

# The serotonin transporter, gender, and 17 $\beta$ oestradiol in the development of pulmonary arterial hypertension

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## Aims

Idiopathic and familial forms of pulmonary arterial hypertension (PAH) predominantly affect females through an unknown mechanism. Activity of the serotonin transporter (SERT) may modulate the development of PAH, and mice overexpressing SERT (SERT+ mice) develop PAH and severe hypoxia-induced PAH. In the central nervous system, oestrogens influence activity of the serotonin system. Therefore, we examined the influence of gender on the development of PAH in SERT+ mice and how this is modulated by female hormones.

## Methods and results

PAH was assessed via measurement of right ventricular systolic pressure (RVSP), pulmonary vascular remodelling (PVR), and right ventricular hypertrophy. Male SERT+ mice did not develop PAH. Female SERT+ mice demonstrated increased RVSP and PVR and this was abolished by ovariectomy. Following exposure to hypoxia, SERT+ mice exhibited severe PAH and this was also attenuated by ovariectomy. Chronic administration of 17 $\beta$  oestradiol re-established the PAH phenotype in ovariectomized, normoxic, and hypoxic SERT+ mice. 17 $\beta$  oestradiol also up-regulated tryptophan hydroxylase-1 (TPH1), 5-hydroxytryptamine<sub>1B</sub> (5-HT<sub>1B</sub>) receptor, and SERT expression in human pulmonary arterial smooth muscle cells (hPASCs). 17 $\beta$  oestradiol stimulated hPASC proliferation and this was inhibited by both the TPH inhibitor para-chlorophenylalanine and the 5-HT<sub>1B</sub> receptor antagonist SB224289.

## Conclusion

17 $\beta$  oestradiol is critical to the development of PAH and severe hypoxia-induced PAH in female SERT+ mice. In hPASCs, 17 $\beta$  oestradiol-induced proliferation is dependant on *de novo* serotonin synthesis and stimulation of the 5-HT<sub>1B</sub> receptor. These interactions between the serotonin system and 17 $\beta$  oestradiol may contribute to the increased risk of PAH associated with female gender.

## Keywords

17 $\beta$  oestradiol • Oestrogen • Gender • Pulmonary arterial hypertension • Serotonin

## 1. Introduction

Pulmonary arterial hypertension (PAH) is characterized by both remodelling and vasoconstriction of the pulmonary vasculature. Mutations in the gene encoding for the bone morphogenetic protein receptor-2 (BMPRII) are accountable for ~80% of familial PAH cases; however, penetrance for this gene is incomplete as only 20% of BMPRII mutation carriers develop PAH.<sup>1</sup> Therefore, it is assumed that other genetic or environmental risk factors are involved.

In both idiopathic and familial forms of PAH, there is a gender bias, with females up to three-fold more likely to present with disease,<sup>2–4</sup> although the reasons for this disparity remain unknown. Oestrogens

are one possible risk factor in PAH. The ingestion of oral contraceptives has previously been associated with PAH<sup>5,6</sup> and female PAH patients show increased expression levels of Estrogen receptor 1 (ESR1), the gene encoding for oestrogen receptor alpha, compared with unaffected females.<sup>7</sup> Decreased expression of the oestrogen-metabolizing enzyme cytochrome P450 1B1 (CYP1B1) leading to altered oestrogen metabolism has also been identified in female PAH patients harbouring a BMPRII mutation compared with unaffected female carriers.<sup>8</sup>

In contrast, experimental models of PAH have repeatedly shown that female rodents exhibit less severe PAH compared with males. For example, female rats exposed to chronic hypoxia develop moderate PAH compared with severe PAH in males.<sup>9</sup> Ovariectomized rats

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exhibit severe PAH following hypoxic insult and this can be attenuated with  $17\beta$  oestradiol treatment.<sup>10</sup> In addition, male apolipoprotein E knockout mice (ApoE<sup>-/-</sup> mice) prescribed a high-fat diet develop a more established PAH phenotype compared against high-fat treated ApoE<sup>-/-</sup> females.<sup>11</sup> This absence of a suitable animal model which replicates the female bias observed in human PAH has limited experimental research to date.

Multiple studies have implicated serotonin, the serotonin transporter (SERT), and 5-hydroxytryptamine<sub>1B</sub> (5-HT<sub>1B</sub>) receptors in the pathobiology of PAH. In mice, the development of hypoxia-induced PAH and dexfenfluramine-induced PAH are dependent on peripheral serotonin synthesis.<sup>12,13</sup> SERT expression is increased in human pulmonary arterial smooth muscle cells (hPASCs) derived from idiopathic PAH (IPAH) patients and this is responsible for increased serotonin-induced proliferation in these cells.<sup>14</sup> The 5-HT<sub>1B</sub> receptor mediates human pulmonary arterial vasoconstriction<sup>15</sup> and is also involved in hPASC proliferation.<sup>16</sup>

In the central nervous system, oestrogens regulate expression of multiple serotonin pathway mediators, including tryptophan hydroxylase<sup>17</sup> (TPH; the rate-limiting enzyme in serotonin synthesis) and SERT.<sup>18</sup> We have previously shown that mice overexpressing the SERT (SERT+ mice) develop PAH and severe hypoxia-induced PAH.<sup>19</sup> Here, we investigated the possible interactions between serotonin and oestrogens *in vitro* in hPASCs, and the effects of gender and  $17\beta$  oestradiol *in vivo* on the development of PAH in SERT+ mice.

## 2. Methods

### 2.1 SERT+ mice

The generation of SERT+ mice (background strain: C57Bl/6JxCBA) has been previously described.<sup>19</sup> Age-matched littermates were studied as controls. All experimental procedures conform with the United Kingdom Animal Procedures Act (1986) and with the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), and ethical approval was also granted by the University Ethics Committee.

### 2.2 Bilateral ovariectomy

To investigate the role of ovarian hormones in the development of PAH, we ovariectomized wildtype (WT) and SERT+ mice at 8–10 weeks of age. Bilateral ovariectomy was performed under inhalational anaesthesia (1.5% isoflurane supplemented with O<sub>2</sub>). A dorsal midline skin incision was performed and the ovaries located and removed by cauterization through the distal uterine tube. Successful removal of the ovaries was confirmed at necropsy by weight measurement of the uterus. *In vivo* measurements were assessed following 12 weeks surgical recovery (5 months of age). Sham-operated mice were studied as controls.

### 2.3 $17\beta$ oestradiol administration

Nine weeks following ovariectomy,  $17\beta$  oestradiol-containing pellets (0.1 mg/21 day pellet, Innovative Research of America, USA) or vehicle pellets were subcutaneously implanted into the dorsal neck and the assessment of PAH was performed after 21 days. Male mice administered  $17\beta$  oestradiol pellets were subject to implant 21 days immediately prior to the assessment of PAH, which was carried out at 5 months of age. The selected dose of  $17\beta$  oestradiol has been previously shown to produce physiologically relevant (<1 nmol/L) concentrations of circulating  $17\beta$  oestradiol.<sup>20</sup>

### 2.4 Haemodynamic measurements

The development of PAH in male and female SERT+ mice was investigated in both normoxia and following 14 days of hypobaric hypoxia. Right ventricular (RV) pressure and systemic arterial pressure measurements were measured and analysed as previously described.<sup>21</sup> Age-matched littermate C57Bl/6JxCBA mice were studied as controls. Haemodynamic measurements from six to nine mice for each group were assessed.

### 2.5 Lung histology

Sagittal sections of lung were elastica-Van Gieson stained and microscopically assessed for the muscularization of pulmonary arteries (<80  $\mu$ m external diameter) in a blinded fashion as previously described.<sup>21</sup> Remodelled arteries were confirmed by the presence of a double elastic laminae. Lung sections from four mice for each group were studied. Approximately 150 arteries from each lung section were assessed.

### 2.6 Right ventricular hypertrophy

Right ventricular hypertrophy (RVH) was assessed by weight measurement of the RV free wall and left ventricle plus septum (LV+S). The ratio expressed is RV/LV+S.

### 2.7 hPASC proliferation

#### 2.7.1 Cell counts

Experimental procedures using hPASCs conform with the principles outlined in the Declaration of Helsinki, and was also approved by the University Ethics review board (approval reference number: 08/H0304/56). As we established an important role for  $17\beta$  oestradiol in the development of PAH *in vivo*, we further examined its mechanisms of action *in vitro*. With relevance to human PAH, we investigated the effects of  $17\beta$  oestradiol on hPASCs. hPASCs (passage 3–7) were seeded in 24-well plates at a density of 20 000 per/well and grown to 60% confluency [DMEM supplemented with 10% foetal bovine serum (FBS)] before quiescence in 0.2% FBS for 24 h as described previously.<sup>16</sup> hPASCs were exposed to a range of concentrations (0.1–1 nmol/L) of  $17\beta$  oestradiol, oestrone, oestriol (Sigma, UK), or progesterone (Tocris, UK) in the presence of 2.5% FBS and proliferation assessed at 4–5 days. All antagonists were exposed to hPASCs for at least 30 min prior to the addition of  $17\beta$  oestradiol. DMEM and drugs were replaced every 48 h. Cell counts were performed in a blinded fashion using a haemocytometer ( $n = 3$  for each experiment and performed in duplicate). The smooth muscle phenotype of the hPASC was confirmed at passages 3–7 by staining for  $\alpha$ -smooth muscle actin.

#### 2.7.2 [<sup>3</sup>H]Thymidine incorporation

To measure DNA synthesis, hPASCs (passage 3–7) were seeded in 24-well plates at a density of 20 000 per/well and grown to 60% confluency (DMEM supplemented with 10% FBS) before quiescence in 0.2% FBS for 24 h. Cells were then exposed to range of concentrations (0.1–1 nmol/L) of  $17\beta$  oestradiol, oestrone, oestriol (Sigma, UK), or progesterone (Tocris, UK) in the presence of 2.5% FBS and proliferation assessed at 4 days. Where appropriate, antagonists were exposed to hPASCs for at least 30 min prior to the addition of  $17\beta$  oestradiol. DMEM and drugs were replaced every 48 h. Twenty four hours prior to the end of each experiment, 0.2  $\mu$ Ci/well [<sup>3</sup>H]thymidine was added to each well. hPASCs were then rinsed with phospho-buffered saline and 5% trichloroacetic acid, then lysed with 0.3 mol/L NaOH. Radioactivity was measured using a liquid scintillation counter, and data expressed as % change compared with 2.5% FBS, as determined by [<sup>3</sup>H]thymidine incorporation.  $n = 3$  for each experiment and counts performed in duplicate.

### 2.7.3 Western blot analysis

hPASCs (passage 3–7) were seeded in 6-well plates at a density of 20 000 per/well and grown to 80% confluency (DMEM supplemented with 10% FBS) before serum starvation for 24 h. Following exposure to 1 nmol/L 17 $\beta$  oestradiol at multiple time points, hPASC lysates were prepared for immunoblot as previously described.<sup>13</sup> Immunoblotting was performed with antibodies against TPH1 (1:200; Chemicon, UK), the 5-HT<sub>1B</sub> receptor (1:200; Abcam, UK) and SERT (1:200; Abcam, UK). Densitometrical analysis was performed using TotalLab TL100 software.  $n = 4$  for each experiment.

### 2.8 Statistical analysis

Data were analysed using a two-way ANOVA followed by Bonferroni's post hoc test, one-way ANOVA followed by Dunnett's post hoc test, or Student's *t*-test as appropriate. Data are expressed as mean  $\pm$  SEM.

## 3. Results

### 3.1 Effects of gender, SERT, ovariectomy, and 17 $\beta$ oestradiol on the development of PAH

In male mice, right ventricular systolic pressure (RVSP), pulmonary arterial remodelling, and RVH were all unaffected following SERT overexpression, this was apparent under both normoxic and hypoxic conditions (Figure 1A–C). In normoxic conditions, sham-operated female SERT+ mice demonstrated increased RVSP and pulmonary arterial remodelling (Figure 2A and B) with RVH being unaffected (Figure 2C). The increased RVSP and remodelling observed in female SERT+ mice was abolished by ovariectomy (Figure 2A and B). The administration of 17 $\beta$  oestradiol in ovariectomized SERT+ mice re-established increases in both RVSP and pulmonary arterial remodelling (Figure 2A and B). RVH was similar in both WT and SERT+ mice and ovariectomy had no further effect. However, ovariectomized SERT+ mice administered 17 $\beta$  oestradiol exhibited a decrease in RVH (Figure 2C). Following exposure to chronic hypoxia, female SERT+ mice developed marked increases in both RVSP and remodelling which were higher than their WT controls. This severe PAH phenotype was attenuated by ovariectomy and subsequently re-established following the administration of 17 $\beta$  oestradiol (Figure 2A and B). RVH was also greater in hypoxic female SERT+ mice and this was unaffected by ovariectomy, however, 17 $\beta$  oestradiol decreased RVH in the ovariectomized group (Figure 2C). There were no differences in mean systemic arterial pressure between any of the groups (data not shown). Comparing male and female differences in WT mice (Figure 1), we observed that hypoxia-induced increases in RVSP and pulmonary arterial remodelling were more severe in male WT mice than female WT mice ( $P < 0.001$ ). These gender differences were not apparent in SERT+ mice. We observed a marked decrease in uterine weight following ovariectomy, indicative of successful removal of the ovaries (Table 1). Furthermore, an increase in uterine weight was observed in ovariectomized female mice following 17 $\beta$  oestradiol administration, as a consequence of 17 $\beta$  oestradiol-mediated hypertrophy. Two additional controls were performed in this study. We examined the effects of ovariectomy in WT mice. Whilst this had no effect on RVH, it increased both RVSP from  $21.31 \pm 0.42$  to  $25.01 \pm 0.84$  mmHg ( $P < 0.01$ ;  $n = 8$ ) and pulmonary arterial remodelling from  $7.95 \pm 0.63$  to  $10.7 \pm 0.54\%$  ( $P < 0.01$ ;  $n = 4$ ). However, in hypoxia, the severity of PAH in WT mice was unaffected following

ovariectomy, as assessed by no changes in RVSP, pulmonary arterial remodelling, and RVH (data not shown). To further establish the role of 17 $\beta$  oestradiol in SERT+ mice, we investigated if its administration in males would uncover a PAH phenotype. Figure 3A–C demonstrates, however, that 17 $\beta$  oestradiol had no effect on RVSP, remodelling, or RVH in male SERT+ mice. We also assessed the effects of 17 $\beta$  oestradiol in male WT mice exposed to hypoxia. Hypoxia-induced elevations in RVSP (Figure 3D), pulmonary arterial remodelling (Figure 3E), and RVH (Figure 3F) were all decreased following 17 $\beta$  oestradiol administration.

### 3.2 Effects of ovarian hormones on hPASC proliferation

As we identified that 17 $\beta$  oestradiol is critical to the development of PAH in female SERT+ mice *in vivo*, we wished to determine possible mechanisms and also examine the relevance of this with respect to human PAH. Therefore, we examined the effects of 17 $\beta$  oestradiol on hPASC proliferation at concentrations of 0.1–1 nmol/L. For comparison, we also examined the effects of oestrone, oestriol, and progesterone on hPASC proliferation (Figure 4). At 1 nmol/L, 17 $\beta$  oestradiol stimulated hPASC proliferation, as assessed by an increase of both cell number and DNA synthesis (Figure 4A and B), whereas oestrone, oestriol, and progesterone had no effects on hPASC proliferation (Figure 4C–H). These proliferative effects of 17 $\beta$  oestradiol are consistent with our *in vivo* findings.

### 3.3 Effects of 17 $\beta$ oestradiol on TPH1, 5-HT<sub>1B</sub> receptor, and SERT expression in hPASCs

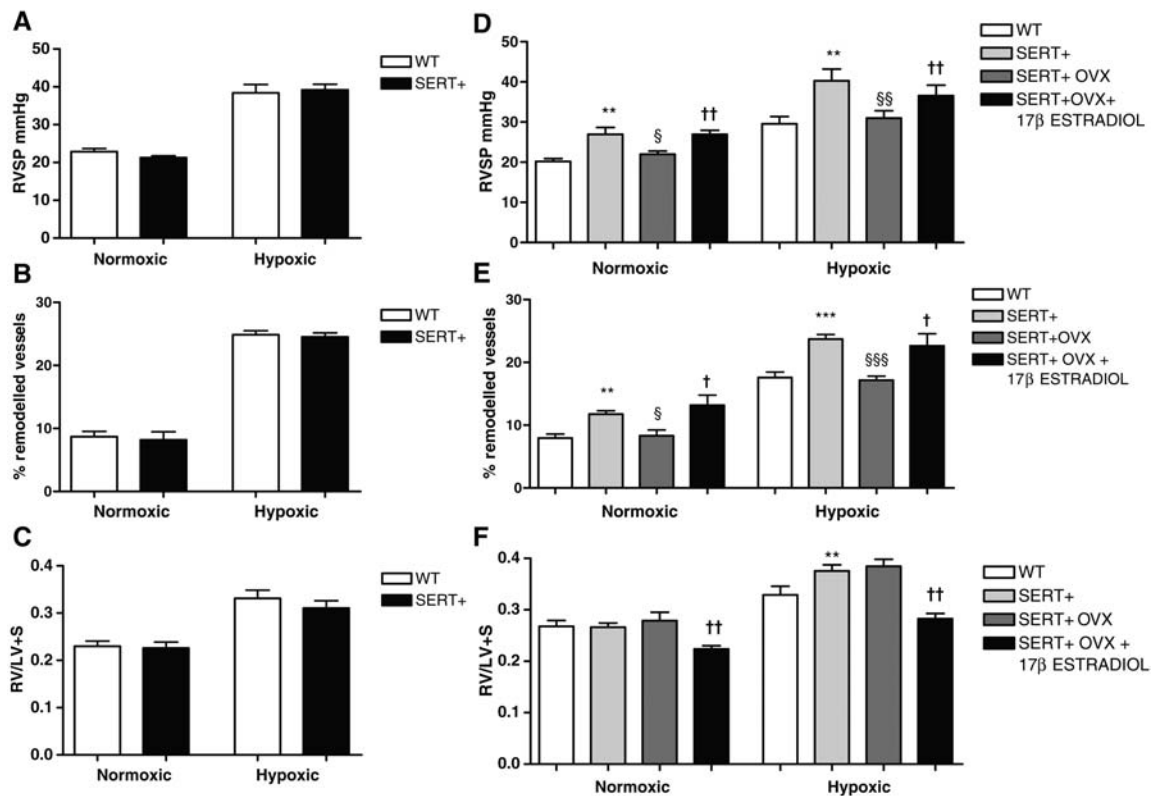
To determine whether 17 $\beta$  oestradiol regulates relevant components of the serotonin system, we exposed hPASCs to 1 nmol/L of 17 $\beta$  oestradiol at multiple time points and investigated the expression of TPH1, the 5-HT<sub>1B</sub> receptors, and SERT. 17 $\beta$  oestradiol increased expression of TPH1 (Figure 5A and B), the 5-HT<sub>1B</sub> receptors (Figure 5C and D), and SERT (Figure 5E and F) at both 4 and 24 h time points.

### 3.4 Effects of 17 $\beta$ oestradiol on 5-HT-induced proliferation in hPASCs

Following the observation that 17 $\beta$  oestradiol increased expression of key serotonin pathway mediators involved in the development of PAH, we investigated if the serotonin system was involved in 17 $\beta$  oestradiol-induced hPASC proliferation. To test this, we investigated the effects of inhibitors for TPH [*p*-chlorophenylalanine (PCPA) 10  $\mu$ mol/L], the 5-HT receptors (5-HT<sub>1B</sub> antagonist, SB224289 300 nmol/L; 5-HT<sub>2A</sub> antagonist, ketanserin 30 nmol/L), and SERT (citalopram 1  $\mu$ mol/L) on 1 nmol/L 17 $\beta$  oestradiol-mediated proliferation of hPASCs. Although none of the inhibitors had any effect on FBS-induced proliferation, both the TPH inhibitor PCPA and the 5-HT<sub>1B</sub> receptor antagonist SB224289 successfully inhibited 17 $\beta$  oestradiol-induced proliferation (Figure 6A and B), whereas the 5-HT<sub>2A</sub> receptor antagonist ketanserin and the SERT inhibitor citalopram had no effect.

## 4. Discussion

Both IPAH and familial PAH occur more in females than in males. For example, in recent epidemiological studies carried out in Scotland,



**Figure 1** Right ventricular systolic pressure ( $n = 6-9$ ), pulmonary arterial remodelling [ $n = 4$  animals ( $> 600$  vessels examined)], and right ventricular hypertrophy ( $n = 6-9$ ) measurements from both female and male WT and SERT+ mice under normoxic and hypoxic conditions. Male SERT+ mice do not develop PAH or exhibit exaggerated hypoxia-induced PAH compared with wildtype mice, as assessed by no change in RVSP (A), pulmonary arterial remodelling (B), or RVH (C). Female SERT+ mice develop PAH and exhibit severe hypoxia-induced PAH compared with wildtype mice (D-F). Under both normoxic and hypoxic conditions, ovariectomy decreases both RVSP (D) and remodelling (E) in SERT+ mice (SERT+ OVX), and increases in these indices are re-established following  $17\beta$  estradiol administration (SERT+ OVX+  $17\beta$  estradiol). Under normoxic conditions, RVH is similar in wildtype and SERT+ mice and unchanged following ovariectomy, however, a decrease is observed in ovariectomized SERT+ mice administered  $17\beta$  estradiol (F). In hypoxia, SERT+ mice exhibit increased RVH compared with wildtype mice. Whilst ovariectomy had no effect on RVH in hypoxic SERT+ mice, a decrease was observed in ovariectomized SERT+ mice administered  $17\beta$  estradiol. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  cf. WT; \$ $P < 0.05$ , \$\$\$ $P < 0.001$  cf. SERT+; † $P < 0.05$ , †† $P < 0.01$  cf. SERT+ OVX. Data are expressed as mean  $\pm$  SEM and analysed by two-way ANOVA followed by Bonferroni's post hoc test.

France, and USA, 60, 65, and 77% of patients studied, respectively, were female.<sup>2-4</sup> The reasons for this increased frequency in females are unclear and under investigated. One reason for this under investigation is the absence of a suitable animal model. Paradoxically, it is observed that male rats exhibit severe hypoxia-induced PAH compared with female rats<sup>9</sup> and oestrogens protect against monocrotaline-induced PAH.<sup>22</sup>

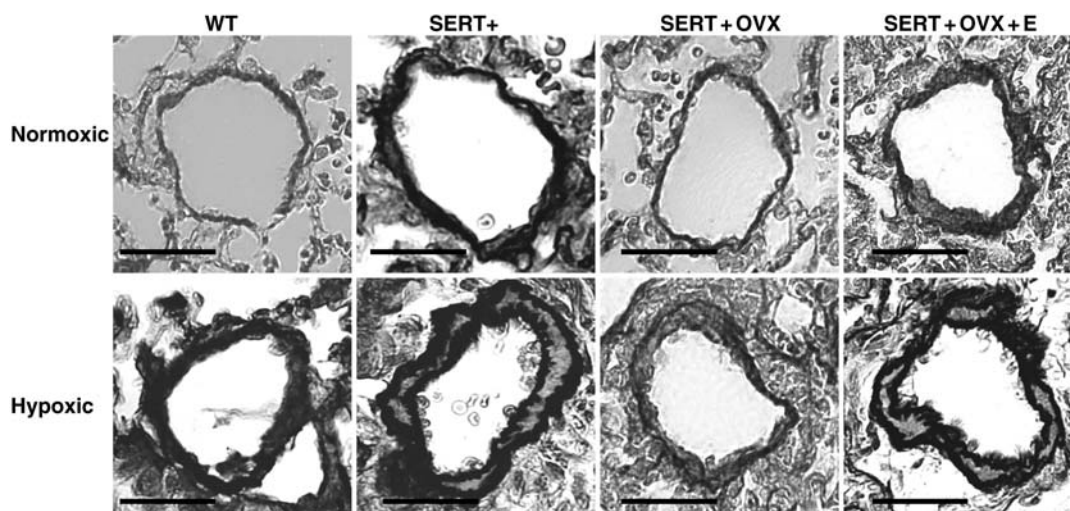
This is the first complete study to describe an animal model of PAH with female susceptibility. Preliminary evidence suggests that increased susceptibility in female mice may also be observed in dexfenfluramine-induced PAH<sup>23,24</sup> and vascular endothelial growth factor (VEGF) receptor antagonist (SU 5416) + hypoxia-induced PAH.<sup>25</sup> In the current study, only female SERT+ mice develop PAH and we provide experimental evidence that interactions between  $17\beta$  oestradiol and the serotonin system may contribute towards this PAH pathogenesis. We demonstrate that female SERT+ mice develop PAH as indicated by elevated RV pressure and pulmonary arterial remodelling, whereas male SERT+ mice remain unaffected. These results suggest that sex hormones are

critical to the development of PAH in this model. To investigate this hypothesis, we ovariectomized these mice and assessed the PAH phenotype after 12 weeks. Ovariectomy completely abolished both the development of PAH and severe hypoxia-induced PAH in SERT+ mice. These results suggest a detrimental role for ovarian-derived hormones in SERT+ mice.

To determine whether  $17\beta$  oestradiol is the hormone critical to the development of PAH *in vivo*, we assessed the effects of its re-introduction into ovariectomized SERT+ mice. As previously mentioned, ovariectomy protects SERT+ mice against the development of PAH; however,  $17\beta$  oestradiol completely re-established a disease phenotype in these mice. Similarly, this hormone also re-established severe hypoxia-induced PAH in ovariectomized SERT+ mice. These results confirm that female gender, via the influence of ovarian-derived  $17\beta$  oestradiol, is critical to the development of PAH in SERT+ mice.

We wished to establish a cellular model to investigate possible mechanisms of action of  $17\beta$  oestradiol and also examine the relevance of our *in vivo* study to human cells. We chose hPASCs as





**Figure 2** Photomicrographs showing representative resistance pulmonary arteries stained with elastica-Van Gieson from both normoxic and hypoxic female WT and female SERT+ mice (Scale bar 50  $\mu$ m). SERT+ mice exhibit remodelling to a greater extent than WT mice and this is attenuated following ovariectomy, however, can be re-established following the administration of 17 $\beta$  oestradiol.

**Table 1** Effects of ovariectomy and 17 $\beta$  oestradiol administration on uterine weight in SERT+ mice

Group	Uterine weight (mg)
Normoxic	
SERT+	182.70 $\pm$ 20.31
SERT+ OVX	17.66 $\pm$ 3.44*
SERT+ OVX+ 17 $\beta$ oestradiol	93.84 $\pm$ 8.93**
Hypoxic	
SERT+	181.85 $\pm$ 12.25
SERT+ OVX	22.33 $\pm$ 0.89*
SERT+ OVX+ 17 $\beta$ oestradiol	119.41 $\pm$ 14.64**

Values are shown as mean  $\pm$  SEM ( $n = 6-9$ ) and analysed by one-way ANOVA followed by Dunnett's post hoc test.

SERT, serotonin transporter; OVX, ovariectomized.

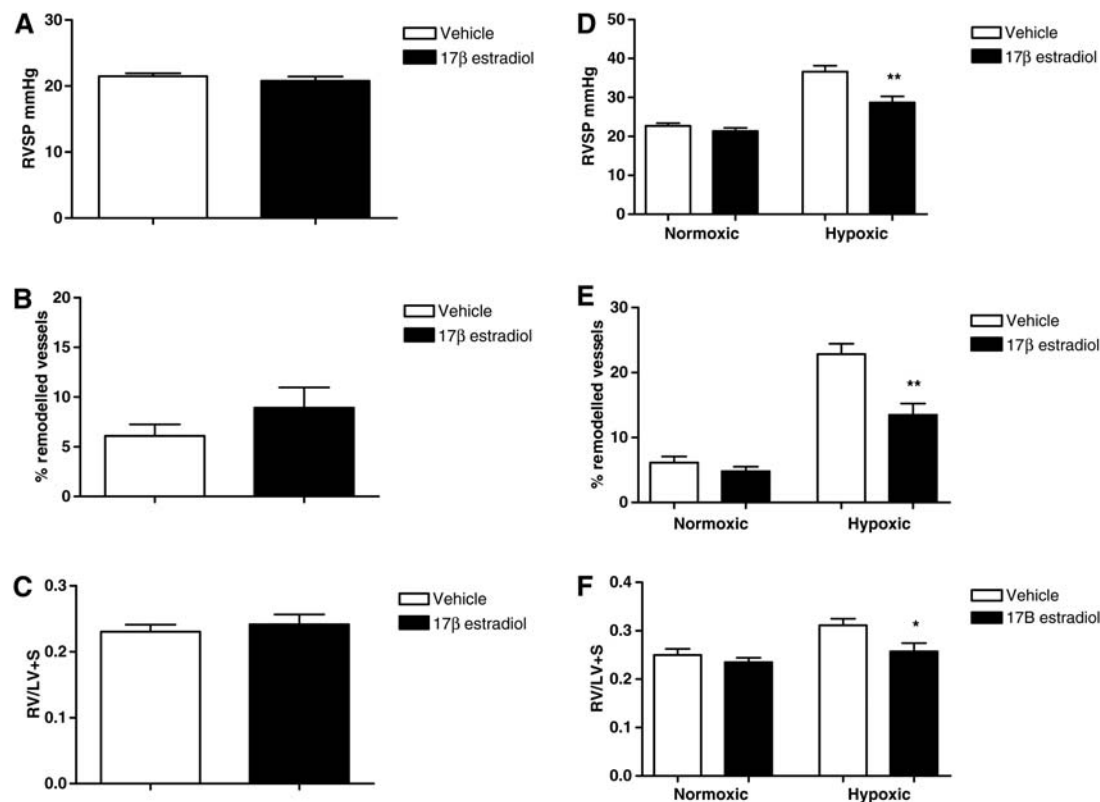
\* $P < 0.001$  cf. SERT+.

\*\* $P < 0.001$  cf. SERT+ OVX.

these have been extensively studied to investigate critical pathways in PAH. For example, hPASCs proliferate to serotonin via stimulation of the 5-HT $_1B$  receptors and SERT,<sup>26</sup> and have also been studied to further investigate BMPRII signalling in PAH.<sup>27</sup> We observed that 17 $\beta$  oestradiol stimulated proliferation, whereas oestrone, oestriol, and progesterone had no effect. This is consistent with our observation that 17 $\beta$  oestradiol re-established a PAH phenotype in ovariectomized SERT+ mice. An aliquot of 1 nmol/L of 17 $\beta$  oestradiol was sufficient to promote hPASC proliferation and similar growth effects have been previously reported in rat PASCs.<sup>28</sup> This is physiologically relevant as 17 $\beta$  oestradiol circulates at concentrations between 0.1 and 1 nmol/L<sup>29</sup> and smooth muscle hyperplasia is a hallmark of PAH.

The suggestion that 17 $\beta$  oestradiol is involved in the pathogenesis of experimental PAH is consistent with recent findings in human PAH. Decreased expression of the 17 $\beta$  oestradiol-metabolizing enzyme CYP1B1 has been reported in female PAH patients harbouring a BMPRII mutation compared with unaffected female carriers.<sup>8</sup> Multiple factors modulate the levels of oestrogen-metabolizing enzymes in the liver and target tissues, and the biological effects of an oestrogen will therefore depend on the profile of metabolites formed and the biological activities of each of these metabolites.<sup>30</sup> 17 $\beta$  oestradiol is metabolized to both pro- and anti-proliferative metabolites and its effects will depend on its metabolism. 17 $\beta$  oestradiol can be converted to oestrone and subsequently metabolized to 16 $\alpha$ -hydroxyestrone (16-OHE1) via CYP3A4. Or alternatively, 17 $\beta$  oestradiol is metabolized to 2-hydroxyestradiol (2-OHE) via the oestrogen-metabolizing enzymes CYP1A1/2 and to a lesser extent via CYP1B1.<sup>31,32</sup> 2-OHE can itself be metabolized to 2-methoxyestradiol (2-ME) via catechol *O*-methyltransferase. Both 2-OHE and 2-ME have anti-proliferative effects on cells,<sup>33</sup> whereas 16 $\alpha$ -OHE1 stimulates proliferation by constitutively activating the oestrogen receptor.<sup>34</sup> Metabolism of 17 $\beta$  oestradiol will therefore be species-, gender-, and strain-dependent and differential disruption in the balance of metabolites may therefore account for the differential effects of female hormones in different models of PAH. Consistent with this, 17 $\beta$  oestradiol did not promote PAH in male SERT+ mice suggesting gender differences in oestrogen metabolism and/or its effects.

As discussed above, in other models of PAH it would appear that male gender predisposes to PAH and 17 $\beta$  oestradiol may actually be protective in PAH. It has, for example, been shown to improve both RVH and pulmonary arterial remodelling in hypoxia-induced and monocrotaline-induced PAH.<sup>10,22</sup> Our studies are consistent with this as we show that male WT mice developed more severe hypoxia-induced PAH compared with female WT mice and 17 $\beta$  oestradiol ablated hypoxia-induced PAH in these males. 17 $\beta$  oestradiol also reduced RVH in ovariectomized SERT+ mice. In addition to



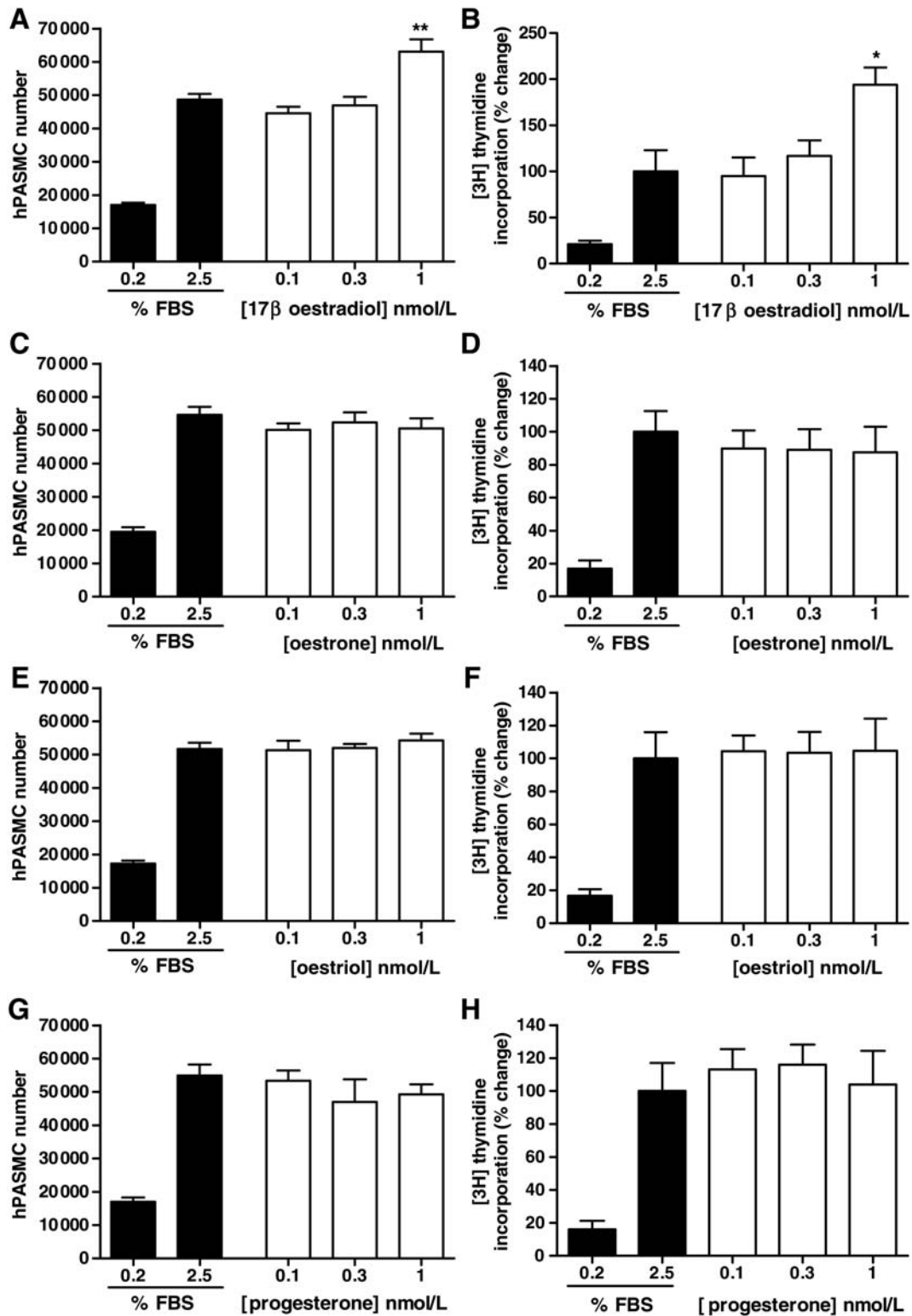
**Figure 3** Effects of 17 $\beta$  oestradiol administration in male SERT+ mice and wildtype mice. RVSP (A;  $n = 9$ ), pulmonary arterial remodelling [B;  $n = 4$  animals (>600 vessels examined)], and RVH (C;  $n = 9$ ) measurements were all unaffected in male SERT+ mice following 17 $\beta$  oestradiol administration. RVSP (D;  $n = 9$ ), pulmonary arterial remodelling (E;  $n = 4$ ) and RVH (F;  $n = 9$ ) were all attenuated in 17 $\beta$  oestradiol-treated wildtype mice exposed to hypoxia. No change was observed in normoxia. \* $P < 0.05$ , \*\* $P < 0.01$  cf. vehicle. Data are expressed as mean  $\pm$  SEM and analysed by one-way ANOVA followed by Dunnett's post hoc test.

the anti-proliferative effects of various 17 $\beta$  oestradiol metabolites, it is also an established nitric oxide-dependent vasodilator in rat pulmonary arteries,<sup>35</sup> up-regulates endothelial nitric oxide synthase expression in pulmonary arterial endothelial cells<sup>36</sup> and suppresses hypoxia-induced endothelin-1 gene expression.<sup>37</sup> These effects may protect against the development of PAH in some species and/or strains. However, our results suggest that when SERT is up-regulated, 17 $\beta$  oestradiol loses these protective effects and this may be via facilitating the mitogenic effects of serotonin. The implications of our study may translate clinically and help explain the inconsistency of the occurrence of PAH which may depend on multiple influences on 17 $\beta$  oestradiol metabolism including age, early menopause, gender, and various other factors that affect 17 $\beta$  oestradiol metabolism.

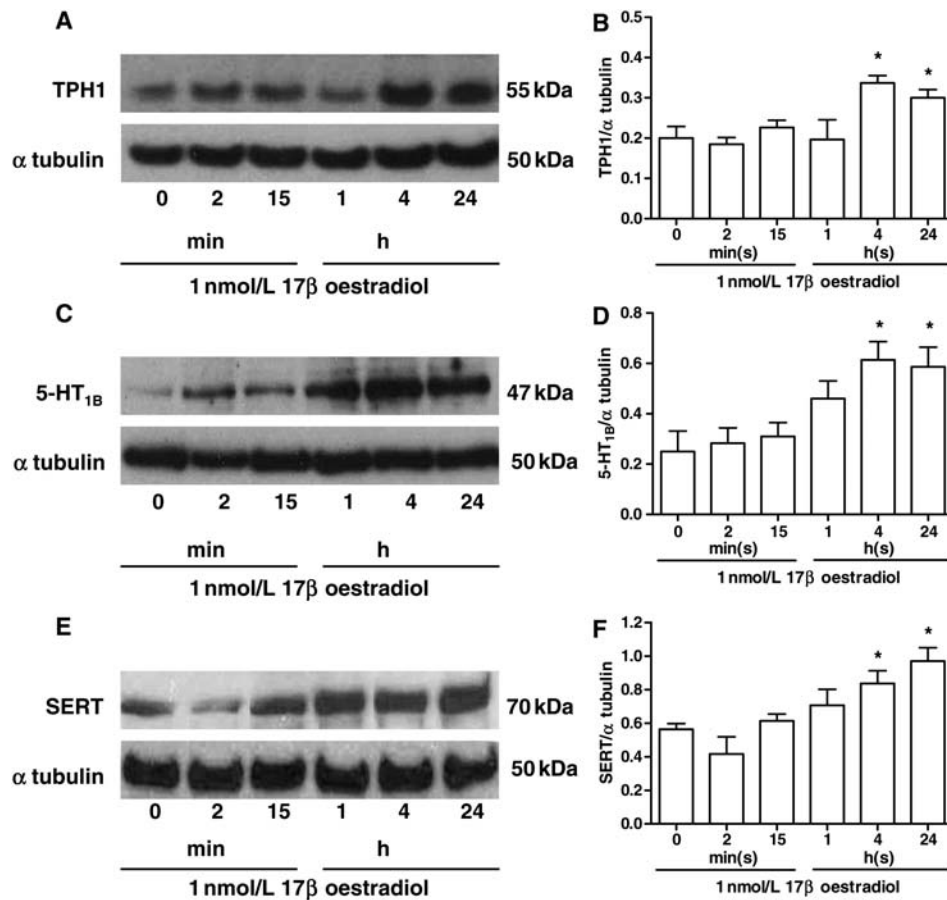
There are multiple serotonin effects within the pulmonary circulation which promote PAH including microthrombosis, arterial vasoconstriction, and proliferation. Indeed, it has been previously shown that TPH1, SERT, and the 5-HT<sub>1B</sub> receptors are all implicated in both human and experimental PAH. For example, the expression of TPH1, the rate-limiting enzyme involved in peripheral serotonin synthesis, is increased in the lungs of IPAH patients.<sup>38</sup> The exogenous administration of serotonin uncovers a PAH phenotype in BMPRII+/- mice<sup>39</sup> and also increases the severity of hypoxia-induced PAH in rats.<sup>40</sup> In addition, mice deficient of

peripheral serotonin (*tph1*-/- mice), achieved through deletion of the *tph1* gene, do not develop hypoxia-induced PAH<sup>12</sup> or dexfenfluramine-induced PAH.<sup>13</sup> Serotonin effects within the pulmonary vasculature are mediated in part via SERT. For example, hPASMCs derived from IPAH patients proliferate to a greater extent than those from controls following serotonin stimulation and this is dependent on SERT activity.<sup>14</sup> A genetic polymorphism leading to increased activity/expression of SERT has been identified in a small cohort of PAH patients.<sup>14</sup> Subsequent studies in larger patient studies have failed to support these findings although patients with the SERT polymorphism may present at an earlier age than those without.<sup>41,42</sup> As previously reported<sup>19</sup> and further observed in the current study, mice overexpressing SERT develop PAH and severe hypoxia-induced PAH. Conversely, mice devoid of the SERT gene are less susceptible to the development of hypoxia-induced PAH.<sup>43</sup> Here, our findings demonstrate that female gender is also a risk factor in the development of PAH in SERT+ mice.

In the central nervous system, oestrogens influence serotonin signalling via up-regulation of multiple pathway mediators including TPH and SERT.<sup>17,18</sup> On this premise, we hypothesized that 17 $\beta$  oestradiol was similarly affecting the serotonin system within the pulmonary circulation and this was the mechanism through which serotonin and 17 $\beta$  oestradiol synergise to facilitate PAH. Thus, we investigated whether 17 $\beta$  oestradiol regulated expression of



**Figure 4** Human pulmonary artery smooth muscle cell (hPASC) proliferation to a range of concentrations (0.1–1 nmol/L) of 17 $\beta$  oestradiol, oestrone, oestriol, and progesterone in the presence of 2.5% FBS. 17 $\beta$  oestradiol increases both cell number and DNA synthesis at 1 nmol/L (A and B). Oestrone (C and D), oestriol (E and F), and progesterone (G and H) do not increase hPASC proliferation at a range of concentrations. \* $P < 0.05$ , \*\* $P < 0.01$  increased proliferation cf. 2.5% FBS.  $n = 3$  for each experiment and performed in duplicate. Data are expressed as mean  $\pm$  SEM and analysed by one-way ANOVA followed by Dunnett's post hoc test.



**Figure 5** Time response effects of 1 nmol/L 17 $\beta$  oestradiol on tryptophan hydroxylase-1 (TPH1), 5-HT<sub>1B</sub> receptor, and serotonin transporter (SERT) expression in hPASMCs. Representative western blots and densitometrical analysis showing that 1 nmol/L 17 $\beta$  oestradiol increases expression of TPH1 (A and B), the 5-HT<sub>1B</sub> receptor (C and D), and SERT (E and F) at 4 and 24 h. \* $P < 0.05$  cf. control.  $n = 4$  for each experiment. Quantitative data are shown as mean  $\pm$  SEM and analysed using one-way ANOVA followed by Dunnett's post hoc test.

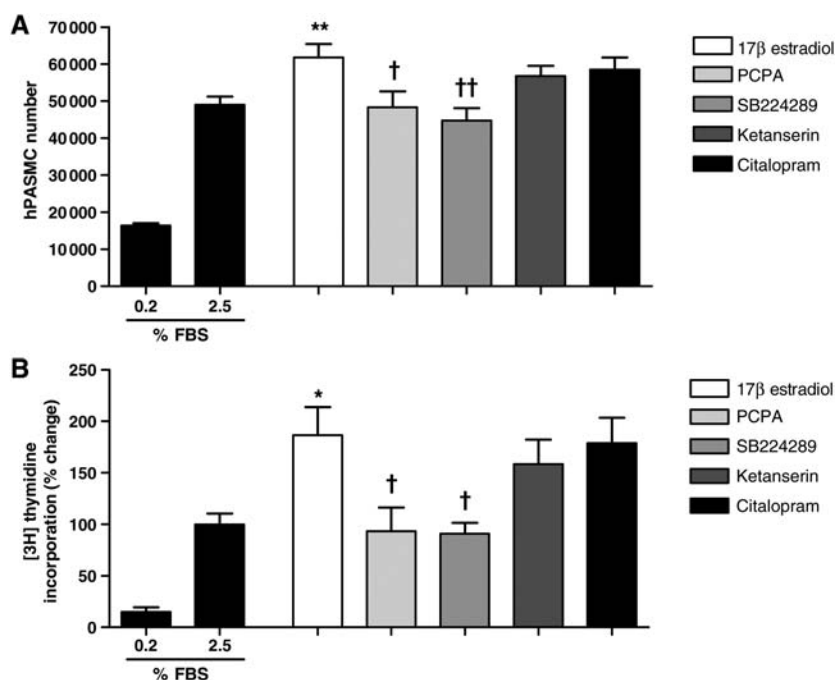
any serotonin pathway mediators in hPASMCs. Here, we report for the first time that TPH1 is present in hPASMCs, and this is markedly increased following stimulation with 17 $\beta$  oestradiol. In addition, 17 $\beta$  oestradiol also increased both 5-HT<sub>1B</sub> receptor and SERT expression in hPASMCs. This may be relevant, as both have previously been shown to interact to promote serotonin-induced hPASMC proliferation.<sup>26</sup> On the basis of these findings, we were interested in determining if 17 $\beta$  oestradiol-mediated hPASMC proliferation via a serotonergic mechanism. Indeed, we report that 17 $\beta$  oestradiol-induced proliferation is completely abolished in the presence of the TPH inhibitor PCPA and the 5-HT<sub>1B</sub> receptor, antagonist SB224289. This suggests that serotonin synthesis, and subsequent activation of the 5-HT<sub>1B</sub> receptor, is essential in mediating the proliferative response of hPASMCs to 17 $\beta$  oestradiol. It could be expected that the increased SERT expression may also increase the SERT-dependent serotonin activation of small GTPases within the cytoplasm ('serotonylation'), to further promote the mitogenic effects of serotonin. However, the observation that the SERT inhibitor citalopram was not sufficient to block proliferation suggests a minor role, although co-operation between the 5-HT<sub>1B</sub> receptors and SERT have previously been shown to mediate serotonin-induced proliferation

and therefore a role for SERT in 17 $\beta$  oestradiol-induced proliferation cannot be ruled out. These findings are consistent with a role for serotonin in PAH as TPH1, the 5HT<sub>1B</sub> receptors, and SERT have all previously been implicated in the pathogenesis of both experimental and human PAH, as discussed above.

We have previously reported that un-dosed SERT<sup>+</sup> mice develop elevated RVSP in the absence of RVH.<sup>19</sup> Conversely, chronically hypoxic  *tph1*<sup>-/-</sup> mice develop RVH in the absence of increased RVSP.<sup>12</sup> The present study confirms dissociation of these indices in SERT<sup>+</sup> mice. Further, we now show that ovariectomy decreased RVSP in SERT<sup>+</sup> mice whilst having no effect on RVH, and the administration of 17 $\beta$  oestradiol to ovariectomized SERT<sup>+</sup> mice increased RVSP whilst decreasing RVH. One explanation for this dissociation is that both 17 $\beta$  oestradiol and serotonin have direct effects on ventricular cardiomyocytes. 17 $\beta$  oestradiol exerts both pro- and anti-hypertrophic effects on these cells.<sup>44</sup> Serotonin is considered a survival factor in cardiomyocytes,<sup>45</sup> and apoptosis is a pre-dominant feature of ventricular remodelling.<sup>46</sup>

In conclusion, here, we have shown that female gender pre-disposes SERT<sup>+</sup> mice to the development of PAH and 17 $\beta$  oestradiol is critical to this. 17 $\beta$  oestradiol appears to increase serotonin synthesis in hPASMCs. This, in combination with 17 $\beta$  oestradiol-mediated





**Figure 6** Effects of inhibitors for serotonin synthesis, 5-HT receptors, and serotonin transporter (SERT) on 1 nmol/L 17 $\beta$  oestradiol-mediated proliferation of hPASM Cs in the presence of 2.5% FBS. 17 $\beta$  oestradiol-mediated proliferation is inhibited by the presence of the TPH inhibitor, PCPA (10  $\mu$ mol/L), and the 5-HT<sub>1B</sub> receptor, antagonist SB224289 (300 nmol/L) but unaffected by the 5-HT<sub>2A</sub> receptor, antagonist ketanserin (30 nmol/L) or the SERT inhibitor citalopram (1  $\mu$ mol/L). Both cell counts and [3H]thymidine incorporation were performed. \* $P < 0.05$ , \*\* $P < 0.01$  increased proliferation cf. 2.5% FBS; † $P < 0.05$ , †† $P < 0.01$  decreased proliferation cf. 1 nmol/L 17 $\beta$  oestradiol.  $n = 3$  for each experiment and performed in duplicate. Data are expressed as mean  $\pm$  SEM and analysed by one-way ANOVA followed by Dunnett's post hoc test.

up-regulation of SERT and the 5-HT<sub>1B</sub> receptor, may act to enhance serotonin-induced proliferation. The SERT and the 5-HT<sub>1B</sub> receptor have previously been shown to mediate both serotonin-induced proliferation of hPASM Cs<sup>16,26</sup> and serotonin-induced constriction of pulmonary arteries.<sup>47</sup> These findings may offer insight into the gender differences apparent in human PAH.

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## References

- Newman JH, Trembath RC, Morse JA, Grunig E, Loyd JE, Adnot S et al. Genetic basis of pulmonary arterial hypertension: current understanding and future directions. *J Am Coll Cardiol* 2004;**43**:33–39.
- Peacock AJ, Murphy NF, McMurray JJ, Caballero L, Stewart S. An epidemiological study of pulmonary arterial hypertension. *Eur Respir J* 2007;**30**:104–109.
- Humbert M, Sitbon O, Chaouat A, Bertocchi M, Habib G, Gressin V et al. Pulmonary arterial hypertension in France: results from a national registry. *Am J Respir Crit Care Med* 2006;**173**:1023–1030.
- Thenappan T, Shah SJ, Rich S, Gomberg-Maitland M. A USA-based registry for pulmonary arterial hypertension: 1982–2006. *Eur Respir J* 2007;**30**:1103–1110.
- Masi AT. Editorial: pulmonary hypertension and oral contraceptive usage. *Chest* 1976;**69**:451–453.
- Morse JH, Horn EM, Barst RJ. Hormone replacement therapy: a possible risk factor in carriers of familial primary pulmonary hypertension. *Chest* 1999;**116**:847.
- Rajkumar R, Konishi K, Richards T, Ishizawa D, Wiechert A, Kaminski N et al. Genome-wide RNA expression profiling in lung identifies distinct signatures in idiopathic pulmonary arterial hypertension and secondary pulmonary hypertension. *Am J Physiol Heart Circ Physiol* 2010;**298**:1235–1248.
- Austin ED, Cogan JD, West JD, Hedges LK, Hamid R, Dawson EP et al. Alterations in oestrogen metabolism: implications for higher penetrance of familial pulmonary arterial hypertension in females. *Eur Respir J* 2009;**34**:1093–1099.
- Rabinovitch M, Gamble WJ, Miettinen OS, Reid L. Age and sex influence on pulmonary-hypertension of chronic hypoxia and on recovery. *Am J Physiol* 1981;**240**:H62–H72.
- Resta TC, Kanagy NL, Walker BR. Estradiol-induced attenuation of pulmonary hypertension is not associated with altered eNOS expression. *Am J Physiol Lung Cell Mol Physiol* 2001;**280**:L88–L97.
- Hansmann G, Wagner RA, Schellong S, Perez VA, Urashima T, Wang L et al. Pulmonary arterial hypertension is linked to insulin resistance and reversed by peroxisome proliferator-activated receptor-gamma activation. *Circulation* 2007;**115**:1275–1284.
- Morecroft I, Dempsey Y, Bader M, Walther DJ, Kotnik K, Loughlin L et al. Effect of tryptophan hydroxylase 1 deficiency on the development of hypoxia-induced pulmonary hypertension. *Hypertension* 2007;**49**:232–236.
- Dempsey Y, Morecroft I, Welsh DJ, Macritchie NA, Herold N, Loughlin L et al. Converging evidence in support of the serotonin hypothesis of dexfenfluramine-induced pulmonary hypertension with novel transgenic mice. *Circulation* 2008;**117**:2928–2937.
- Eddahibi S, Humbert M, Fadel E, Raffestin B, Darmon M, Capron F et al. Serotonin transporter overexpression is responsible for pulmonary artery smooth muscle hyperplasia in primary pulmonary hypertension. *J Clin Invest* 2001;**108**:1141–1150.
- Morecroft I, Heeley RP, Prentice HM, Kirk A, MacLean MR. 5-hydroxytryptamine receptors mediating contraction in human small muscular pulmonary arteries: importance of the 5-HT<sub>1B</sub> receptor. *Br J Pharmacol* 1999;**128**:730–734.

16. Morecroft I, Pang L, Baranowska M, Nilsen M, Loughlin L, Dempsey Y et al. In vivo effects of a combined 5-HT1B receptor/SERT antagonist in experimental pulmonary hypertension. *Cardiovasc Res* 2010;**85**:593–603.
17. Pecins-Thompson M, Brown NA, Kohama SG, Bethea CL. Ovarian steroid regulation of tryptophan hydroxylase mRNA expression in rhesus macaques. *J Neurosci* 1996;**16**:7021–7029.
18. Lu NZ, Eshleman AJ, Janowsky A, Bethea CL. Ovarian steroid regulation of serotonin reuptake transporter (SERT) binding, distribution, and function in female macaques. *Mol Psychiatry* 2003;**8**:353–360.
19. MacLean MR, Deuchar GA, Hicks MN, Morecroft I, Shen SB, Sheward J et al. Overexpression of the 5-hydroxytryptamine transporter gene: effect on pulmonary hemodynamics and hypoxia-induced pulmonary hypertension. *Circulation* 2004;**109**:2150–2155.
20. Karas RH, Schulten H, Pare G, Aronovitz MJ, Ohlsson C, Gustafsson JA et al. Effects of estrogen on the vascular injury response in estrogen receptor alpha, beta (double) knockout mice. *Circ Res* 2001;**89**:534–539.
21. Keegan A, Morecroft I, Smillie D, Hicks MN, MacLean MR. Contribution of the 5-HT1B receptor to hypoxia-induced pulmonary hypertension: converging evidence using 5-HT1B-receptor knockout mice and the 5-HT1B/1D-receptor antagonist GR127935. *Circ Res* 2001;**89**:1231–1239.
22. Farhat MY, Chen MF, Bhatti T, Iqbal A, Cathapermal S, Ramwell PW. Protection by oestradiol against the development of cardiovascular changes associated with monocrotaline pulmonary hypertension in rats. *Br J Pharmacol* 1993;**110**:719–723.
23. Dempsey Y, Macritchie NA, Morecroft I, Nilsen M, Loughlin L, MacLean MR. The effects of gender on the development of dexfenfluramine-induced pulmonary arterial hypertension in mice. *Am J Respir Crit Care Med* 2009;**179**:A1808.
24. Dempsey Y, White K, Nilsen M, Loughlin L, MacLean MR. The effects of endogenous female sex hormones on dexfenfluramine-induced pulmonary arterial hypertension in mice. *Am J Respir Crit Care Med* 2010;**181**:A6314.
25. Tofovic SP, Rafikova O. Preventive and therapeutic effects of 2-methoxyestradiol, but not estradiol, in severe occlusive pulmonary arterial hypertension in female rats. *Am J Respir Crit Care Med* 2009;**179**:A1802.
26. Lawrie A, Spiekerkoetter E, Martinez EC, Ambartsumian N, Sheward WJ, MacLean MR et al. Interdependent serotonin transporter and receptor pathways regulate S100A4/Mts1, a gene associated with pulmonary vascular disease. *Circ Res* 2005;**97**:227–235.
27. Yang J, Davies RJ, Southwood M, Long L, Yang X, Sobolewski A et al. Mutations in bone morphogenetic protein type II receptor cause dysregulation of Id gene expression in pulmonary artery smooth muscle cells: implications for familial pulmonary arterial hypertension 3. *Circ Res* 2008;**102**:1212–1221.
28. Farhat MY, Vargas R, Dinggaan B, Ramwell PW. In vitro effect of oestradiol on thymidine uptake in pulmonary vascular smooth muscle cell: role of the endothelium. *Br J Pharmacol* 1992;**107**:679–683.
29. Rosselli M, Imthurn B, Macas E, Keller PJ, Dubey RK. Circulating nitrite/nitrate levels increase with follicular development: indirect evidence for estradiol-mediated no release. *Biochem Biophys Res Commun* 1994;**202**:1543–1552.
30. Zhu BT, Conney AH. Functional role of estrogen metabolism in target cells: review and perspectives. *Carcinogenesis* 1998;**19**:1–27.
31. Hanna IH, Dawling S, Roodi N, Guengerich FP, Parl FF. Cytochrome P450 1B1 (CYP1B1) pharmacogenetics: association of polymorphisms with functional differences in estrogen hydroxylation activity. *Cancer Res* 2000;**60**:3440–3444.
32. Tsuchiya Y, Nakajima M, Yokoi T. Cytochrome P450-mediated metabolism of estrogens and its regulation in human. *Cancer Lett* 2005;**227**:115–124.
33. Tofovic SP, Zhang XC, Jackson EK, Dacic S, Petrusevska G. 2-Methoxyestradiol mediates the protective effects of estradiol in monocrotaline-induced pulmonary hypertension. *Vascul Pharmacol* 2006;**45**:358–367.
34. Swaneck GE, Fishman J. Covalent binding of the endogenous estrogen 16 alpha-hydroxyestrone to estradiol receptor in human breast cancer cells: characterization and intranuclear localization. *Proc Natl Acad Sci USA* 1988;**85**:7831–7835.
35. Lahm T, Crisostomo PR, Markel TA, Wang M, Wang Y, Weil B et al. Exogenous estrogen rapidly attenuates pulmonary artery vasoreactivity and acute hypoxic pulmonary vasoconstriction. *Shock* 2008;**30**:660–667.
36. MacRitchie AN, Jun SS, Chen Z, German Z, Yuhanna IS, Sherman TS et al. Estrogen upregulates endothelial nitric oxide synthase gene expression in fetal pulmonary artery endothelium. *Circ Res* 1997;**81**:355–362.
37. Earley S, Resta TC. Estradiol attenuates hypoxia-induced pulmonary endothelin-1 gene expression. *Am J Physiol Lung Cell Mol Physiol* 2002;**283**:L86–L93.
38. Eddahibi S, Guignabert C, Barlier-Mur AM, Dewachter L, Fadel E, Darteville P et al. Cross talk between endothelial and smooth muscle cells in pulmonary hypertension: critical role for serotonin-induced smooth muscle hyperplasia. *Circulation* 2006;**113**:1857–1864.
39. Long L, MacLean MR, Jeffery TK, Morecroft I, Yang XD, Rudarakanchana N et al. Serotonin increases susceptibility to pulmonary hypertension in BMPR2-deficient mice. *Circ Res* 2006;**98**:818–827.
40. Eddahibi S, Raffestin B, Pham I, Launay JM, Aegerter P, Sitbon M et al. Treatment with 5-HT potentiates development of pulmonary hypertension in chronically hypoxic rats. *Am J Physiol Heart Circ Physiol* 1997;**41**:H1173–H1181.
41. Machado RD, Koehler R, Glissmeyer E, Veal C, Suntharalingam J, Kim M et al. Genetic association of the serotonin transporter in pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2006;**173**:793–797.
42. Willers ED, Newman JH, Loyd JE, Robbins IM, Wheeler LA, Prince MA et al. Serotonin transporter polymorphisms in familial and idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2006;**173**:798–802.
43. Eddahibi S, Hanoun N, Lanfumey L, Lesch KP, Raffestin B, Hamon M et al. Attenuated hypoxic pulmonary hypertension in mice lacking the 5-hydroxytryptamine transporter gene. *J Clin Invest* 2000;**105**:1555–1562.
44. Kilic A, Javadov S, Karmazyn M. Estrogen exerts concentration-dependent pro-and anti-hypertrophic effects on adult cultured ventricular myocytes. Role of NHE-1 in estrogen-induced hypertrophy. *J Mol Cell Cardiol* 2009;**46**:360–369.
45. Nebigil CG, Etienne N, Messaddeq N, Maroteaux L. Serotonin is a novel survival factor of cardiomyocytes: mitochondria as a target of 5-HT2B-receptor signaling. *FASEB J* 2003;**17**:1373–1375.
46. Williams RS. Apoptosis and heart failure 2. *N Engl J Med* 1999;**341**:759–760.
47. Morecroft I, Loughlin L, Nilsen M, Colston J, Dempsey Y, Sheward J et al. Functional interactions between 5-hydroxytryptamine receptors and the serotonin transporter in pulmonary arteries. *J Pharmacol Exp Ther* 2005;**313**:539–548.