transcription factors to regulate the expression of numerous genes.

The inhibitor of DNA binding family of proteins (Id proteins) are major downstream mediators of BMP signalling. These proteins bind to the ubiquitously expressed E protein family members with high affinity and inhibit their binding to target DNA. Id1 and Id3 are major targets of BMP signalling in PASMCs and induction of both are
dependent on intact BMPR-II (4). In particular, Id3 regulates PASMC cell cycle (4), suggesting BMP signalling via Id genes in the regulation of PASMC proliferation, loss of which likely contributes to the abnormal growth of vascular cells in PAH. Furthermore, reduced phospho-Smad1 levels and Id gene levels have been observed in lung tissues from patients with idiopathic and heritable PAH (5) and it was recently shown that mice with targeted deletion of Smad1 in the pulmonary endothelium develop PAH (6).

Major PAH Registries report a greater incidence of PAH in females (7, 8). Indeed, altered estrogen metabolism has been implicated in the increased penetrance in female PAH patients harbouring a BMPR-II mutation (8-10). In addition, estrogen has been shown to decrease BMPR-II signalling via the estrogen receptor 1 (11). Aromatase (CYP19A1), a member of the cytochrome P450 superfamily, synthesizes estrogens through the aromatization of androgens. We recently demonstrated that isolated hPASMCs and the medial layer of intact pulmonary arteries express aromatase and that expression was greatest in female hPASMCs. Indeed inhibition of aromatase protects female mice and rats from developing experimental PH by restoring BMPR-II signalling (12). These observations confirm that there are sex differences in cell-cycle regulation (e.g. (13)) and we hypothesised that fundamental differences exist in the activity of BMP signalling pathways in male and female non-PAH hPASMCs that may favour proliferation in female PASMCs and predispose women to PAH. Some of the results of these studies have been previously reported in the form of an abstract (14, 15).
Methods

An expanded methods section is available in the online supplement.

non-PAH hPASMCs

The peripheral human non-PAH PASMCs were isolated by microdissection from peripheral segments of artery (0.3 to 1.0mm external diameter) from macroscopically normal tissue removed from control patients undergoing pneumonectomy with no reported presence of PAH, as previously described (5). Proliferation studies were carried out as previously described (5). See online supplement for details.

siRNA transfection in pulmonary artery smooth muscle cells

Synthetic small interference RNA (siRNA) targeting human BMPRII, SMAD1 and Id3 (20nmol/L, SCBT) were used for targeted gene knockdown in male hPASMCs pulmonary as described previously (4). See online supplement for details.

Quantitative Reverse Transcription–Polymerase Chain Reaction

At necropsy, lung tissue from each rodent was removed and snap frozen. mRNA expression in lung and hPASMCs was assessed by quantitative reverse transcription–polymerase chain (RT-PCR) reaction as described previously and in online supplement (16).

Immunoblotting

Protein expression was assessed in mouse whole lung and hPASMCs as described previously and in online supplement (16).
Smad1+/- mice.
The Smad1 conditional knockout mouse were generated using the Cre-loxP system (17). Age and sex matched wildtype littermates were studied as controls. Methods for isolating PASMCs from Smad1+/- mice are described in the online supplement.

Hemodynamic Measurements
Heart rate, right ventricular systolic pressure (RVSP) and systemic arterial pressure were measured and analyzed as previously described (16, 18) and in online supplement.

Right Ventricular Hypertrophy
Right ventricular hypertrophy (RVH) in mice was assessed by weight measurement of the right ventricular free wall and left ventricle plus septum. The ratio expressed is $\text{RV/LV+S}$.

Lung Histopathology
3¼m lung sagittal sections were stained with ±-smooth-muscle actin (<80 µm external diameter) and microscopically assessed for degree of muscularisation in a blinded fashion, as previously described (19) and in online supplement.

Bilateral ovariectomy
To investigate the role of ovarian hormones in the development of PH, we ovariectomized wildtype (WT) and Smad1+/- mice at 8-10 weeks of age as previously described (20) and in online supplement.
Measurement of 17β-estradiol concentrations.

Circulating 17β-estradiol levels were quantified in mouse plasma samples by ELISA (Estradiol ELISA, Life Technologies, UK).

Statistics

All data are expressed as mean±SEM. Data were analysed using one-way ANOVA with Bonferroni post-hoc analyses and Student's unpaired t-test (as indicated in figure legends) to determine significance of differences. A P value of less than 0.05 was considered statistically significant.
Results

Proliferation of male vs female hPASMCs
Female non-PAH hPASMCs proliferated consistently to serotonin (1µM), PDGF (5ng/ml) and BMP4 (10ng/ml). In contrast, male cells exhibited no significant proliferation to these mitogens (Figure 1A).

BMPR-II signalling in male vs female hPASMCs
BMPR-II, Id1 and Id3 mRNA transcripts were significantly decreased in female hPASMCs compared to male (Figure 1B). Protein levels of BMPR-II, pSmad1/5/8, Id1 and Id3 were also lower in female hPASMCs compared to male (Figure 1C and 1D), (we confirm these observations in later figures using different cohorts of cells (see Figures 6 and 8). Stimulation with BMP4 resulted in a significantly lower induction of pSmad1/5/8 and Id3 in female hPASMCs than male hPASMCs (Figure 2A and B).

Effect of the BMPR-II/Id axis on proliferation in male PASMCs
To examine further the role of the BMPR-II/Id axis on proliferation to BMP4, we silenced BMPR-II, SMAD1 and Id3 (as it regulates PASMC cell cycle (4)) using siRNA (Figure 3A-C). Whilst the siRNA control, BMPR-II and Id3 had no effect, silencing of Smad1 enabled male hPASMCs to proliferate to BMP4 (Figure 3D).

Female Smad1+/- mice spontaneously develop PH.
We examined gender differences in the development of spontaneous PH in Smad1+/- mice at 5-6 months as we have previously shown that PH in transgenic mice can be age-related and the PH phenotype can take 5-6 months to emerge (21).
Female Smad1 +/- mice developed PH whereas the male mice did not (Figure 4). Female Smad1 +/- mice display significantly elevated RVSP and pulmonary vascular remodelling compared to males (Figure 4A-C). No significant difference in RV hypertrophy or systemic blood pressure were observed between the groups (Figure 4D and 4E). PASMCs isolated from female Smad1+/- mice proliferated faster than those of female WT mice (Figure 4F).

To define the role of female sex hormones in the susceptibility of females Smad1+/- mice, mice were subjected to sham or ovariectomy surgery. Lack of female sex hormones was confirmed by the decreased uterine weight (Figure E1). Ovariectomy attenuated the elevated RVSP observed in female Smad1+/- mice (Figure 5A). Ovariectomy itself slightly increased RVSP and RVH in wild type mice (Figure 5A and B). Ovariectomy also attenuated the increase in pulmonary vascular remodelling observed in the Smad1+/- mice (Figure 5C and D).

**The BMPR-II signalling axis is downregulated in female mouse lung.**

Smad1 mRNA levels were significantly reduced in both male and female Smad1+/- mice compared to wildtype animals (Figure E2A). Female wildtype mice display significantly lower basal levels of Smad1 mRNA in lungs than males and Smad1 mRNA expression was lower in female Smad1+/- mice than male Smad1+/- mice (Figure E2A). Further investigation revealed that the mRNA levels of BMPR-II and Id3 were also significantly lower in female mice than males (Figure E2B and C). Plasma estrogen E2 levels were not affected by Smad1 deficiency (Figure E2D). There was an equal expression of aromatase and CYP1B1 mRNA and protein in the
lungs of both Smad1+/− mice and wildtype mice (Figure E2E-G). Liver and kidney tissues from these animals were also assessed and no gender differences in BMPR-II, Smad1, Id1 or Id3 mRNA were observed in these tissues (Figure E3).

**Effect of estrogen on BMPRII/Smad/Id axis in hPASMCs**

Stimulation of hPASMCs with estrogen had no effect on BMPR-II mRNA or pSmad1/5/8 protein expression in male or female PASMCs (Figure 6A-C). Estrogen did however significantly down-regulate both Id1 and Id3 transcript and protein levels in male PASMCs (Figure 6D-H). pERK2 expression (not pERK1) was elevated in female hPASMCs compared to in male hPASMCs (Figure E4A, B). We investigated the expression of CYP1B1 as well as catechol-O-methyl transferase (COMT), the enzyme that metabolises 4- and 2OHE metabolites to methoxy-metabolites. Expression levels of these enzymes were equal in male and female hPASMCs (Figure E4B).

**Effect of estrogen metabolites on BMPRII/Smad/Id axis in hPASMCs**

Estrogen induced proliferation of hPASMCs (Figure 7A). The proliferative response to estrogen was greatest in female hPASMCs and inhibited by the estrogen receptor alpha (ERα) antagonist MPP (Figure 7A). ERβ and GPER antagonists have no effect on estrogen-induced proliferation (data not shown). 4OHE2 is a major product of CYP1B1 and its effects on hPASMCs have not previously been reported. 4OHE2 did not cause proliferation in female hPASMCs but caused a decrease in proliferation in male cells (Figure 7B). To investigate these differences further we examined the influence of 4OHE2 on BMPR-II signalling. In line with its differential effect on proliferation in male and female hPASMCs, it had a differential effect on BMPR-II
signalling in male and female hPASMCs. In males, consistent with its anti-proliferative effect, it induced an increase in the expression of pSmad1/5/8, Id1 and Id3 whilst in female hPASMCs it reduced these (Figure 8 A-E).
Discussion

In hPAH families, penetrance of PAH in BMPR-II mutation carriers is low (20-30%), suggesting other risk factors must influence the emergence of the PAH phenotype. Of all known risk factors, the risk of developing PAH in families with BMPR-II mutations is affected most by sex with the occurrence of PAH being approximately 3-fold higher in females than males (22). Polymorphisms in the aromatase gene associated with increased estradiol production have also been associated with increased risk of portopulmonary hypertension in patients with liver disease (23). We also recently provided evidence that endogenous estrogen may be a causative factor in PAH and may suppress endogenous BMPR-II signaling axis (12). Thus, in the present study we addressed the ensuing hypothesis that, even before being exposed to any PAH risk factor, women may be predisposed to PAH due to reduced basal BMPR-II signalling in their hPASMCs.

Serotonin, PDGF and BMP4 are all ERK-dependent mitogens in the pulmonary circulation (24-26) and, under the conditions used, all could induce proliferation in female hPASMCs but not male cells. Loss of BMPR-II can reduce the anti-proliferative influence of Smad signalling and can allow unopposed pro-proliferative ERK1/2 signalling (27). Hence we interrogated gender differences in the BMPR-II signalling pathway and pERK expression. Compared with male cells, cohorts of non-PAH female hPASMCs expressed significantly reduced levels of BMPR-II, Id1 and Id3 mRNA levels as well as significantly lower protein levels of BMPR-II, pSmad1/5/8, Id1 and Id3. pERK2 expression was, however, higher in female hPASMCs compared to male hPASMCs. As pERK2 has a positive role in controlling cell proliferation (28, 29) this observation is consistent with the proliferative phenotype of female hPASMCs. This is also consistent with previous findings
showing that BMP4-induced Id expression is negatively regulated by ERK1/2 activation (27). Given the anti-proliferative nature of BMPR-II mediated Smad signaling in hPASMCs (30), the reduced expression of this axis may predispose female PASMCs to a pro-proliferative effects of persistent pERK signaling. Indeed, BMPR-II signaling can prevent PDGF-induced proliferation of human hPASMCs (25). Hence the reduced BMPR-II/Smad/Id gene signaling in female hPASMCs may contribute to the increased proliferation of female cells in response to mitogens. Stimulation of female hPASMCs with BMP4, resulted in a markedly reduced activation of pSmad1/5/8 and Id3 compared to male. Furthermore, in female hPASMCs, the addition of BMP4 results in proliferation, whilst no proliferation was observed in male hPASMCs. We knocked down BMPR-II, Smad1 and Id3 in male hPAMSCs to investigate if this could induce a more proliferative ‘female’ phenotype. Knockdown of Smad1 alone reversed the non-proliferative phenotype in the male cells. This is consistent with differential regulation of Smad1. Indeed, it has recently been reported that Smad1 can be differentially regulated by modifiers including BMPR-II itself (31). These finding suggest that the reduction in Smad1 results in an altered phenotype in male cells, inducing a pro-proliferative phenotype similar to that observed in female cells. These results may suggest that increasing BMPR-II signaling in female PASMCs may reduce a proliferative phenotype. Consistent with this we show here that proliferation is reduced in PASMCs derived from female Smad +/+ mice compared to PASMCs with reduced Smad1 derived from Smad -/+ mice. In vivo, we have shown that the BMPR-II signalling pathway is reduced in the lungs of mice pulmonary hypertension (PH) but that decreasing endogenous estrogen synthesis restores this signalling pathway and reverses PH (32). We have also previously shown that that rescue of BMPR-II signalling with
ataluren reduces the hyperproliferative phenotype of pulmonary artery endothelial and smooth muscle cells derived from PAH patients (33). Only female Smad1+/- mice demonstrated elevated RVSP and increased pulmonary vascular remodelling. We have previously studied normoxic female mice that develop PH (mice over-expressing the serotonin transporter (SERT+ mice), over-expressing mts1 (S100A4) or dosed with dexfenfluramine) and have shown that such normoxic mice do not develop RVH despite elevated RSVP and pulmonary vascular remodelling (20, 34-36). This suggests that under normoxic conditions, the mouse right ventricle is resistant to hypertrophy in the face of moderate RVSP elevation. Consistent with this, the female Smad1+/- mice did not develop RVH. The levels of Smad1 mRNA in both male and female Smad1+/- mice were reduced by 50% compared to their WT counterparts. Neither BMPR-II nor Ild3 mRNA expression was affected by the reduction of Smad1. However, in both wildtype and Smad1+/- mice, BMPR-II, Smad1 and Ild3 expression were all expressed at lower levels in the female lung than the male lung. This down-regulation of the BMPR-II axis was confined to the lung as no significant gender differences were observed in liver or kidney. Smad1 expression was most markedly reduced in the female Smad1+/- mice. We suggest that in female Smad1+/- mice, Smad1 expression in the lung has decreased below a threshold required for the normal functioning of the BMPR-II pathway and thus predisposing PASMCs from female Smad1+/- to a proliferative phenotype resulting in the onset of PH in these animals. Consistent with this, PASMCs from female Smad1+/- mice proliferated significantly more than PASMCs from female WT mice.

In the female Smad1+/- mice we demonstrate that the PH phenotype (RVSP and vascular remodelling) is markedly reduced following ovariectomy. There was an
increase in RVSP, RVH and vascular remodelling in ovariectomised female wildtype mice. This does suggest that in absence of other risk factors, sex hormones can be protective against vascular and ventricular changes consistent with previous studies. Indeed, estrogen has recently been shown to be protective against hypoxic-induced PAH in male rats (37) and progesterone can protect against monocrotaline-induced PAH (38).

Serotonin has been implicated in the PH observed in the SERT+ mouse (39), hypoxic rodents (39-41), the sugen/hypoxic rodent (42) and in dexamfluramine-treated female mice (18). In these models, estrogen plays a causative role in the development of PH. For example, in SERT+ mice the major circulating estrogen 17β-estradiol can restore the PH phenotype in ovariectomized SERT+ mice (36). 17β-estradiol can also induce proliferation of hPASMCs and may therefore contribute to the pulmonary artery remodelling observed in PAH (16, 36). In addition, endogenous estrogen plays a key role in the development of PH in female hypoxic mice and in the female sugen/hypoxic rat (12). We have also previously demonstrated that CYP1B1 can influence the development of PH in animal models (16, 20). Plasma E2 levels were unaffected by Smad1 deficiency in the Smad1 +/- mice. However we confirmed that there is aromatase and CYP1B1 expression in the lungs of the mice used in this study and this is unaffected by Smad1 knockdown, allowing the possibility that intact local estrogen synthesis and metabolism to active metabolites may influence BMPR-II signalling in the lungs of these mice. 17²-estradiol induced proliferation of female, but not male hPASMCs, in an ERα-dependant fashion. Consistent with this, we have previously demonstrated that the ERα-antagonist MPP can reverse hypoxia-induced PH in female but not male mice and this therapeutic effect is associated with an increase in BMPR-II and Id1
expression (12). Stimulation of hPASMCs with 17²-estradiol had no effect on BMPR-II or pSmad1/5/8 expression in males or females. In male cells, 17²-estradiol did however significantly reduce the levels of Id1 and Id3 at both the mRNA and protein level, whilst having no effect on female hPASMCs. These findings suggest that estrogen down-regulates Id1 and Id3 expression. The lack of effect in female cells may be because Id1 and Id3 levels are already significantly reduced in these cells. Whilst there was no effect of acute administration of 17²-estradiol on BMPR-II and pSmad1/5/8 expression, there was decreased expression of these proteins in female hPASMCs. This may be via epigenetic silencing as it has been shown that SMAD1 can be epigenetically silenced through aberrant DNA methylation and such an effect could be mediated by via estrogen (43, 44). The average age of the patients from whom the cells were derived was 65 for the male cells and 61 for the female cells. Hence these were age-matched. However, as the average age of menopause in the UK is 51 years, this means that all the women were post-menopausal. In a recent study however, we examined the expression of aromatase in hPASMCs from men and women of a similar average age as those studied here. We demonstrated that cells derived from post-menopausal women expressed twelve-fold higher levels of aromatase than PASMCs derived from men of a similar age (32). This suggests that female hPASMCs can synthesise more estrogen than male cells. Therefore, we believe that endogenous local estrogen production influences BMPR-II signalling. Consistent with this, we have previously shown in mouse whole lung that inhibition of estrogen production by anastrozole can significantly elevate Id1 and Id3 levels in females but not males indicating that in an in vivo setting local vascular estrogen may be responsible for the reduced levels of Id1 and Id3 observed in females (12).
We have previously shown in PH models that increased CYP1B1-mediated estrogen metabolism may lead to the formation of mitogens, including 16±-hydroxyestrone (16). Sex did not influence CYP1B1 or COMT expression suggesting that all hPASMCs have the potential to metabolise estrogen to 4- and 2-OHE metabolites. We examined 4OHE2 further as this metabolite is one of the major products of CYP1B1 activity and has been shown to be mutagenic in breast epithelial cells and pro-proliferative in human ovarian cancer cells (45, 46). Whilst 4OHE2 had no proliferative effect in female hPASMCs, it inhibited proliferation in male hPASMCs. We were interested to determine if these differential effects were due to changes in BMPR-II signalling. We present the novel observation that 4OHE2 increased expression of pSmad 1/5/8, Id1 and Id3 in male hPASMCs but decreases the expression of pSmad 1/5/8, Id1 and Id3 further in female hPASMCs. The results suggest that locally derived estrogen metabolites such as 4OHE2 can exert differential effects on BMPR-II signalling and cell proliferation which may contribute to gender differences in proliferation.

One of the limitations of this study is the availability of age matched lysates from non-PAH hPASMCs from men and women with no cardiovascular disease. However we have countered these limitations by repeating experiments in each lysate from 2-3 times. In addition, our observation that BMPR-II signalling is depressed in female hPASMCs was reproducible across separate experiments depicted in Figures 1, 6 and 8. In addition the in vivo studies confirm the observation across species (Figure E2).

We conclude that female hPASMCs have decreased BMPR-II signalling compared with male hPASMCs and that estrogenic-driven suppression of BMPR-II signalling
may contribute to a pro-proliferative phenotype in female hPASMCs predisposing women to PAH.

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Figure 1

Proliferation and BMPR-II signaling in male (M) and female (F) non-PAH hPASMCs. (A) Female cells were more proliferative than male cells in response to three days of administration of serotonin (5HT), PDGF and BMP4 (B4) compared to 1% FBS control. (B) Female cells had significantly lower basal levels of BMPR-II (BRII), Id1 and Id3 mRNA after 48hr in 0.1% FBS. (C) Representative immunoblots. (D) Female hPASMCs express significantly lower basal levels of BRII, phospho-Smad 1/5/8 (p-S1/5/8), Id1 and Id3 protein. *p<0.05, **p<0.01 vs male (n=4-7, repeated 2-3 times per isolate). Student's unpaired t-test with two-tailed distribution.

E2/0.1µM; 5-HT/1µM; 4-OHE2/1µM; PDGF/5ng/ml, BMP4/10ng/ml) for 3 days unless stated otherwise.
Figure 2

Effect of BMP4 (stimulation on BMPR-II signaling in male (M) and female (F) non-PAH hPASMCs. Western blot analysis demonstrates that female PASMCs had significantly lower BMP4-stimulated protein levels of p-smad1/5/8 (p-S1/5/8) and Id3 after 2 hour treatment with BMP4 (10ng/ml) following 48 hours growth in 0.1% FBS. (A) Representative Western blot. (B) Expression levels quantified by densitometry. *p<0.05, **p<0.01 vs male (n=6 (M) and n=7 (F), all isolates repeated 2-3 times). Student’s unpaired t-test with two-tailed distribution.
Figure 3

Proliferation in male non-PAH hPASMCs following knockdown of BMPR-II signaling. (A-C) Knockdown of BMPR-II (siBRII), Smad1 (siSmad1) and Id3 (siId3) by siRNA in male hPASMCs. (D) Male hPASMCs do not normally proliferate to BMP4 (B4) however proliferation to B4 (open bars) was restored following knockdown of Smad1 (n=3). *p<0.05, **p<0.01, Student’s unpaired t-test with two-tailed distribution.
Figure 4
Development of pulmonary hypertension in Smad1 heterozygous knockout mice (+/-) compared to wildtype controls (+/+). (+/-) mice developed PAH after 6 months of age. (A) Right ventricular systolic pressure is elevated in female (+/-) mice. n=6-7. (B) Small pulmonary vessels were assessed for degrees of circumferential ±-smooth muscle actin (±-SMA)-positive staining, indicative of muscularization. Vessels were classified as non-muscular (no ±-SMA-positive immunoreactivity), partially muscular or fully muscularized. There was a decrease in non-muscular vessels and an increase in muscularized vessels in the female +/- mice. n=3-4. (C) Resistance pulmonary arterial wall thickness (as measured by a-SMA (dark brown) staining) was increased in female +/- mice. Scale bar (-) indicates 50µm. (D, E) There were no differences in the right ventricular hypertrophy (RV/LV+S) (+/+, n=6-8; +/-, n=5) and mean systemic arterial pressure (mSAP) in +/- vs +/- mice (n=6). (F) PASMCs from female +/- mice were more proliferative than cells from female +/-+ mice (n=3-4). *p<0.05, **p<0.01, ***p<0.001 vs +/-, one-way ANOVA with Bonferroni post-hoc analyses.
Figure 5
Effect of ovariectomy (OVX) or sham operation (sham) on development of pulmonary hypertension in Smad1 heterozygous knockout mice (+/-) compared to wildtype controls (+/+). (A) Effect of OVX on right ventricular systolic pressure (RVSP). (B) Effect of OVX on right ventricular hypertrophy (RV/LV+S) (n=6-8). (C,D) Small pulmonary vessels were assessed for degrees of circumferential ±-smooth muscle actin (±-SMA)-positive (dark brown) staining, indicative of muscularization. Scale bar (-) indicates 50µm. Vessels were classified as non-muscular (no ±-SMA-positive immunoreactivity), partially muscular or fully muscularized. OVX attenuated the increase in the number of muscularised pulmonary arteries observed in the +/- mice (n=3-4). *p<0.05, **p<0.01, ***p<0.001 vs +/-, #p<0.05 , ###p<0.001 vs +/- intact, one-way ANOVA with Bonferroni post-hoc analyses.
Figure 6

Effects of 17β-estradiol (E₂, 1µM) on BMPR-II signaling in male and female non-PAH hPASMCs. (A) E₂ had no effect on BMPR2 (BRII) mRNA expression (n=4). (B) Representative Western blot for p-smad1/5/8 (p-S1/5/8). (C) E₂ had no effect on p-smad1/5/8 protein expression (n=3). (D, E) In male cells, E₂ significantly reduced the levels of Id1 and Id3 mRNA (n=4). (F-H) In male cells, E₂ significantly reduced the levels of Id1 and Id3 protein resulting in expression levels similar to those observed in female (n=3). Expression levels were quantified by densitometry. *p<0.05, **p<0.01 vs vehicle control (C) (n=4). All isolates were repeated 2-3 times.
Figure 7

Proliferation to 17β-estradiol (E₂, 0.1µM) in non-PAH hPASMCs. (A) E2 induced more proliferation in female hPASMCs than male cells (n=4) and E2-induced proliferation was abolished by the ERα-antagonist MPP. ** p<0.01 vs 1% FBS/male. (B) 4OHE₂ (0.1mM) decreased proliferation male hPASMCs only (n=4, all isolates repeated 2-3 times). *p<0.05, Student’s unpaired t-test with two-tailed distribution.
Figure 8
Differential effect of 4OHE2 in male and female non-PAH hPASMCs. (A) Representative Western blots for phospho-smad1/5/8 (p-S1/5/8, Id1 and Id3 in male and female hPASMCs. P-S1/5/8, Id1 and Id3 expression is less in female hPASMCs than male hPASMCs and 4OHE2 increases expression of these in male hPASMCs whilst reducing expression in female hPASMCs. Densitometric analysis of protein for p-S1/5/8 (B), total-Smad1 (t-Smad1) (C), Id1 (D) and Id3 (E) (n=4, all isolates repeated 2-3 times). The expression levels were quantified by densitometry. # p<0.05, ## p<0.01 vs baseline (0); *p<0.05, **p<0.01 vs Male. Student’s unpaired t-test with two-tailed distribution.
A

Male hPASM C (+ BMP4 (10 ng/ml, 2 hours))

p-S1/5/8/60kDa

Id1/20kDa

Id3/17kDa

β-Actin/42kDa

Female hPASM C (+ BMP4 (10 ng/ml, 2 hours))

B

<table>
<thead>
<tr>
<th>Protein</th>
<th>M</th>
<th>F</th>
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<tr>
<td>p-S1/5/8</td>
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<td>60</td>
</tr>
<tr>
<td>Id1</td>
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<td>40</td>
</tr>
<tr>
<td>Id3</td>
<td>80</td>
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</tbody>
</table>

Denstomeric measurement

+ BMP4 (10 ng/ml, 2 hours)