A sex-specific microRNA-96/5HT$_{1B}$ axis influences development of pulmonary hypertension.

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At a glance commentary: Females develop pulmonary arterial hypertension more frequently than males and loss-of-function BMPR-II mutations underlie heritable PAH. Our research shows that, in female pulmonary artery smooth muscle cells from BMPR-II mutant mice and patients, there is down regulation of microRNA-96 associated with concomitant up-regulation of the 5-HT1BR, a direct microRNA-96 target. We show that this contributes to a proliferative phenotype of female human

pulmonary artery smooth muscle cells and that a microRNA-96 mimic prevents the development of pulmonary hypertension in a model of pulmonary arterial hypertension. These findings suggest that down-regulation of microRNA-96 and associated increases in serotonin-induced proliferation may contribute to the pathology of pulmonary arterial hypertension, especially in females.

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Abstract

Rational: Females are pre-disposed to pulmonary arterial hypertension (PAH); evidence suggests that serotonin, mutations in the bone morphogenetic protein receptor II (*BMPR-II*) gene and estrogens influence development of PAH. The 5-HT_{1B} receptor (5-HT_{1B}R) mediates human pulmonary artery smooth muscle cell (hPASMC) proliferation.

Objectives: We aimed to determine whether selected microRNAs (miRNAs) expressed in PASMCs are influenced by sex, BMPR-II mutations and estrogens and contribute to PASMC proliferation in PAH.

Methods: Expression levels of miRNAs targeting genes related to PAH, estrogen and serotonin were determined by quantitative RT-PCR in hPASMCs and mouse PASMCs harbouring a heterozygous mutation in BMPR-II (BMPR-$II^{R899X+/-}$ PASMCs). MiRNA-96 targets the 5-HT_{1B}R and was selected for further investigation. MiRNA target validation was confirmed by luciferase reporter assay. Precursor-miRNA-96 was transfected into hPASMCs to examine effects on proliferation and 5-HT_{1B}R expression. The effect of a microRNA-96 mimic on the development of hypoxic PH in mice was also assessed.

Measurements and Main Results: MiRNA-96 expression was reduced in female BMPR-$II^{R899X+/-}$ PASMCs and hPASMCs from female PAH patients; this was associated with increased 5-HT_{1B}R expression and serotonin mediated proliferation. The 5-HT_{1B}R was validated as a target for miRNA-96. Transfection of precursor-miRNA-96 into hPASMCs reduced 5-HT_{1B}R expression and inhibited serotonin-induced proliferation. Restoration of miRNA-96 expression in pulmonary arteries *in vivo* via administration of a miRNA-96 mimic reduced the development of hypoxia-induced PH in the mouse. We conclude that increased 5-HT_{1B}R expression may be

a consequence of decreased miRNA-96 expression in female patient PASMCs and this may contribute to the development of PAH.

250 words

Introduction

Mutations within the Bone Morphogenetic Protein Type II Receptor (BMPR-II) gene are associated with ~70% heritable PAH (hPAH) cases and ~10-40% idiopathic PAH (1). Loss of function of the BMPR-II receptor results in a decrease in BMPR-II signalling translating into aberrant proliferation of pulmonary arterial smooth muscle cells (PASMCs) (2). Restoration of the BMPR-II signalling pathway represents a novel therapeutic strategy (3). We recently demonstrated, in the hypoxic mouse and Sugen 5416/hypoxic (Su/Hx) rat models, that the therapeutic effect of anastrozole, an inhibitor of estrogen synthesis, was only observed in females and related to restoration BMPR-II signalling (4). BMPR-II mutations exhibit a relatively low frequency of disease penetrance (20-30%) suggesting that other factors also contribute to the development of PAH.

Epidemiological studies report a greater incidence of PAH in females; the female to male ratio ranges from 3-4:1 (5). Hence, female sex hormones, primarily estrogens, may play a causative role in the development of PAH. 17² -estradiol, the main circulating estrogen, increases proliferation of human PASMCs (hPASMCs) and anomalous synthesis and metabolism of estrogen have been associated with the disease (4, 6, 7). Within the promoter region of the BMPR-II gene there is an estrogen response element which may play a role in the suppression of the BMPR-II receptor expression (8).

Evidence suggests that serotonin is involved in the pathogenesis of PAH. Endothelial expression of tryptophan hydroxylase-1 (TPH-1), the rate limiting enzyme in the synthesis of serotonin, is increased in remodelled pulmonary arteries

from idiopathic patients (9). In addition, TPH-1 knockout mice are protected from hypoxia induced pulmonary hypertension (PH) (10, 11). We have shown that only female mice develop PH via a serotonin-dependent mechanism, for example mice over-expressing the serotonin transporter (SERT), mts1 over-expressing mice and mice dosed with dexfenfluramine (6, 12, 13). This PH phenotype is estrogen-dependent and suggests that female sex and estrogen influence the development of PH in these serotonin-dependent models. Serotonin is a potent mitogen and vasoconstrictor in the pulmonary vasculature acting through the SERT and 5-hydroxytryptamine$_{1B}$ receptor (5-HT$_{1B}$R)(14, 15). The expression of TPH1, SERT and the 5-HT1BR in hPASMCs is increased by 17β-estradiol (6).

MicroRNAs (miRNAs) are non-coding RNA sequences that have the ability to direct expression of genes in a post-transcriptional manner through either degradation of target mRNA or silencing of mRNA translation. Several miRNAs have recently been shown to play a role in PAH pathophysiology (16). Literature suggests that sex hormones i.e. estrogens have the ability to regulate the expression of miRNAs (17) which led us to speculate whether sex can also influence expression of miRNAs. Thus, in the present study we investigated whether sex and/or BMPR-II expression affects miRNA expression within PASMCs and hence the expression of genes that may influence the development of PAH.

Methods

Pulmonary Artery Smooth Muscle Cells

PASMCs derived from knock-in mice harbouring a heterozygous mutation in BMPR-II (R899X) and littermate wild type (WT) control mice were prepared as described previously (18) and in online supplement. These mice develop spontaneous pulmonary hypertension (19). Human PASMCs were prepared as described previously and in online supplement (6).

Cell Proliferation Assay

To assess proliferation, PASMCs were counted using the haemocytometer approach as described previously (6).

hPASMCs transfection with miRNA-mimic

HPASMCs were transfected with either 1nM precursor (pre)-miR-96 (Ambion, PM10422) or pre-miRNA-negative control (Ambion, AM17110 #1) as described in the online supplement. For expression analysis each condition was performed in biological replicates to allow for RNA and protein harvest 48hrs and 72 hrs post-transfection respectively. For proliferation analysis PASMCs were then quiesced in 0.2% FBS and the proliferation protocol followed as per online supplement.

Taqman quantitative-PCR Analysis of miRNAs and mRNAs

Total RNA extraction and Taqman quantitative RT-PCR were conducted as previously described (16, 20) and in online supplement.

Western Blot Analysis of Protein

Protein extraction and Western blot analysis was carried out as described previously (7, 16) and in online supplement.

Pulmonary arterial remodelling

Remodelled pulmonary arteries were investigated by elastin-picrosirius red staining as previously described (16, 20) and in online supplement.

Luicferase Reporter Assay

The psi-CHECK-2 dual luciferase reporter vector (Promega) was utilised for the reporter assay as described in online supplement. Luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega) and luminescence detection performed via LUMIstar OPTIMA microplate reader (BMG Labtech).

Chronic Hypoxia

Female WT C57/Bl (Charles River) mice aged 2 months old were exposed to 14 day hypobaric hypoxia (10% O_2, 550mbar) as described previously (21). Mice maintained in normoxic conditions (21% O_2, 1000mbar) were studied as controls.

Administration of miRNA-96 mimic

To assess whether miRNA-96 was involved in the pathology of pulmonary hypertension, the miRNA-96 mimic (Applied Biosystems, #MC10422) was administered intravenously via the tail vein once a week for the 2 weeks of hypoxic exposure. See online supplement for further details.

Anastrozole-treated mice

Lungs from mice treated with anastrozole 3mg/kg, or vehicle 1% carboxymethylcellulose via subcutaneous injection for 14 days (4) were studied to examine the influence of endogenous estrogen depletion on miRNA-96 regulation.

In Vivo Assessment of PH

Measurements of right ventricular systolic pressure (RVSP), right ventricular hypertrophy (RVH) and pulmonary vascular remodelling were performed as previously described (4, 7) and in the online supplement.

In-situ Hybridisation

Localisation of miRNA-96 within pulmonary arteries was performed by in-situ hybridisation as previously described (22) and in the online supplement.

Analysis of Data

Values are expressed as mean ± standard error of the mean (SEM). A t-test or 1-way ANOVA followed by Tukey's post-hoc test was performed to evaluate the statistical significance between all groups where appropriate. A probability level of $p<0.05$ was defined as being statistically significant.

Results

Proliferation and miRNA expression in BMPR-II$^{R899X+/-}$ PASMCs

Female BMPR-II$^{R899X+/-}$ PASMCs were more proliferative to PDGF and serotonin compared to male BMPR-II$^{R899X+/-}$ PASMCs (Figure 1A). We examined miRNAs that are associated with PAH, hypoxia, serotonin and estrogen systems in the female

and male BMPR-II$^{R899X+/-}$ PASMCs. We examined miRNA-503 [targets e.g. fibroblast growth factor 2], miRNA-145 [targets krueppel-like factor 5 (23)], miRNA-155 [targets e.g. hypoxia-inducible factor ±](24). We also studied miRNAs targeting ERα (miRNA-22, miRNA-206 (25)), CYP1B1 (miRNA-27b (26)), the 5-HT$_{1B}$R (miRNA-96 (27)) or SERT (miRNA-16 (28)).

There was no significant difference between the expression of miRNAs 16, 22 and 27b in male or female BMPR-II$^{R899X+/-}$ PASMCs vs. WT cells (Figure E1). MiRNA-145 and miRNA-206 were significantly increased in both female and male BMPR-II$^{R899X+/-}$ PASMCs compared to WT. MiRNA-155 was down-regulated in female BMPR-II$^{R899X+/-}$ PASMCs compared to WT and up-regulated in male BMPR-II$^{R899X+/-}$ PASMCs compared to WT. There was reduced expression of miRNA-503 in female BMPR-II$^{R899X+/-}$ PASMCs compared to WT (Figure E1).

Expression of miRNA-96 was reduced by ~80% in female BMPR-II$^{R899X+/-}$ PASMCs compared with their WT controls; expression was equal in male BMPR-II$^{R899X+/-}$ PASMCs compared with their WT controls (Figure 1B). In WT PASMCs, there was no significant difference in miRNA-96 expression between female and male. In the BMPR-II$^{R899X+/-}$ PASMCs there was decreased expression of miRNA-96 in females compared to males. *In silico* analysis (miRwalk http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) suggested miRNA-96 may target the 5-HT$_{1B}$R. Therefore we analysed the expression of the 5-HT$_{1B}$R in female and male BMPR-II$^{R899X+/-}$ PASMCs. 5-HT$_{1B}$R expression was only increased in female BMPR-II$^{R899X+/-}$ PASMCs (Figure1C and D) and accompanied by a decrease in miRNA-96 expression.

Our rationale for focussing our studies on miRNA-96 was that this is the only known miRNA to target the 5-HT$_{1B}$R (27); the 5-HT$_{1B}$R mediates pathogenic effects of

serotonin in PAH(14, 21). In addition, a role for miRNA-96 in PAH has not previously been described, hence this novel sex-specific microRNA-96/5-HT_{1B}R axis/BMPR-II was worthy of further investigation.

MiRNA-96 expression in hPASMCs

MiRNA-96 was also down-regulated in PASMCs from female PAH patients compared to non-PAH patient controls (Figure 2A). Consistent with findings from the BMPR-$II^{R899X+/-}$ PASMCs, there was no change in male patient PASMCs (Figure 2A). Notably, 5-HT_{1B}R expression was only increased in female PAH patients (Figure 2B and C).

Validation of 5-HT_{1B}R as a direct target of microRNA-96

To assess transfection conditions in HeLa cells we performed qRT-PCR analysis to examine the mature form of miRNA-96 following pre-miR-96 and pre-miRNA-negative control transfections. A significant increase in miRNA-96 expression in the pre-miRNA-96 transfected cells was observed compared to control with no increase observed in the pre-miRNA-negative control group (Figure 3A). Luciferase reporter assay confirmed a miRNA-96 binding site in the 3'UTR of 5-HT_{1B}R mRNA as pre-miRNA-96 was able to significantly reduce luciferase activity at a concentration of 25nM with pre-miRNA-negative control showing no significant reduction in luciferase activity of the 5-HT_{1B}R construct (Figure 3B). Importantly pre-miRNA-96 had no effect on reducing luciferase activity of the psi-CHECK-2 control vector and of the mutated 5-HT_{1B}R construct (Figure 3B).

miRNA-96 and human PASMC proliferation

The proliferative response to serotonin after 72 hours was only observed in female patient PASMCs and was inhibited by the 5-HT_{1B}R selective antagonist SB224289 (Figure 4A-D). The selective 5-HT_{1B}R agonist, CP94253, induced proliferation only in female patient PASMCs and this was inhibited by SB224289 (Figure 4A-D). Direct over-expression of miRNA-96 (Figure 5A) did not affect 5-HT_{1B}R mRNA expression (Figure 5B) but did significantly decrease endogenous 5-HT_{1B}R protein expression (Figure 5C). Pre-miRNA-96 abolished serotonin-induced proliferation (Figure 5D). Moreover, the effect of pre-miRNA-96 was comparable to the effects of the 5-HT_{1B}R antagonist SB224289 (Figure 5D). These results suggest that miRNA-96 expression can regulate 5-HT_{1B}R-mediated proliferation in female patient PASMCs.

The influence of estrogen on miRNA-96

17β-estradiol decreased miRNA-96 expression (Figure 6A) but did not significantly affect 5-HT_{1B}R mRNA expression (Figure 6B). To determine if endogenous 17β-estradiol also influences miRNA-96 expression we assessed miRNA-96 and 5-HT_{1B}R protein expression in lung from female and male mice that had been dosed with an aromatase inhibitor anastrozole. These mice have depleted circulating and local lung synthesis of estrogen and elevated BMPR-II signalling (4). MiRNA-96 was elevated in the lungs from the estrogen-depleted female mice (Figure 6C) and this was accompanied by a decrease in 5-HT_{1B}R mRNA expression (Figure 6D). No changes in miRNA-96 and 5-HT_{1B}R mRNA expression were observed within male lung tissue (Figure E2).

In vivo effect of a miRNA-96mimic in the hypoxic mouse

We determined if a miRNA-96 mimic would prevent the development of hypoxia-induced PH (associated with decreased BMPR-II signalling (4, 29)) and if any therapeutic effect was related to 5-HT_{1B}R expression. Firstly we confirmed that MaxSuppressor™ In Vivo RNA-LANCEr II delivered miRNA-96 mimic to the pulmonary arteries and specifically their medial layer. Figure 7A demonstrates that miRNA-96 expression was increased in the pulmonary arteries following administration of the miRNA-96 mimic. From in situ hybridisation we report that the miRNA-96 expression is localised within the smooth muscle medial layer of the pulmonary artery (Figure 7B). The miRNA-96 mimic reduced hypoxia-induced increases in RVSP (Figure 7C) and RVH (Figure 7D) and inhibited pulmonary vascular remodelling (Figure 7E and F). Importantly, the miRNA-96 mimic had no effect under normoxic conditions (Figures 7C-F) and did not affect mean systemic arterial pressure and heart rate amongst experimental groups (Figure E3). We confirmed that the hypoxic mouse demonstrates decreased BMPR-II mRNA expression (Figure 8A). MiRNA-96 expression was decreased by hypoxia and restored following the administration of the miRNA-96 mimic (Figure 8B). 5-HT_{1B}R protein expression was markedly increased in the hypoxic lung and the miRNA-96 mimic returned 5-HT_{1B}R expression to normoxic levels (Figure 8C).

Discussion

Sexual dimorphism of miRNA expression has been recognised within both physiological and pathological processes (30, 31). However, this is the first study to observe sex differences in miRNA expression with regards to PAH. Through *in vitro* cell culture models and *in vivo* animal studies we present novel data demonstrating how miRNA-96, under the potential influence of estrogen, plays a role in the

development of PH in a sex-dependent manner, by regulating 5-HT_{1B}R expression and hence serotonin-induced proliferation.

We have previously demonstrated that female mouse lung displays decreased BMPR-II and Id1 expression compared to male lung (4). We have also demonstrated that, as a consequence of mutation in BMPR-II, there is unopposed p38MAPK/ERK signalling and increased proliferation in hPASMCs (2). PDGF induced proliferation only in female non-PAH hPASMCs, and we attribute this to decreased BMPR-II signalling in these cells (32). These observations are consistent with our observation that the female BMPR-$II^{R899X+/-}$ PASMCs proliferate to a greater extent to PDGF and serotonin compared to male BMPR-$II^{R899X+/-}$ PASMCs. We have previously shown that administration of serotonin unmasks a PH phenotype in BMPR-$II^{+/-}$ mice (26). This is consistent with our novel observation that there is an increase in the proliferative response to serotonin and 5-HT_{1B}R expression in female BMPR-$II^{R899X+/-}$ PASMCs, associated with a decrease in miRNA-96 expression.

To examine if these observations translated to human tissue, we demonstrated that serotonin-induced proliferation of hPASMCs from female patients was mediated by the 5-HT_{1B}R as the effect was replicated by a 5-HT_{1B}R agonist and abolished by a 5-HT_{1B}R selective antagonist. These results are consistent with previous studies showing the importance of the 5-HT_{1B}R mediating hPASMC proliferation (21, 23). MiRNA-96 expression was decreased in female PAH patient PASMCs correlating with an increase in 5-HT_{1B}R expression. This is consistent with our observations in female mouse BMPR-$II^{R899X+/-}$ PASMCs, where miRNA-96 expression is decreased and 5-HT_{1B}R expression is increased compared with male mouse BMPR-$II^{R899X+/-}$ PASMCs where there was no differential effect.

Interestingly, proliferation to serotonin in hPASMCs was only observed in female patient PASMCs, despite low expression levels of the 5-HT_{1B}R in male patient and control PASMCs. The 5-HT_{1B}R is Gi-linked and Gi-linked responses are regulated by synergistic influences such that a threshold for activation is required (33, 34). Indeed, activity and /or expression of the 5-HT_{1B}R can be increased synergistically by Gq-linked receptor stimulation (14, 35-37), estrogen (6), co-activation of the serotonin transporter (14, 38, 39) and pERK (40, 41). Estrogen synthesis is increased in female hPASMCs (4) providing a stimulus for over-expression of SERT and 5-HT_{1B}R (6) and SERT expression is increased in patient PASMCs (42). In addition, pERK2 expression is elevated in female hPASMCs and further enhanced in PASMCs from PAH patients (2, 41). In addition there is increased expression of Gq-linked receptors (e.g. PDGF, endothelin-1(43)) in PASMCs from patients with PAH. Hence female hPASMC from PAH patients are influenced by a unique combination of synergins (SERT, 5-HT_{1B}R, Gq-stimulation, estrogen) that can facilitate 5-HT_{1B}R-mediated responses.

In silico bioinformatic analysis suggests the 5-HT_{1B}R is a putative target of miRNA-96. In addition, a polymorphism in the 3'UTR of the 5-HT_{1B}R mRNA disrupts binding of miRNA-96 (27). We validated 5-HT_{1B}R was a target of miRNA-96 through luciferase reporter assay. Additionally, we demonstrated direct over-expression of miRNA-96 (via transfection of pre-miRNA-96) in hPASMCs could decrease expression of target 5-HT_{1B}R protein in *in vitro* cell culture. This acute over-expression of the miRNA-96 had no effect on 5-HT_{1B}R mRNA suggesting that after 48 hours the miRNA-96 is binding to the 3'UTR of 5-HT_{1B}R mRNA, silencing translation to protein without actual degradation of the mRNA.

We demonstrated direct over-expression of miRNA-96 ablated the ability of serotonin to induce proliferation of female PAH patient PASMCs which accompanied a decrease in 5-HT_{1B}R expression. Thus within the female PAH patient PASMCs, down-regulation of miRNA-96 may lead to over-expression of 5-HT_{1B}R which subsequently increased the capacity of serotonin to induce proliferation. This is the first report that miRNA-96 expression influences distal hPASMCs proliferation and that manipulation of miRNA-96 can influence proliferation through targeting the 5-HT_{1B}R. It is distal PASMCs that contribute to pulmonary vascular remodelling but it is of interest that down-regulation of miRNA-96 may mediate an increase in BMP-4 signalling in proximal PASMCs which demonstrate a distinct phenotype and are less involved in the pathobiology associated with PAH.

Little is currently known about the role miRNA-96 plays in vascular physiology and pathology. Aberrant expression of miRNA-96 has been observed in cancer biology where it has been describes as being either oncogenic (44) or anti-oncogenic (45) and miRNA-96 has previously been associated with breast cancer (46). Breast cancer is a disease strongly influenced by estrogen and suggests that there could be a link between estrogen and miRNA-96 expression. Indeed estrogen can alter miRNA expression through three mechanisms, firstly through estrogen-response-elements (ERE) in the promoter element of the pri-miRNA gene (47), secondly through transcription of estrogen related genes e.g. c-MYC which can in turn interact with transcription of primary-miRNA gene (48) and thirdly through interaction with miRNA biogenesis as there is an ER± binding site in the promoter region of the Dicer gene (17). We have previously demonstrated that estrogen increases the protein expression of the 5-HT_{1B}R in hPASMCs (8). It was therefore

of interest to investigate if estrogen can influence 5-HT_{1B}R expression through modulating epigenetic control. Stimulation of hPASMCs with estrogen decreased the expression of miRNA-96 suggesting that estrogen could influence 5-HT_{1B}R expression via regulation of miRNA-96. This is consistent with bioinformatic analysis showing the primary-miRNA-96 gene harbours an ERE in the promoter region (49). It is equally possible that estrogen could influence miRNA-96 expression indirectly though reduction in BMPR-II signalling (8) as Smads are multifunctional proteins that modify gene expression themselves, transcriptionally through DNA binding and post-transcriptionally through pri-miRNA binding and regulation of miRNA processing. For example, it has been reported that the expression of miRNA-21 is influenced by BMP signalling. Here, SMAD proteins interact with DROSHA to increase the biogenic processing of pri-miRNA-21 into pre-miRNA-21 and finally into mature miRNA-21 (50). It is also known that pre-miRNAs may compete with their mature miRNA and hence serve as post-transcriptional regulators of miRNA activity (51). Regulation of gene activity by miRNAs may therefore be affected by changes in BMPR-II/Smad signalling; decreased BMPR-II signalling by estrogen may facilitate the gene-silencing effects of miRNA-96 indirectly via decreased pre-miRNA processing.

Previous studies have shown that *exogenously* administered estrogen can actually protect against PH in male mice (52). We recently demonstrated expression of aromatase (the estrogen synthesising enzyme) in human, rat and mouse pulmonary artery smooth muscle and that the expression of aromatase was greatest in females (4). In addition we showed that inhibition of endogenous aromatase by anastrozole increases lung BMPR-II expression and can prevent and reverse PH in the hypoxic mouse and rat Su/Hx model but only in females (6). Anastrozole

depletes all endogenous estrogen, both circulating (4) and vascular. This suggests that *endogenous* estrogen plays a role in the development of PH in female mice and rats via restoration of BMPR-II signalling, but not the males and that the combination of circulating estrogen and local endogenous synthesis of estrogen in pulmonary arteries drives a PH phenotype in females. Lungs from mice treated with anastrozole demonstrated a significantly higher expression of miRNA-96 compared to control lung. This increase in miRNA-96 was associated with a decrease in 5-HT_{1B}R expression and was only observed in lungs from female mice not males. Collectively, our results suggest that endogenous estrogen regulates BMPR-II and miRNA-96 expression only in female lung. This supports the hypothesis that sex plays an important role in the regulation of miRNAs and BMPR-II. Indeed we have recently demonstrated that hPASMCs from non-PAH females have reduced BMPR-II signalling compared with male hPASMCs and this contributes to increased proliferative responses in the female hPASMCs (32). As PAH is more frequently presented in women and endogenous estrogens may influence this, it also raises the question as to whether or not female and male PAH patients may require different therapeutics. This concept has previously been addressed with regards to current PAH treatments where it was observed that women responded better to endothelin-1 receptor antagonists compared to men (53).

To investigate if restoration of miRNA-96 expression in vivo can protect against PH via the 5-HT_{1B}R, we performed an *in vivo* study to examine the effects of a miRNA-96mimic. BMPR-II expression is reduced in the hypoxic mouse, the monocrotaline rat and the Sugen/hypoxic rat (4, 54, 55). Here we utilised the hypoxic mouse model of PH which produces a robust disease phenotype including decreased BMPR-II signalling. We confirmed that BMPR-II mRNA expression was reduced in

the lungs of the hypoxic mice. We report that 5-HT_{1B}R protein expression is markedly elevated in the lungs from the hypoxic mice whilst miRNA-96 expression is reduced. In situ analysis demonstrated that the miRNA-96 expression is primarily in the medial smooth muscle cell layer of the pulmonary arteries. We showed i.v. administration of the miRNA-96 mimic delivered the miRNA-96 to the pulmonary arteries and also that the miRNA-96 mimic reduced hypoxic-induced increases in RVSP and RVH and prevented pulmonary vascular remodelling. This was associated with an increase in lung miRNA-96 expression and decrease in 5-HT_{1B}R expression. This substantiates our hypothesis that PH is associated with increased 5-HT_{1B}R-mediated remodelling/proliferation under the control of miRNA-96. The miRNA-96 mimic had no effect on mean systemic arterial pressure or heart rate suggesting that this therapeutic strategy could be pulmonary selective.

In summary, the present work suggests that estrogen and BMPR-II deficiency can decrease miRNA-96 expression in PASMCs causing an increase in 5-HT_{1B}R expression and this may influence the pathobiology of PAH in females. In addition, a miRNA-96 mimic can reduce lung 5-HT_{1B}R expression *in vivo* and can prevent the development of experimental PH (see Figure 8D for summary). Further study into the potential of a miRNA-96-mimic as a novel therapeutic strategy in PAH is warranted.

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Figure 1. Characteristics of pulmonary artery smooth muscle cells (PASMCs) in female and male BMPR2$^{R899X+/-}$ mice (BMPR-II$^{R899X+/-}$ PASMCs) and wildtype (WT) controls. (A) Proliferative responses after 72 hours to 10% FBS, PDGF and serotonin (5-HT) in male and female BMPR-II$^{R899X+/-}$ PASMCs (n=3, in triplicate). (B) Expression of miRNA-96 in male and female BMPR-II$^{R899X+/-}$ PASMCs and WT control cells (n=8, all isolates repeated 3 times). The expression of 5-HT$_{1B}$R mRNA (C) and protein (D) in male and female BMPR-II$^{R899X+/-}$ PASMCs and WT control cells (n=6, all isolates repeated 3 times) and representative immunoblot of 5-HT$_{1B}$R protein expression in male and female BMPR-II$^{R899X+/-}$ PASMCs and WT control cells. Data displayed as mean SEM. *P<0.05, **, $^{++}$P<0.01, ***P<0.001 determined by one-way analysis of variance with Tukey's post test.

Figure 2. Expression of miRNA-96 and 5-HT_{1B}R in human pulmonary artery smooth muscle cells (PASMCs). (A) Expression of miRNA-96 in human PASMCs from female and male PAH patients and non-PAH patient control (n=4-6, all isolates repeated 3 times). The expression of 5-HT_{1B}R mRNA (B) and protein (C) in human PASMCs from female and male PAH patients and non-PAH patient control (n=4-6, all isolates repeated 3 times) and representative immunoblot of 5-HT_{1B}R protein expression in human PASMCs from female and male patient and non-patient control. Data displayed as mean SEM. *P<0.05, determined by one-way analysis of variance with Tukey's post test.

Figure 3. Validation of miRNA-96 gene target 5-HT_{1B}R. (A) The effect of pre-miRNA-96 transfection on the expression of miRNA-96 (n=3). (B) The effect of pre-miRNA-96 on the luciferase activity of the psi-check-2 control construct, the 5-HT_{1B}R construct and mutated 5-HT_{1B}R construct (n=3, all isolates repeated 3 times). Data displayed as mean SEM. **P<0.01, ****P<0.001 determined by one-way analysis of variance with Tukey's post test.

Figure 4. The effect of serotonin (5-HT) on proliferation in human PASMCs. The proliferative response to serotonin in female non-PAH patient control (A), female PAH patient (B), male non-PAH patient control (C) and male PAH patient (D) human PASMCs (n=3, in triplicate). SB224289 is the selective 5-HT1BR antagonist and CP94253 is the selective 5-HT_{1B}R agonist. Data displayed as % of 2.5% FBS negative control. *P<0.05, **P<0.01, ****P<0.001, determined by one-way analysis of variance with Tukey's post test.

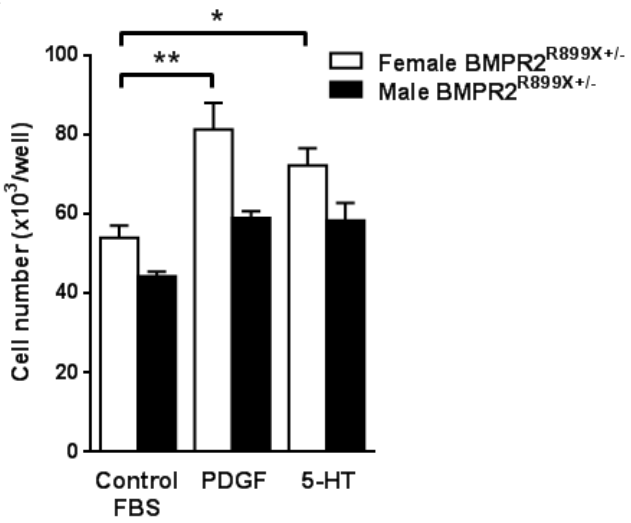
Figure 5. The effect of over-expressing miRNA-96 in human PASMCs from female patients. The effect of pre-miRNA-96 transfection in human PASMCs from female patients on miRNA-96 expression (A), 5-HT_{1B} mRNA (B) and 5-HT_{1B}R protein (C) (n=3, all isolates repeated 3 times). (D) The effect of over-expression of miRNA-96 on serotonin (5-HT) - induced proliferation in human PASMCs from female PAH patients (n=3, all isolates repeated 3 times). Data displayed as mean SEM. *P<0.05, **P<0.01, determined by one-way analysis of variance with Tukey's post test.

Figure 6. The effect of 17² -estradiol (E2) on miRNA-96 and 5-HT_{1B}R mRNA expression. The effect of E2 stimulation on miRNA-96 expression (A) and 5-HT_{1B}R mRNA expression (B) in human PASMCs (n=6, all isolates repeated 3 times). The effect of depleting endogenous E2 levels by inhibiting aromatase on miRNA-96 expression (C) and 5-HT_{1B}R mRNA expression (D) in whole female mouse lung homogenate (n=5, all isolates repeated 3 times). Data displayed as mean SEM. *P<0.05, **P<0.01, determined by one-way analysis of variance with Tukey's post test.

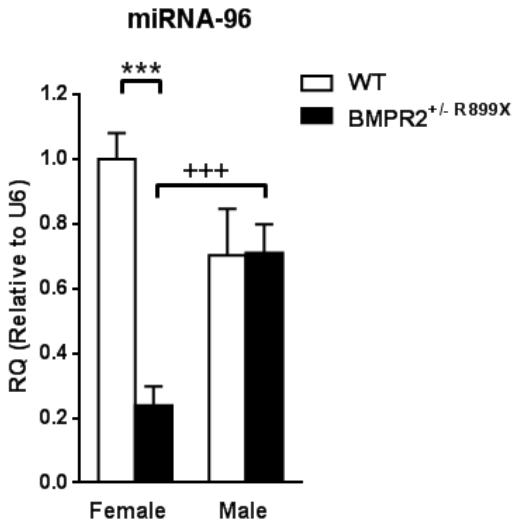
Figure 7. The effect of a miRNA-96 mimic on hypoxia-induced pulmonary hypertension in female mice. (A) The effect of 30µg dose (i.v.) of miRNA-96 mimic on miRNA-96 expression in pulmonary artery (n=5). (B) Representative images of miRNA-96 in situ hybridisation in pulmonary arteries from hypoxic mice (Scale bar 40µ). Effects of miRNA-96 mimic 30µg dose on (C) right ventricular systolic pressure (RVSP) (n=9-10), (D) right ventricular hypertrophy (RVH) (n=10) and (E) the % of remodelled pulmonary arteries (n=6) in normoxic and hypoxic female mice. (F) Representative images of pulmonary arteries (Elastin Van Giesen stain, scale bar 50µ). Data displayed as mean SEM. *P<0.05, **P<0.01, ***P<0.001determined by unpaired t-test and one-way analysis of variance with Tukey's post test.

Figure 8. The effect of a miRNA-96 mimic on miRNA, mRNA and protein level. (A) The effect of hypoxia and miR-96 mimic on BMPR2 expression (n=6), (B) miR-96 expression (n=6) and (C) 5-HT_{1B}R protein expression (n=6) in whole lung homogenate. (D) Schematic outlining proposed pathway: MiRNA-96 normally down-regulates the 5-HT_{1B}R which decreases serotonin-induced proliferation of pulmonary artery smooth muscle cells (PASMCs). Estrogen, hypoxia and BMPR-II deficiency in females can decrease miRNA-96 expression causing an increase in 5-HT_{1B}R expression which increases serotonin-induced proliferation and this may contribute to the pathobiology of PAH in females. Data displayed as mean SEM. *P<0.05, ***P<0.001, determined by one-way analysis of variance with Tukey's post test.

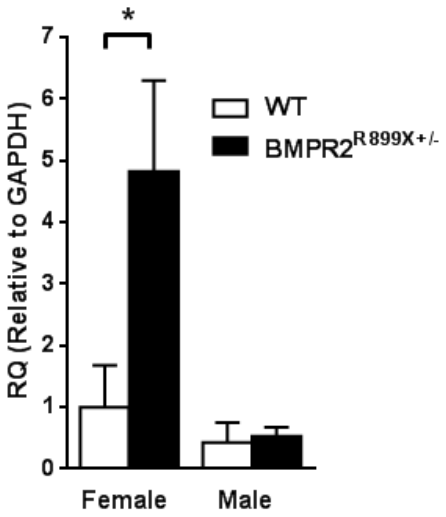
A


*
**
Female BMPR2R899X+/-
Male BMPR2R899X+/-
Cell number (x10^3/well)
100
80
60
40
20
0
Control FBS
PDGF
5-HT

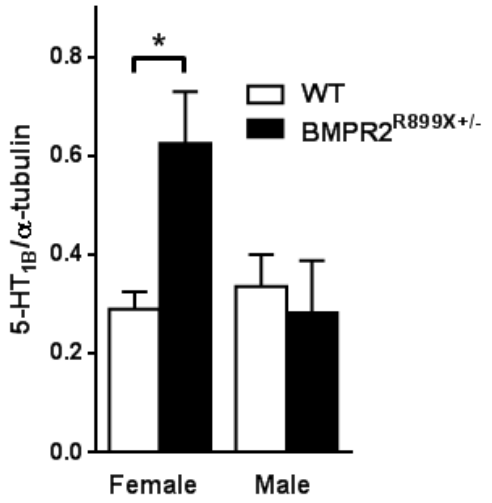
B

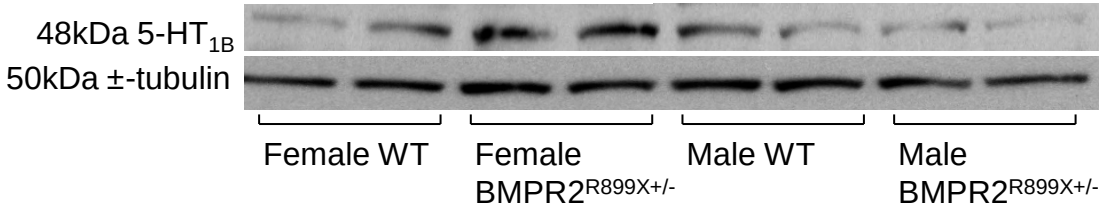

miRNA-96
***
+++
WT
BMPR2+/- R899X
RQ (Relative to U6)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Female
Male

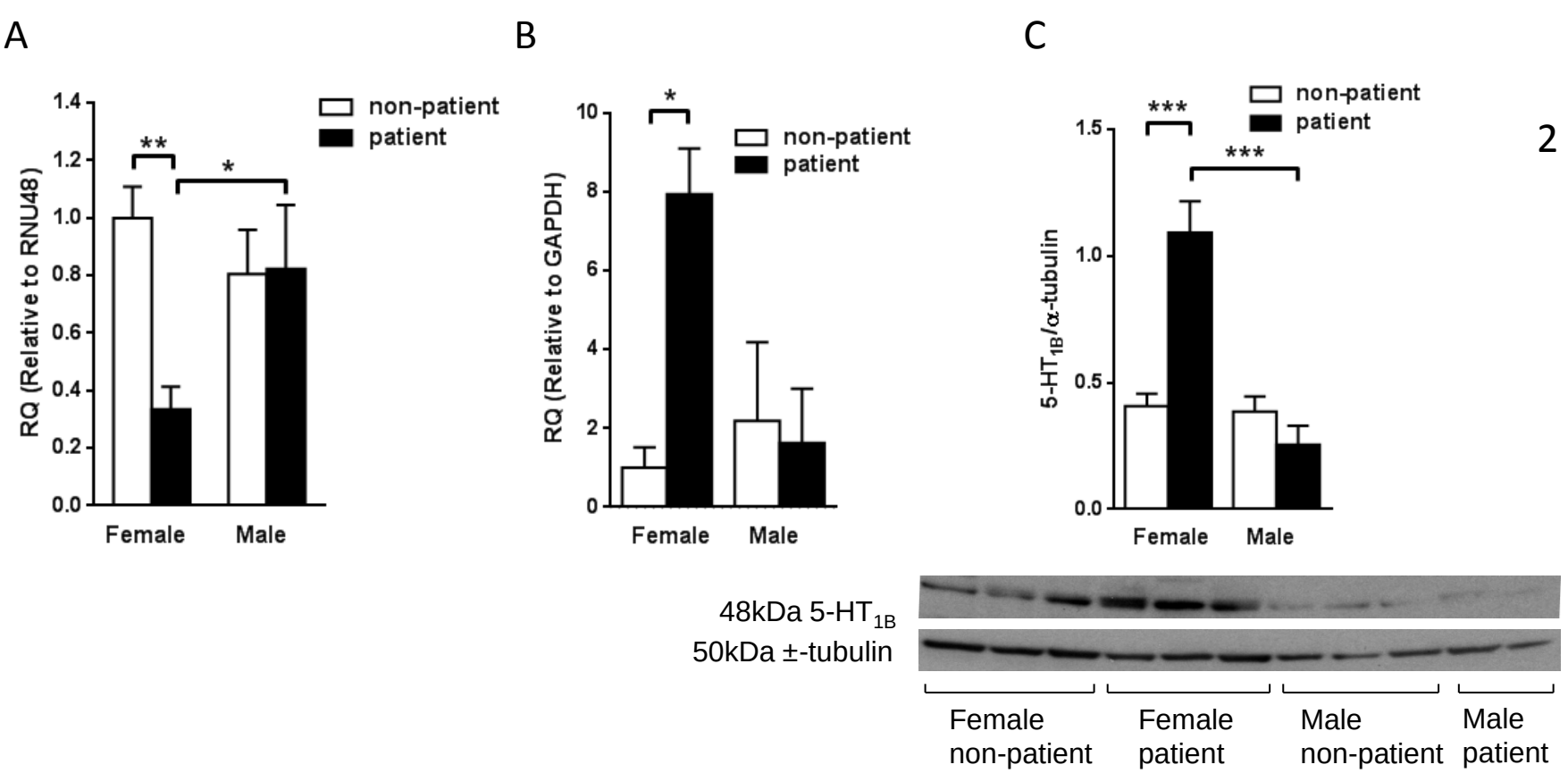
C


*
WT
BMPR2R899X+/-
RQ (Relative to GAPDH)
7
6
5
4
3
2
1
0
Female
Male

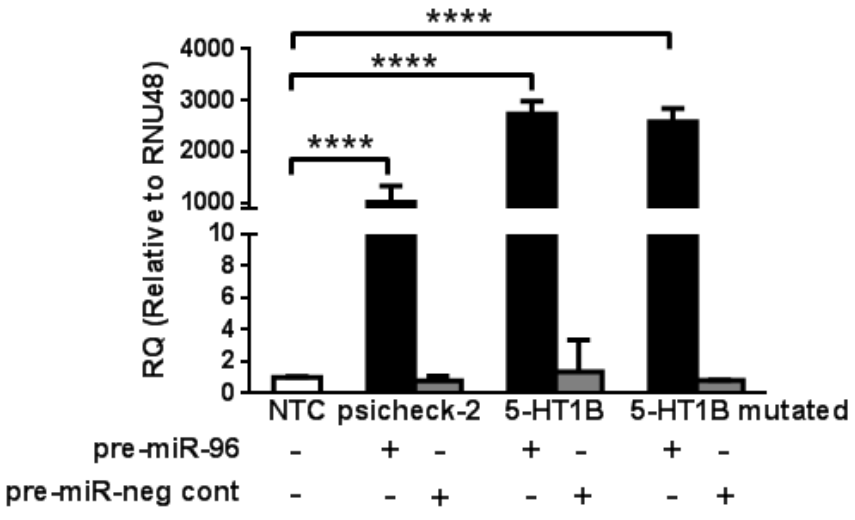
D


*
WT
BMPR2R899X+/-
5-HT1B/α-tubulin
0.8
0.6
0.4
0.2
0.0
Female
Male

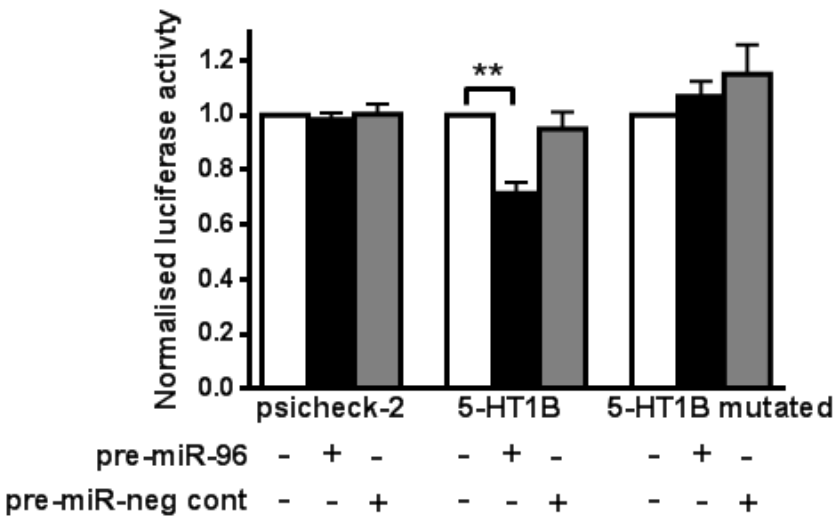

48kDa 5-HT1B
50kDa ±-tubulin
Female WT
Female BMPR2R899X+/-
Male WT
Male BMPR2R899X+/-

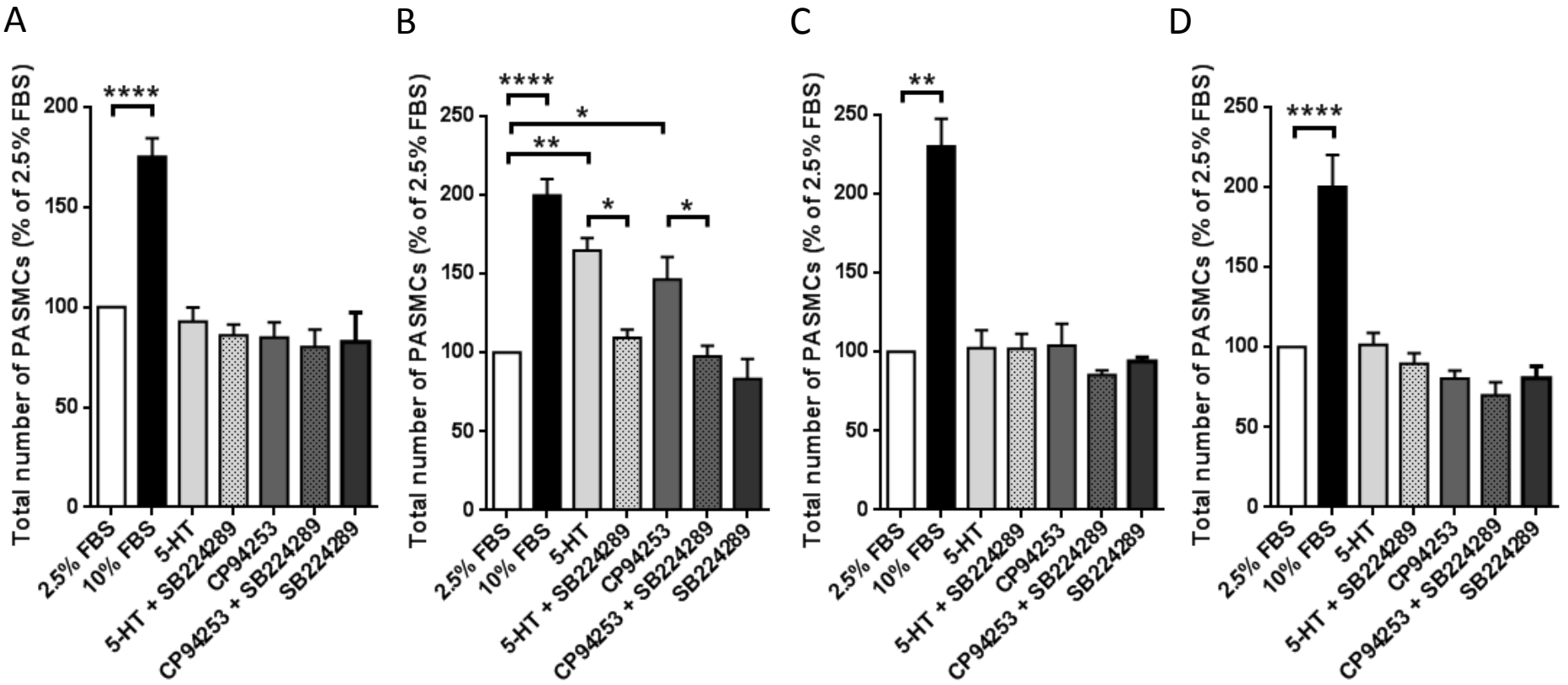

A
B
C
2
non-patient
patient
RQ (Relative to RNU48)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
Female
Male
RQ (Relative to GAPDH)
10
8
6
4
2
0
*
5-HT1B/α-tubulin
1.5
1.0
0.5
0.0
***
***
48kDa 5-HT1B
50kDa ±-tubulin
Female non-patient
Female patient
Male non-patient
Male patient

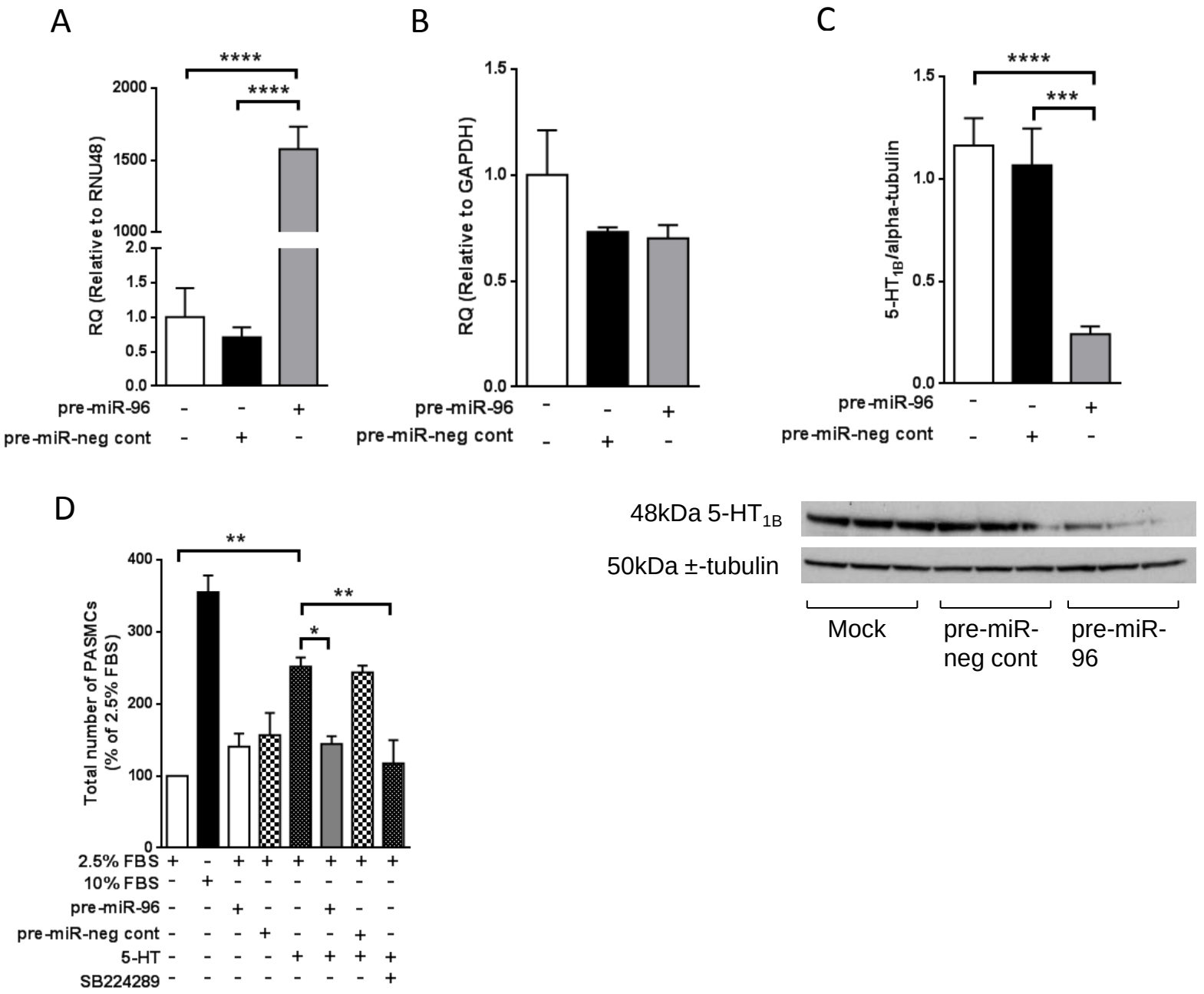
A

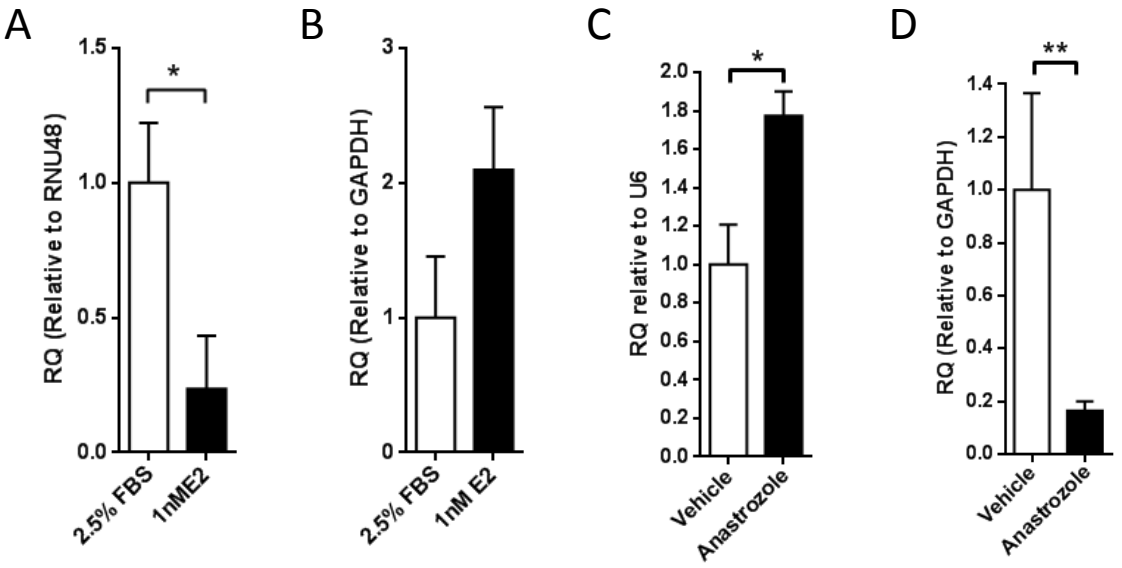

****
****
****
4000
3000
2000
1000
10
8
6
4
2
0
RQ (Relative to RNU48)
NTC
psicheck-2
5-HT1B
5-HT1B mutated
pre-miR-96 - + - + - + -
pre-miR-neg cont - - + - + - +

B

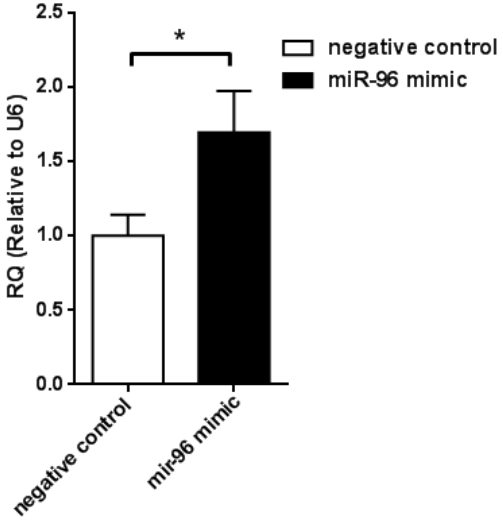

**
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Normalised luciferase activty
psicheck-2
5-HT1B
5-HT1B mutated
pre-miR-96 - + - - + - - + -
pre-miR-neg cont - - + - - + - - +


A
B
C
D
Total number of PASMCs (% of 2.5% FBS)
****
**
*
2.5% FBS
10% FBS
5-HT
5-HT + SB224289
CP94253
CP94253 + SB224289
SB224289

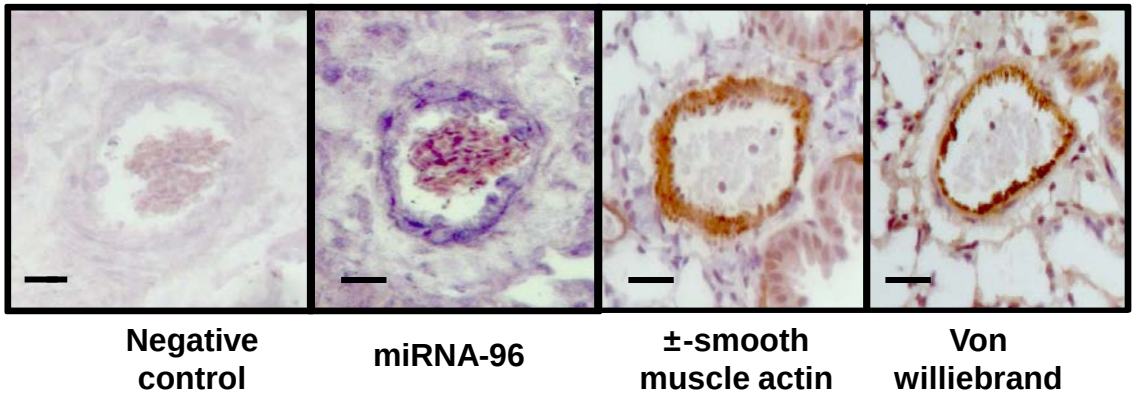

A
B
C
D
RQ (Relative to RNU48)
RQ (Relative to GAPDH)
5-HT1B/alpha-tubulin
pre-miR-96
pre-miR-neg cont
48kDa 5-HT1B
50kDa ±-tubulin
Mock
pre-miR-neg cont
pre-miR-96
Total number of PASMCs (% of 2.5% FBS)
2.5% FBS
10% FBS
5-HT
SB224289


A
RQ (Relative to RNU48)
1.5
1.0
0.5
0.0
*
2.5% FBS
1nME2
B
RQ (Relative to GAPDH)
3
2
1
0
2.5% FBS
1nM E2
C
RQ relative to U6
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Vehicle
Anastrozole
D
RQ (Relative to GAPDH)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Vehicle
Anastrozole

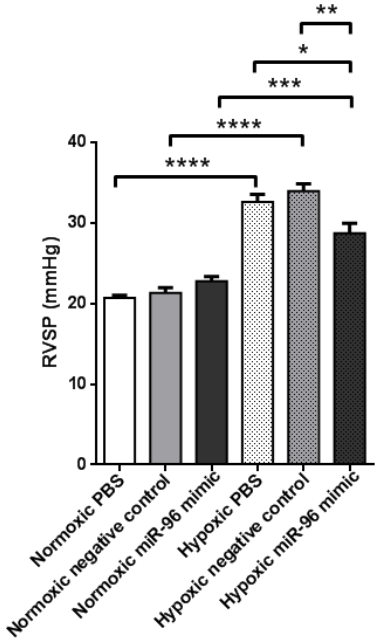
A


2.5
2.0
1.5
1.0
0.5
0.0
RQ (Relative to U6)
*
negative control
miR-96 mimic
negative control
mir-96 mimic

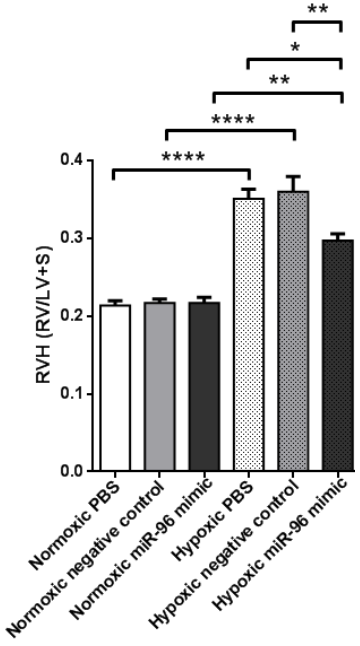
B


Negative control
miRNA-96
±-smooth muscle actin
Von williebrand

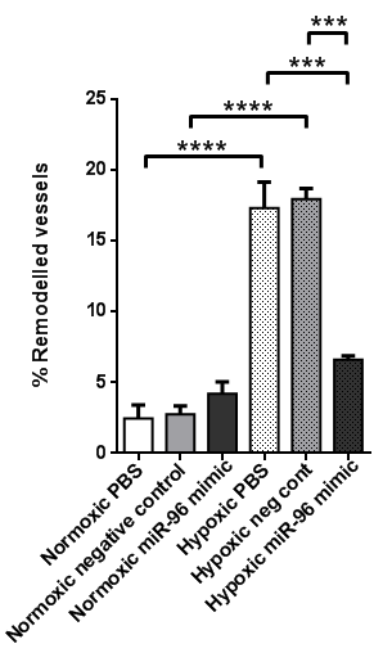
C


40
30
20
10
0
RVSP (mmHg)
****
****
***
*
**
Normoxic PBS
Normoxic negative control
Normoxic miR-96 mimic
Hypoxic PBS
Hypoxic negative control
Hypoxic miR-96 mimic

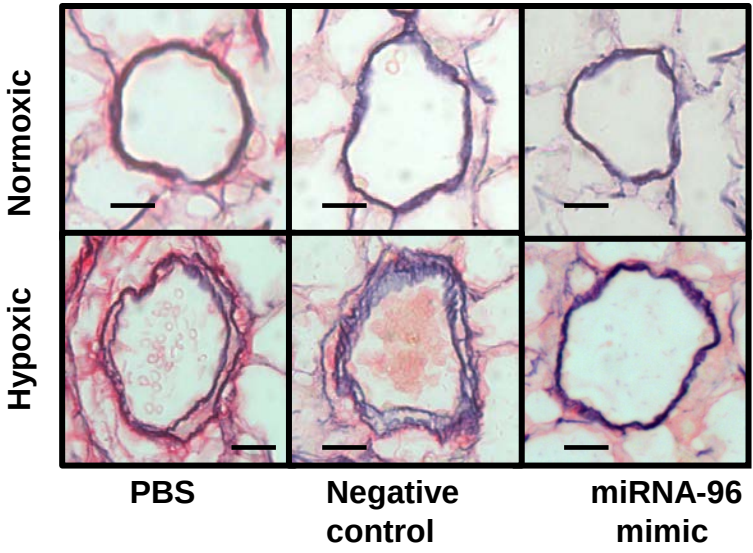
D

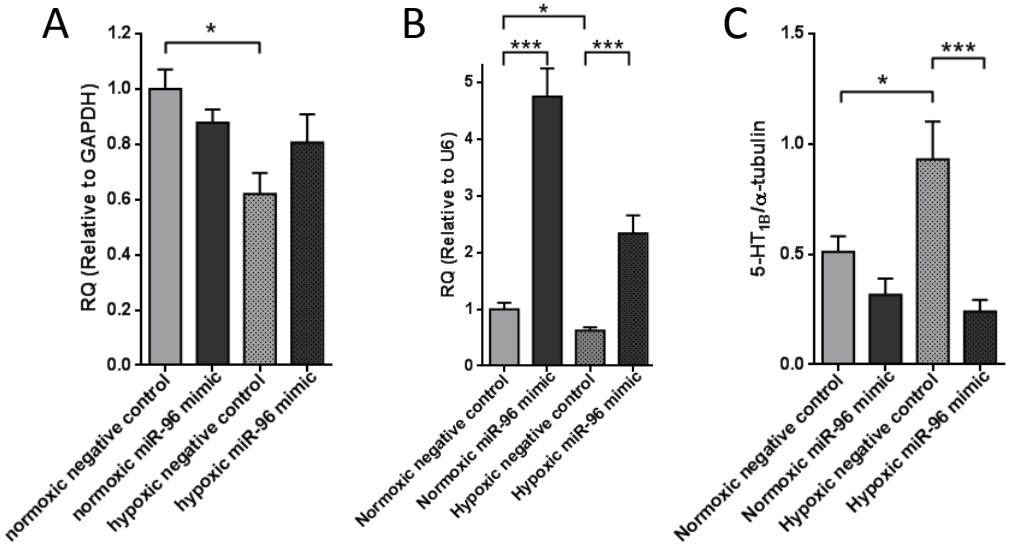

0.4
0.3
0.2
0.1
0.0
RVH (RV/LV+S)
****
****
**
*
**
Normoxic PBS
Normoxic negative control
Normoxic miR-96 mimic
Hypoxic PBS
Hypoxic negative control
Hypoxic miR-96 mimic

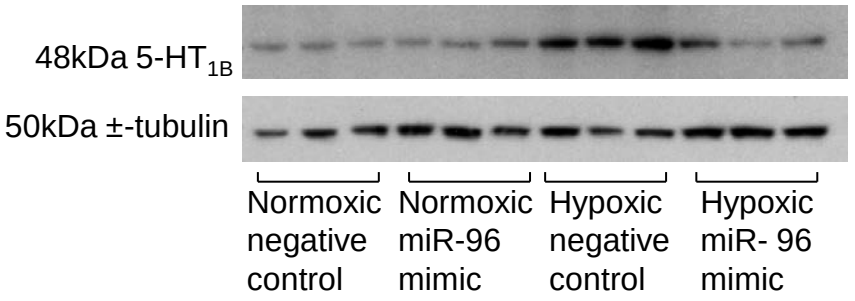
E


25
20
15
10
5
0
% Remodelled vessels
****
****
***
***
Normoxic PBS
Normoxic negative control
Normoxic miR-96 mimic
Hypoxic PBS
Hypoxic neg cont
Hypoxic miR-96 mimic

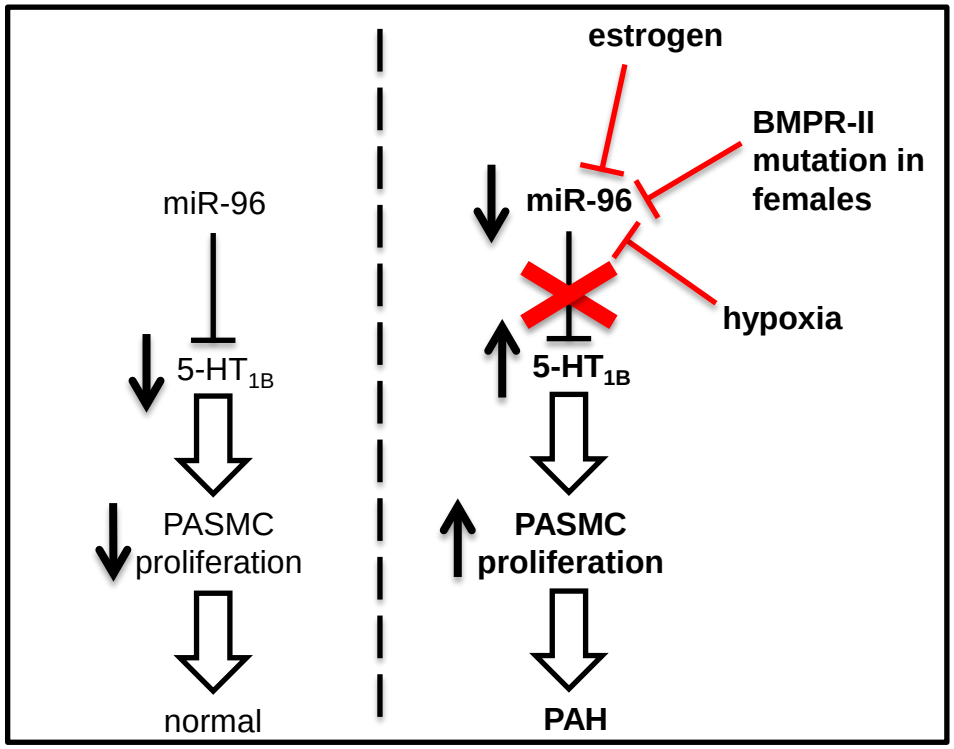
F


Normoxic
Hypoxic
PBS
Negative control
miRNA-96 mimic


A
RQ (Relative to GAPDH)
normoxic negative control
normoxic miR-96 mimic
hypoxic negative control
hypoxic miR-96 mimic
B
RQ (Relative to U6)
Normoxic negative control
Normoxic miR-96 mimic
Hypoxic negative control
Hypoxic miR-96 mimic
C
5-HT1B/α-tubulin
Normoxic negative control
Normoxic miR-96 mimic
Hypoxic negative control
Hypoxic miR-96 mimic


48kDa 5-HT1B
50kDa ±-tubulin
Normoxic negative control
Normoxic miR-96 mimic
Hypoxic negative control
Hypoxic miR- 96 mimic

D


miR-96
5-HT1B
PASMC proliferation
normal
estrogen
BMPR-II mutation in females
miR-96
hypoxia
5-HT1B
PASMC proliferation
PAH

A sex-specific microRNA-96/5HT$_{1B}$ axis influences development of pulmonary hypertension.

Online supplement

Emma Wallace, Nicholas W. Morrell, Xudong D. Yang, Lu Long., Hannah Stevens, Margaret Nilsen, Lynn Loughlin, Kirsty M. Mair, Andrew H. Baker and Margaret R. MacLean.

Methods

Ethical Information

All animal procedures conform to the UK Animal Procedures Act (1986) and 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). Experimental procedures using human lung tissue and human PASMCs (hPASMCs) conform to the principles outlined in the Declaration of Helsinki and were approved by Cambridgeshire 1 Research Ethics committee (REC reference: 08/H0304/56). All non-PAH human lung biopsies were confirmed as macroscopically normal and collected from patients undergoing pneumonectomy with no reported presence of PAH.

Pulmonary Artery Smooth Muscle Cell Culture

Female and male pulmonary artery smooth muscle cells (PASMCs) derived from $BMPR2^{+/-\ R899X}$ mice and littermate wild type control mice were provided by Prof. N. W. Morrell (University of Cambridge, Cambridge, UK). $BMPR2^{+/-\ R899X}$ mice have a heterozygous knock-in mutation of the BMPR2 gene. Briefly the main pulmonary artery was dissected from 3-6 month old mice and PASMCs were explanted, derived and grown in 20% FBS (Foetal Bovine Serum) DMEM Dulbecco's Modified Eagle Medium) with supplement Pack (C-39262, PromoCell, UK) before passage. The smooth muscle phenotype was confirmed by positive immunofluorescent staining using an antibody to smooth muscle specific ±-actin (Sigma, UK). The cells were used between passages 4~6 for experiments.

Female human PASMCs were provided by Prof. N. W. Morrell (University of Cambridge, Cambridge, UK). Briefly human PASMCs were explanted from the small

distal pulmonary arteries of the pulmonary vasculature from ten PAH patients, four with HPAH, three with IPAH, one with APAH and two with unknown forms of PAH. PASMCs derived from non-PAH subjects were utilised as controls. Pulmonary hypertension was not suspected on clinical or radiological grounds in these subjects and cells were derived from macroscopically normal tissue. HPASMCs were cultured in DMEM supplemented with 10% FBS in the presence of antibiotic antimycotic solution (containing penicillin, streptomycin and amphotericin). All hPASMCs were used between passage 4 and 8 and phenotype was confirmed via smooth muscle cell morphology. Details of hPASMCs utilised can be found in Table 1.

Cell Proliferation Assay

PASMCs were plated on 24-well or 96-well plates at equal density in 10% FBS DMEM. Once the cells reached ~60% confluency they were quiesced in 0.2% FBS phenol free DMEM for a period of 24 hours. PASMCs were then brought to 2.5% FBS for hPASMCS and 1% for mouse PASMCS and pre-incubated with 300nM of the selective 5-HT_{1B} receptor antagonist, SB224289 hydrochloride (Tocris, #1221), for 1 hour prior to 72 hour stimulation with either 1µM of serotonin hydrochloride (5-HT) (Tocris, #3547) or 1µM of the selective 5-HT_{1B} receptor agonist CP94253 hydrochloride (Tocris, #1317). To asses proliferation PASMCs were counted using the haemocytometer approach.

hPASMCs Transfection with miR-mimic

PASMCs were plated on 6-well and 24-well plates at equal density in 10% FBS DMEM. 50% confluent cells underwent transfection with 1nM pre-miR-96 (Ambion, PM10422) or pre-miR-negative control (Ambion, AM17110 #1) using Lipofectamine 2000 (Invitrogen) and optimem (Invitrogen) for a total of 6 hours before the media

was replaced with 10% FBS DMEM. For expression analysis each condition was performed in duplicate to allow RNA harvest 48 hours and protein harvest 72 hours post-transfection for Taqman and western blot analysis respectively. For proliferation analysis PASMCs were then quiesced in 0.2% FBS and the above proliferation protocol followed.

Taqman quantitative-PCR Analysis of miRNAs and mRNAs

Total RNA was extracted using the miRNeasy kit (Qiagen) according to the manufacturer's instructions and RNA purity quantified using NanoDrop-1000 Spectrophotometer. Expression of miR and mRNA was assessed by Taqman quantitative-PCR as previously described (1, 2). To obtain a fold change miR expression data was normalised to U6 (Applied Biosystems, #001973) for mouse and RNU48 (Applied Biosystems, #001006) for human. mRNA expression data was normalised to GAPDH (Applied Biosystems, #Mm03302249 and #Hs02758991).

Western Blot Analysis of Protein

Protein was extracted using the Radio-Immunoprecipitation Assay (RIPA) lysis method. The RIPA buffer was supplemented with proteases inhibitors phenylmethanesulfonylfluoride (PMSF), soybean trypsin inhibitor and benzamidine. Briefly, supplemented RIPA buffer was added to PASMCs which were then agitated on ice for 10 minutes. Cell lysates were collected via scraping and left on ice for 30 minutes to promote disassociation of proteins. Lysates were then briefly sonicated before being spun for 10 minutes at 10000rpm 4° to achieve a cell pellet. Protein concentration was determined by colorimetric bicinchoninic acid (BCA) assay system (Pierce). Protein expression data was normalised to GAPDH and quantified using densitometry.

Lung Immunohistochemistry

Mouse lungs inflated with 10% neutral buffered formalin (NBF) were cut into 5 micron frontal sections for immunohistochemical staining as previously described (3, 4). Lung sections were stained with elastin and picosirius and remodelling of the pulmonary vasculature confirmed by the presence of a double elastic laminae.

Luicferase Reporter Assay

The psi-CHECK-2 dual luciferase reporter vector (Promega) was utilised for the reporter assay. Briefly, a fragment of the 3'UTR (un-translated region) of the 5-HT1B receptor gene was generated by polymerase chain reaction (PCR) from genomic mouse DNA and was cloned via the in-fusion cloning method into the multiple cloning site of the psi-CHECK-2 vector at the XhoI and the NotI restriction sites. The psi-CHECK-2 5-HT1B vector was then sequenced to confirm there was no unwanted mutations within the 3'UTR region of the 5-HT1B. Site-directed mutagenesis was performed to create a single point substitution mutation in the $5\text{-}HT_{1B}$ 3'UTR to further assess miRNA-96 binding. This was confirmed by sequencing analysis. 1ug of psi-CHECK-2 control vector, psi-CHECK-2 5HT1B vector or psi-CHECK-2 mutated 5-HT1B vector along with either pre-miR-96 or pre-miR negative control were co-transfected into HeLa cells using Lipofectamine 2000 and optimem for a total of 6 hours before being replaced with 10% FBS DMEM. HeLa cells were left for 48 hours and the luciferase activity measured using the Dual-Glo Luciferase Assay System (Promega) and luminescence detection performed via LUMIstar OPTIMA microplate reader (BMG Labtech). The renilla luciferase activity was normalised to the internal firefly luciferase activity and data expressed as a percentage of the internal control.

Administration of miRNA-96 mimic

To assess whether miRNA-96 was involved in the pathology of pulmonary hypertension, the miRNA-96 mimic (Applied Biosystems, #MC10422) was administered intravenously via the tail vein once a week for 2 weeks using the MaxSuppressor™ In Vivo RNA-LANCEr II delivery method as previously described to facilitate delivery to the lung (31). The mimic was prepared in the delivery reagent as per manufacturing instructions at a dose of 1.5mg/kg per injection i.e. ~30ug per mouse per injection. Negative miRNA mimic (Applied Biosystems) and PBS dosed animals were used as controls.

To confirm that this delivery protocol delivered miRNA-96 to the pulmonary artery, miRNA-96 levels were determined in isolated main pulmonary arteries following administration of the miRNA-96 mimic. In situ analysis was also performed to visualize distribution of miRNA-96 in the lung.

In Vivo Haemodynamics

Hameodynamic measurements were performed in mice as previously described (3, 4). Briefly, the left common carotid artery was cannulated to allow continuous observation of systemic mean arterial blood pressure (MAP). The right ventricle of the heart was catheterised to allow measurement of right ventricular systolic pressure (RVSP) via a transdiaphragmatic technique. Data was acquired using Biopac (Biopac Systems, CA, USA). After in vivo measurements were obtained mice were killed by anaesthetic overdose. Whole hearts were excised and measurement of right ventricular hypertrophy (RVH) determined by the ratio of the free right ventricle (RV) wall weight and the left ventricle (LV) wall plus septum (S) weight (RV/LV+S). Tissues were excised and snap frozen for further analysis.

In situ Hybridisation

In situ hybridization was performed as previously described (5). Briefly 5µm frontal plane lung sections were deparaffinised in an ethanol gradient. Sections were then treated with proteinase K (20µg/ml) at 37°C for 20 minutes before being fixed with 4% formaldehyde and ethyl–3– (3–dimethylaminopropyl) carbodiimide (EDC) solution. To inactivate endogenous enzymes, lung sections were treated with acetylation buffer. Next lung sections were pre-hybridised for 1 hour at probe annealing temperature before overnight probe annealing in hybridisation oven. Double DIG-labelled (3' and 5') miRCURY LNA hsa-miR-96 probe (50nmol/L) and miRCURY LNA scramble-miR probe (negative control) were used at hybridisation temperature 52°C and 57°C respectively. Post-hybridisation stringency washes with saline sodium citrate buffer were performed before inactivation of endogenous peroxidise activity by 3% hydrogen peroxide. Immunodetection was carried out by the DIG nucleic acid detection kit (ROCHE ~ 11175041910) as per manufacturer's instructions. Positive staining was evident by a purple colour.

Table 1. PAH Patient and non-PAH patient information.

Patient group	Sex	Age	Disease Status
Non-PAH	Female	58yrs	Mild emphysema
	Female	64yrs	N/A
	Female	N/A	N/A
	Female	71yrs	Adenocarcinoma
	Female	59yrs	N/A
	Female	70yrs	N/A
	Male	72yrs	N/A
	Male	62yrs	Emphysema
	Male	N/A	N/A
	Male	68yrs	N/A
	Male	60yrs	Squamous cell carcinoma
	Male	75yrs	N/A
PAH	Female	N/A	HPAH (N903S)
	Female	33yrs	IPAH
	Female	24yrs	IPAH

	Female	**N/A**	**PAH**
	Female	**30yrs**	**HPAH (R899X)**
	Female	**N/A**	**IPAH**
	Male	**43yrs**	**APAH**
	Male	**N/A**	**HPAH (C347R)**
	Male	**17yrs**	**HPAH (W9X)**
	Male	**N/A**	**PAH**

Table 1. Known characteristics of patients from whom cells were derived. n/a, not available; HPAH, heritable pulmonary arterial hypertension (gene mutation in parenthesis); IPAH idiopathic pulmonary arterial hypertension; APAH, associated pulmonary arterial hypertension. N/A: information not available.

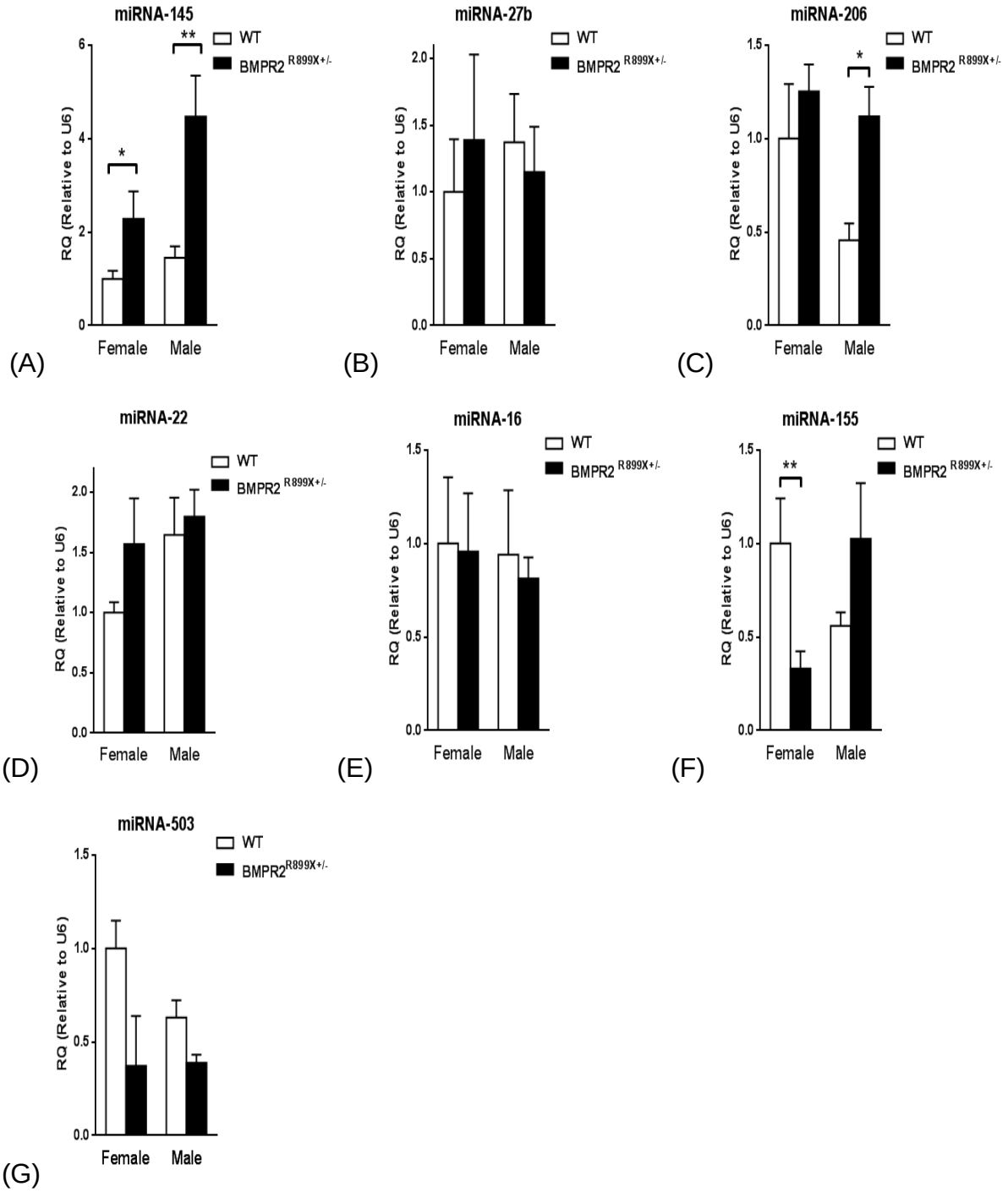
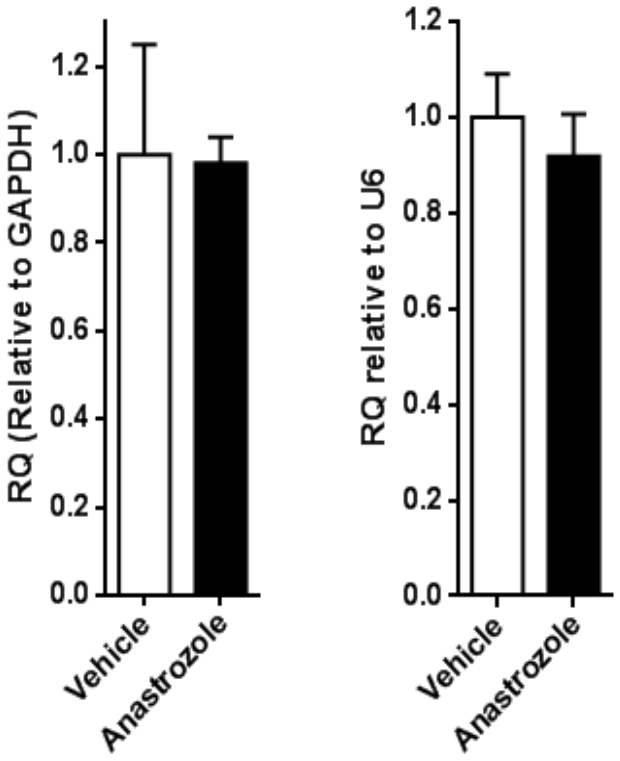
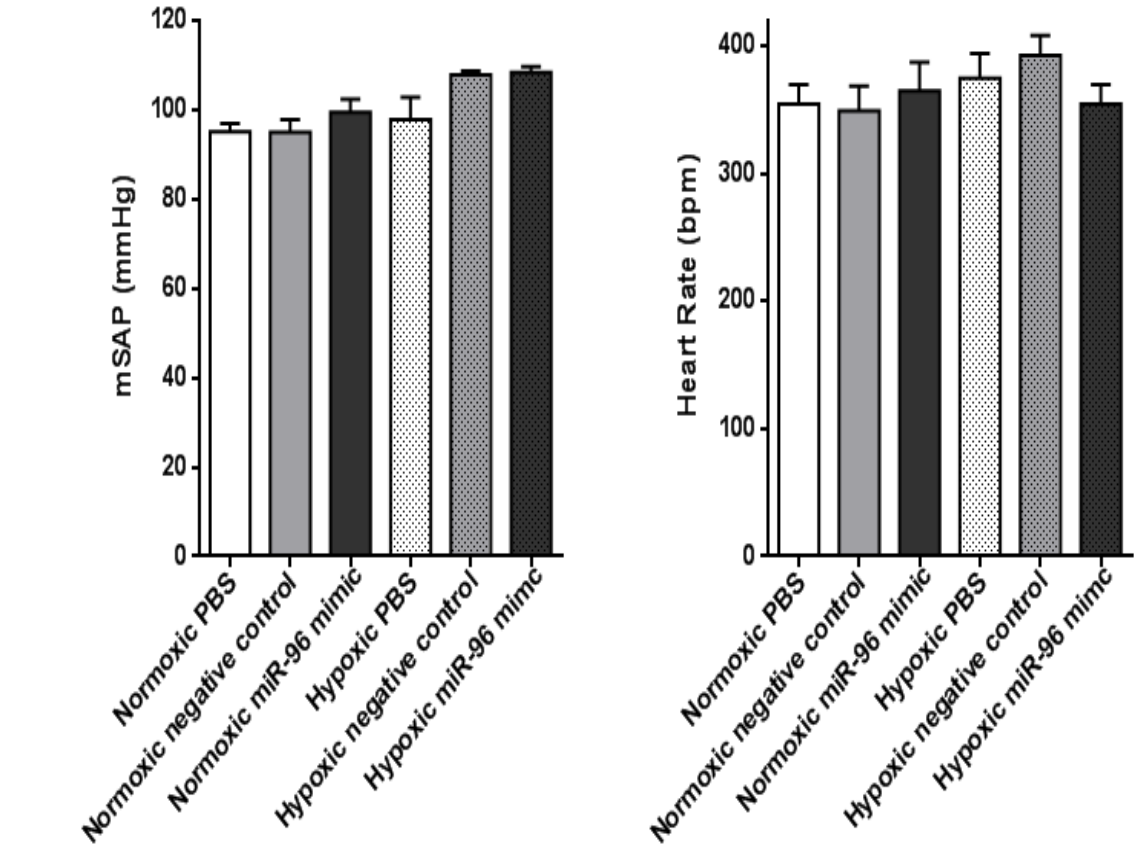


Figure E1. MiRNA expression in pulmonary artery smooth muscle cells (PASMCs) from female and male BMPR2$^{R899X+/-}$ and wild-type (WT) mice. Expression of (A) miRNA-16, (B) miRNA-22, (C) miRNA-27b, (D) miRNA-155, (E) miRNA-206, (F) miRNA-145 and (G) miRNA-503 in PASMCs from female and male BMPR2$^{R899X+/-}$ and WT mice (n=8, all isolates repeated 3 times). Data displayed as mean SEM. *P<0.05, ** P<0.01, ***P<0.001 determined by one-way analysis of variance with Tukey's post test.



(A) **(B)**

Figure E2. The effect of estrogen on miRNA-96 and 5-HT_{1B} mRNA expression. The effect of depleting endogenous 17² -estradiol levels by inhibiting aromatase on miRNA-96 expression (A) and 5-HT_{1B} mRNA expression (B) (n=5, all isolates repeated 3 times). Data displayed as mean SEM.



(A) **(B)**

Figure E3. The effect of a miRNA-96 mimic on hypoxia-induced pulmonary hypertension in female mice. The effect of 30µg dose (i.v.) of miRNA-96 mimic on (A) mean systemic arterial pressure (mSAP) and (B) heart rate (HR) (n=9-10). Data displayed as mean SEM.

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