SUPPLEMENTAL MATERIAL

Data S1.

Expanded Materials and Methods

Animal studies

All experimental procedures were carried out in accordance 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 of Glasgow Ethics Committee. All *in vivo* studies and subsequent analysis were carried out blinded. There were two reasons for selecting the hypoxic rat model in this study. One was that we were focusing on the hypothesis that 2ME2 may exert its therapeutic effect via HIF1α and hence hypoxia on its own was the stimulus of choice. Secondly, we were interested in *in vivo* sex differences and many studies have shown that hypoxia-induced PH in rats is more severe in males than females and we wished to determine the effects of 2ME2 on these *in vivo* sex-dependent differences. Male and female Sprague Dawley rats weighing 200g were purchased from Envigo (Germany). Rodents were housed in a 12-hour light dark cycle with access to food and water ad libitum. For hypoxic studies, rats were placed in a hypobaric chamber for two weeks at an atmospheric pressure of 550 mbar after which they were removed for dosing. Animals were anaesthetised using 3% (v/v) isoflurane and a small incision was made in the scruff of the neck. A 3 mm slow-release pellet containing either 2ME2 (1.26 mg pellet/21 days; 60ug/kg/day) (Abcam, UK/ Innovative research of America, USA) or vehicle carrier (Innovative research of America, USA) was implanted using a 10 gauge stainless steel precision trochar

(MP-182; Innovative research of America, USA). The wound was then closed using medical grade glue (3M Vetbond, USA). Animals were allowed to recover fully before being placed back into hypobaric conditions for a further two weeks. Age and weight matched animals were also dosed in normoxic conditions for the control portion of the study using an identical procedure. No obvious irritation at implant site was observed for any animal in this study. Dosage of 2ME2 was based on previous published studies [1].

Haemodynamic measurements

Animals were anaesthetically induced in 3% (v/v) isoflurane and then maintained at approximately 2% (v/v) isoflurane supplemented with a constant flow of medical oxygen (1L/min). Right ventricular systolic pressure (RVSP) measurements were taken using a Miller (UK) SPR-869 catheter and measurements recorded using the corresponding software (LabChart Pro). The catheter was inserted into the exposed right jugular vein and guided into the right ventricle of the heart to measure right ventricular pressure.

Right ventricular hypertrophy

In order to assess right ventricular hypertrophy the right ventricular (RV) free wall was removed and weighed. This was then expressed as a ratio to the left ventricular wall plus septum (LV+S) weight (RV/LV+S).

Culture and isolation of PASMCs

Female and male PASMCs were isolated from pulmonary arteries of patients undergoing a pneumonectomy procedure (0.3 – 1 mm diameter) from distal portions of macroscopically normal lung tissue or from lungs of patients undergoing lung transplantation, as described previously. All human cells were isolated in Professor Nicholas Morrell's laboratory, Cambridge, UK. Experimental procedures using hPASMCs conform to the principles outlined in the declaration of Helsinki. All cells used within this study are detailed in Table S1 and were used between passages 4 and 8. Female rat PASMCs were isolated by enzymatic digestion of intra-lobar pulmonary arteries. Briefly, connective tissue/parenchyma/adventitia was removed from the pulmonary arteries. Under sterile conditions, the pulmonary arteries were washed with PBS, cut into smaller pieces and placed into enzymatic dissociation buffer pre-heated to 37°C (Ham's F-12 culture medium (Sigma, UK) containing 1% gentamycin, 1mg/ml type 1 collagenase, 0.125mg/ml elastase, 0.375mg/ml soybean trypsin inhibitor and 2mg/ml bovine serum albumin (Sigma, UK)). The tissue mixture was agitated gently at 37°C for 1-2 hours. Digestion mix was then passed through a 100 µm strainer and centrifuged at 1200 rpm for 3 minutes. The supernatant was removed and cells were re-suspended in fresh F12 medium (1% Penicillin-Streptomycin, 10% FBS) for passage 0. At passages 1-4 cells were grown in 4.5g/l DMEM (1% Penicillin-Streptomycin, 10% FBS; Sigma, UK). Rat PASMCs were utilised for experiments between passages 2 and 4.

Cellular proliferation experiments

Cellular proliferation was assessed manually using a haemocytometer. Briefly, cells in 12-well dishes were grown to 50-60% confluency. Cells were then quiesced in 0.2% charcoal-striped serum (CSS) [2] for 24 hours. Media was then changed to 5% CSS and 2ME2 (Abcam, UK) or vehicle (ethanol) added to the well. After 48 hours cells were trypsinized and counted. Cellular proliferation/viability was also measured using the CCK-8 kit (Dojindo, Japan). Cells were grown in 96-well plates to 50% confluency then quiesced for 24 hours in 0.2% CSS. Cells were stimulated with 2ME2 or vehicle in 5% CSS. After for 46 hours the CCK-8 substrate was added to each well and incubated at 37°C for 2 hours. A plate reader was then used to measure the O.D. at 450nm.

Caspase activity assay

Cells were grown in 96-well culture dishes and stimulated for 6 hours. After 6 hours the media was removed and Cell Event reagent added for 30 mins. Fluorescent signal was then detected using a SpectraMax M2 plate reader with absorption/emission maxima of ~502/530 nm.

Immunoblotting

Protein expression was assessed by immunoblotting in whole lung tissue and hPASMCs. Whole lung rat samples were homogenized and lysed in RIPA buffer (Sigma, UK) containing HALT protease and phosphatase inhibitors (Thermo-Fisher, UK). hPASMCs were lysed in ice-cold 1% (v/v) lauryl maltoside/PBS (Abcam, UK) containing HALT protease and phosphatase inhibitors. Protein concentrations were

determined using BCA assay (Thermo-Fisher, UK) or by nanodrop (ND-1000 spectrophotometer (Thermo-Fisher, UK). 20µg of protein was loaded for hPASMCs and whole lung lysates, for protein identification by SDS-PAGE and immunoblotting. Protein expression was quantitated in immunoblots probed the relevant antibody by overnight incubation at 4°C. Membranes were then incubated with anti-rabbit or anti-mouse secondary antibodies. Immunoblots were developed using Pierce™ ECL Western Blotting Substrate (Thermo-Fisher, UK) or EMD Millipore Immobilon™ Western Chemiluminescent HRP Substrate (ECL) (Fisher Scientific) and normalized to beta actin (Sigma, A5441). Densitometric analysis was performed using TotalLab TL100 software. A full list of antibodies and dilutions are detailed in Table S2. In some instances blots were cut and probed for two separate antibodies. This was only carried out where proteins of interest exhibited a large difference in molecular weight and where antibodies have been validated and shown to be specific for their target. Blots where appropriate may be stripped and re-probed using loading controls.

Imaging

Cellular localisation of α-tubulin in hPASMCs was assessed by immunofluorescence. Briefly, cells were grown on glass coverslips until 50-60% confluent. Cells were then washed in PBS and fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature. Cells were then permeabilized with 0.2% Triton X-100/PBS (Sigma, UK) and blocked with 2%BSA/PBS with 0.05% Tween-20 (Sigma, UK). α-tubulin primary antibody (1:500; Sigma, UK) was added to the coverslips and incubated overnight at 4ºC. Cells were then washed in PBS and incubated with secondary

antibody for 1 hour at room temperature (Alexa goat anti-mouse 594, ThermoFisher Scientific, UK). After a further PBS wash, ProLong™ Diamond antifade mountant with DAPI (ThermoFisher Scientific, UK) was applied to the coverslips and mounted on glass slides for imaging. Images were acquired using an LSM-510 or LSM-800 laser-scanning confocal microscope (Zeiss, Germany). Brightfield images were captured using an EVOS XL core microscope (ThermoFisher Scientific). All subsequent cellular measurements were performed using ImageJ (NIH) software.

TaqMan™ RT-PCR

RNA was extracted by finely mincing tissue with a scalpel then placing into QIAzol solution (Qiagen, UK). Tissue was then lysed using a Qiagen Tissue lyser and RNA was extracted with miRNeasy® mini kit following the manufacturers protocol (Qiagen, UK). Cells were scraped and also lysed using QIAzol solution. RNA was quantified using a nanodrop (ND-1000 spectrophotometer (Thermo-Fisher, UK) and normalised to approximately 300ng total RNA. RNA was then reverse transcribed to cDNA using the TaqMan™ reverse transcription kit (Applied Biosystems). Semi-quantitative real-time PCR was performed using an Applied Biosystems Viia 7 real-time PCR system. Specific dual labelled TaqMan™ primer-probe sets were purchased from Thermo-Fisher, UK (Table S3). Results are expressed as a ratio to a reference gene using the $2^{-\Delta Ct}$ comparison method or as fold change when comparing drug treatments.

Histopathology

Pulmonary vascular remodelling was assessed using 5μm sections cut and stained using Millers elastin/Picro Sirius Red for identification of vascular remodelling, characterized by a distinctive double elastic lamina. The total number of remodelled vessels was expressed over the total number of vessels present in a lung section as assessed in a blinded fashion.

Table S1. Patient details of non-PAH male and female hPASMCs used within this study.

Cell type	Patient Sex	Patient details	Patient age
Non-PAH	Female	Mild emphysema	64
Non-PAH	Female	Adenocarcinoma	71
Non-PAH	Female	Emphysema	57
Non-PAH	Female	Lung carcinoma	70
Non-PAH	Female	Squamous carcinoma	57
Non-PAH	Female	n/a	56
Non-PAH	Female	Squamous carcinoma	59
Non-PAH	Female	Mild emphysema	58
Non-PAH	Female	n/a	64
Non-PAH	Male	Emphysema	62
Non-PAH	Male	n/a	72
Non-PAH	Male	Squamous carcinoma	60
Non-PAH	Male	Adenocarcinoma	78
Non-PAH	Male	n/a	n/a
Non-PAH	Male	Adenocarcinoma	52
Non-PAH	Male	Lung cancer (unknown)	75

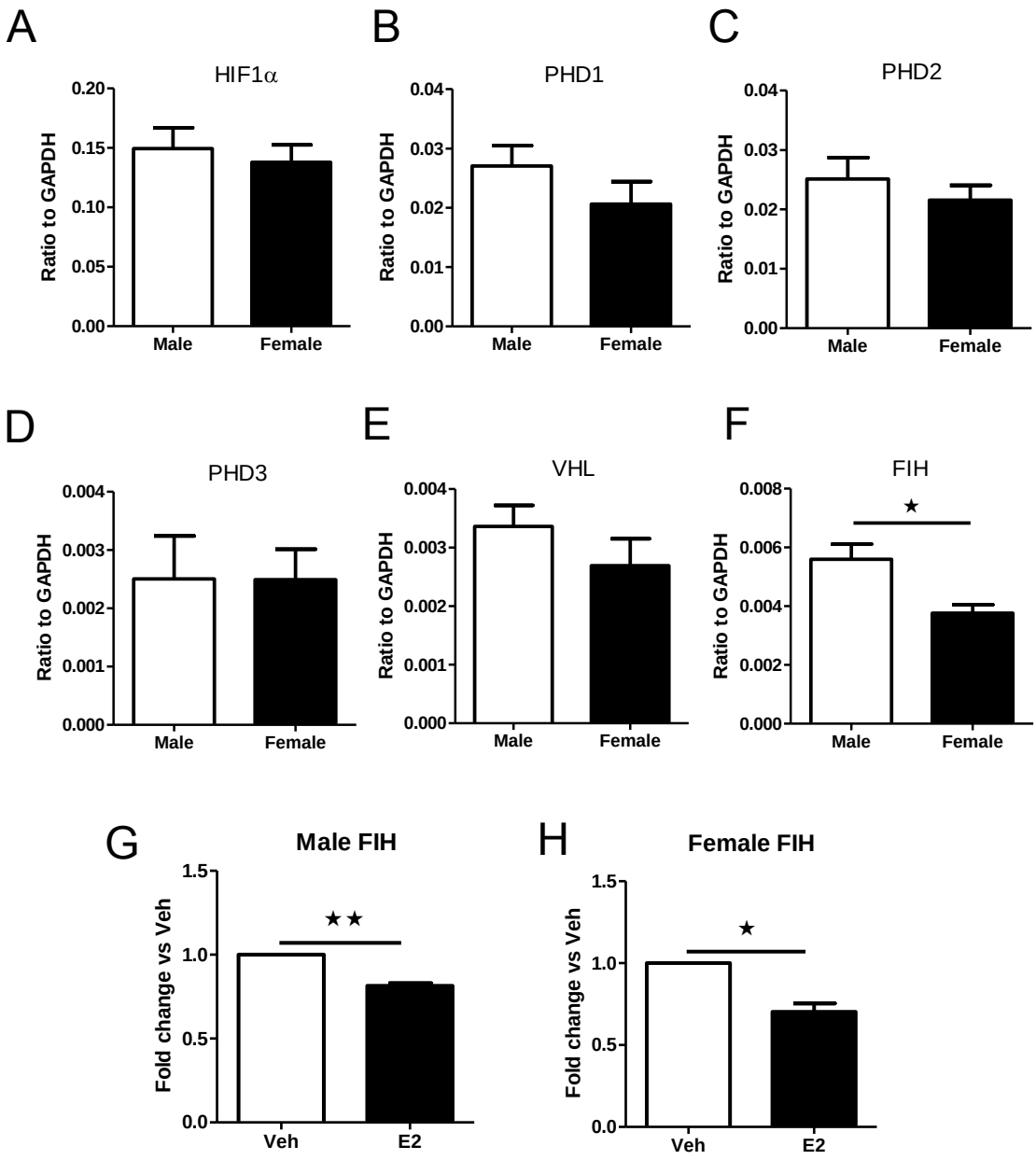
Table S2. Antibodies and dilutions used for Western blotting (WB) or immunofluorescence (IF).

Antibody	Type	Supplier (catalogue #)	Dilution
HIF1α	Rabbit	Novus Biologicals (NB100-105)	WB 1:500
HIF1α	Mouse	BD Bioscience (610958)	WB 1:500
PCNA	Rabbit	Abcam (2426)	WB 1:200
Hexokinase 2	Rabbit	Cell Signaling (2867)	WB 1:1000
PHD2	Rabbit	Cell Signaling (4835)	WB 1:1000
FIH	Rabbit	Novus Biologicals (NB100-428)	WB 1:500
BMPR2	Mouse	BD Bioscience (612292)	WB 1:500
α-tubulin	Mouse	Abcam (7294)	WB 1:5000 IF 1:500
β-actin	Mouse	Sigma (A5441)	WB 1:5000

Table S3. Probe sets used within this study and Taqman™ assay ID details.

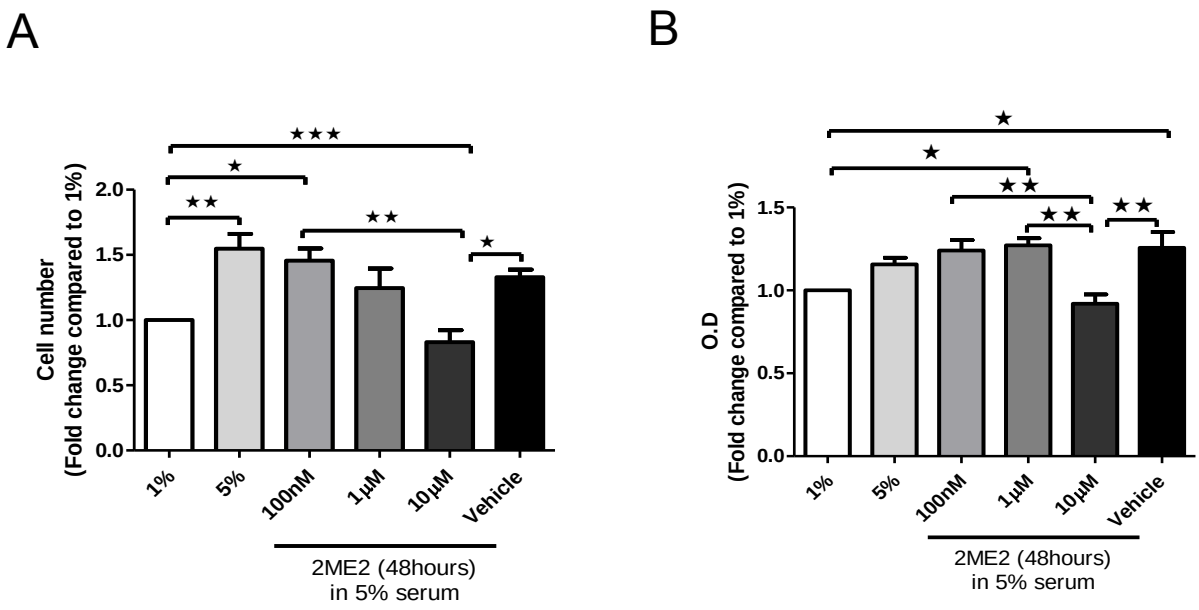
Gene	Target	Assay ID
HIF1α	Rat	Rn01472831
HIF1α	Human	Hs00153153
PHD1	Human	Hs00914594
PHD2	Human	Hs00990001
PHD3	Human	Hs00420085
VHL	Human	Hs00184551
FIH	Human	Hs00215495
Bax	Human	Hs00180269
RIPK1	Human	Hs01041869
BMPR2	Human	Hs00176148
Caspase9	Human	Hs00962278
TP53	Human	Hs01034249

Figure S1. HIF1α signaling in female hPASMCs.



mRNA transcript expression levels of HIF1α (A) Prolyl hydroxylase 1 (PHD1; B), Prolyl hydroxylase 2 (PHD2; C), Prolyl hydroxylase 3 (PHD3, D), Von-Hippel Lindau tumour suppressor (VHL; E) and Factor inhibiting HIF (FIH, F). Effect of 72 hours treatment with 100nM E2 on FIH mRNA expression in male (G) and female (H) hPASMCs. Data is shown as mean ± SEM. ★ $p<0.05$; ★ ★ $p<0.01$ determined by unpaired or paired t-test. hPASMC, human pulmonary arterial smooth muscle cells; Veh, vehicle.

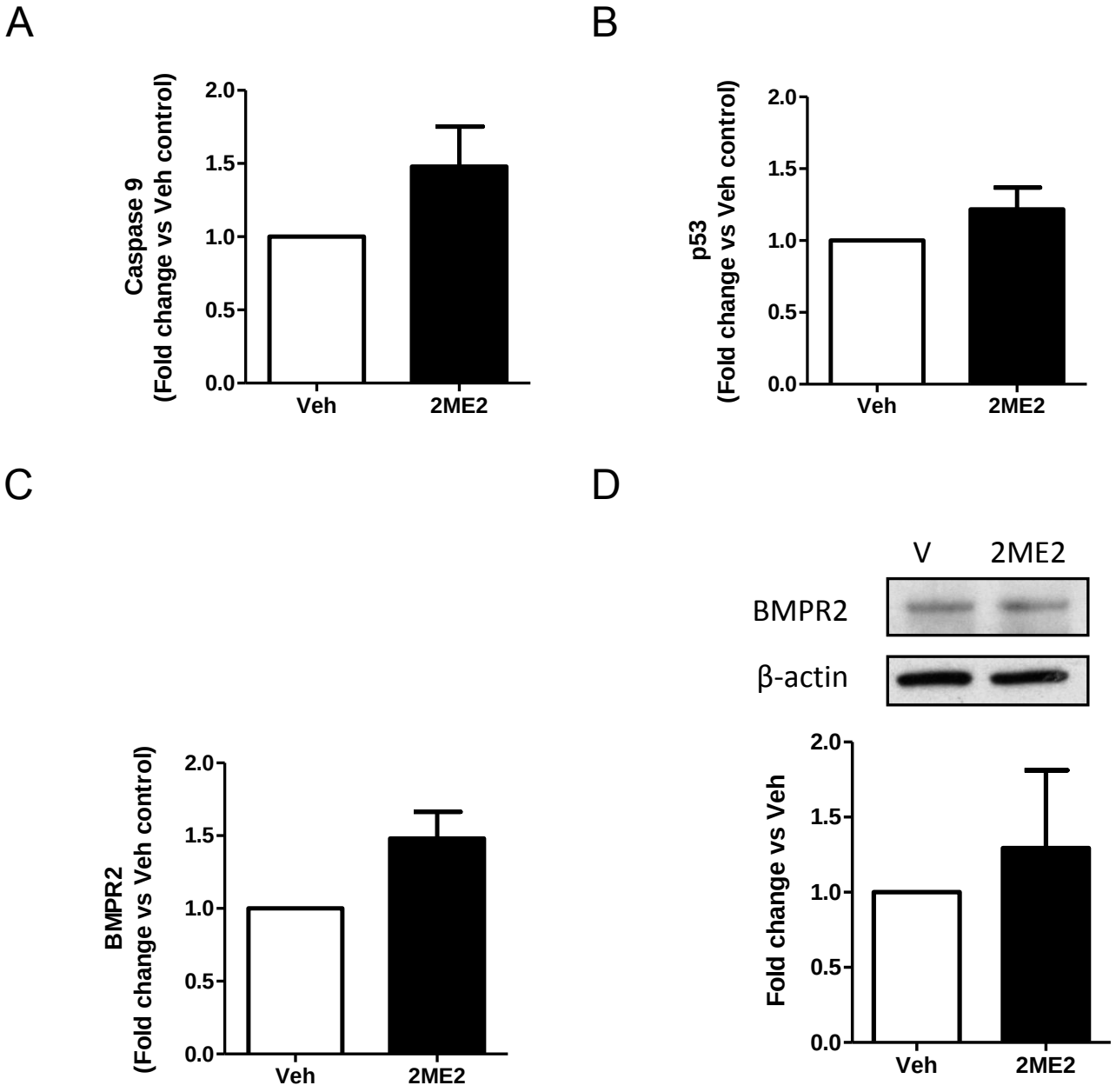
Figure S2. Effect of 2ME2 on the cellular proliferation of rat PASMCs.



Effect of 2ME2 (100nm-10µM) on FBS-induced proliferation in isolated rat female PASMCs assessed by manual cell counts (A) and CCK8 assay (B) after 48 hours (n=5 and 4, respectively). Data is shown as mean ±SEM. ★ $p<0.05$; ★ ★ $p<0.01$; ★ ★ ★ $p< 0.001$ determined by one way ANOVA followed by Tukey's post-hoc test. Veh, vehicle.

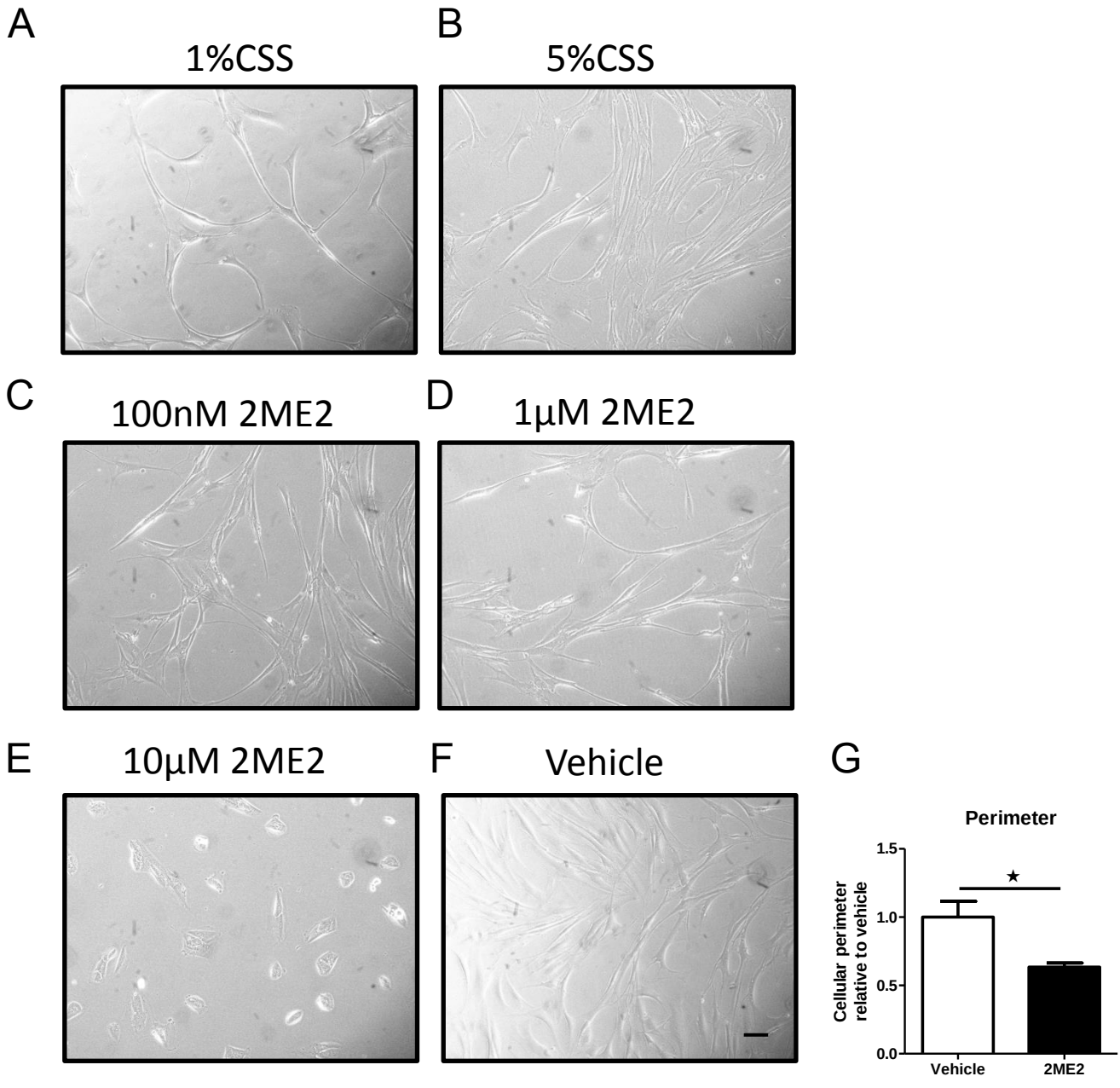
Figure S3

Figure S3. Effects of 2ME2 on pro-apoptotic genes in female hPASMCs.



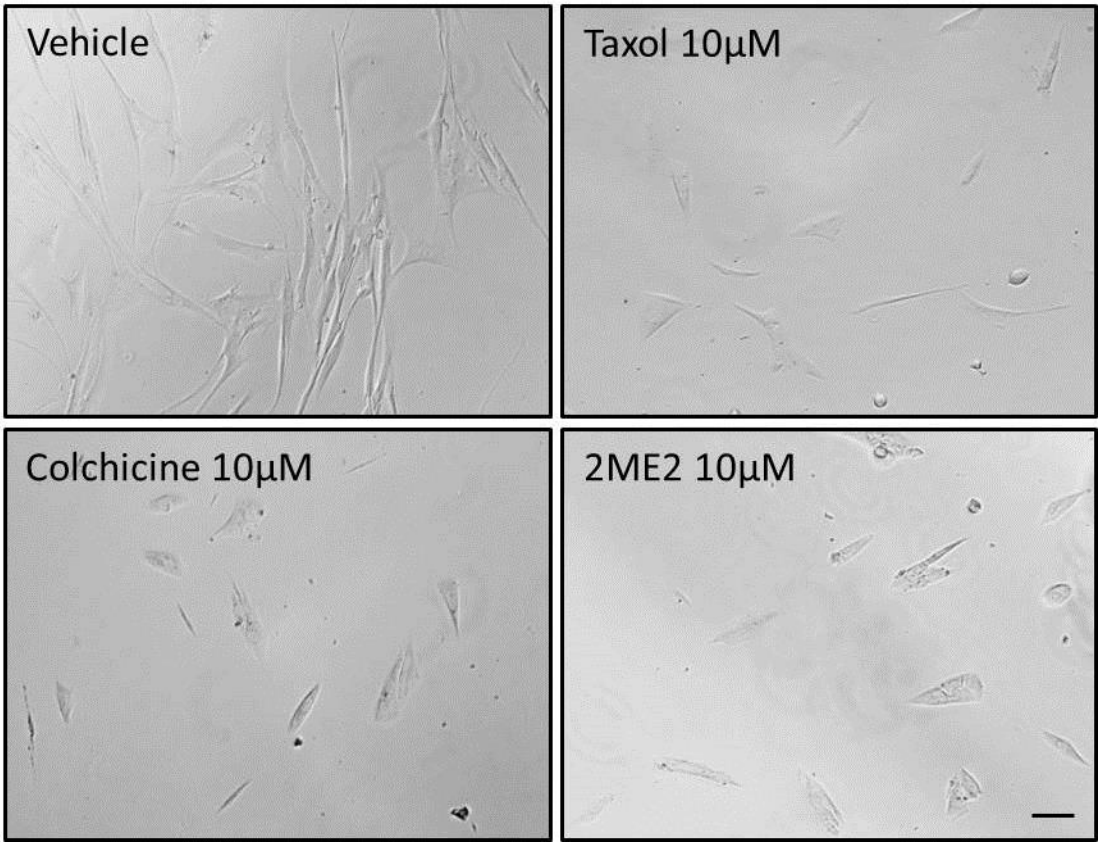
Effect of 2ME2 (10μM, 48 hrs) on mRNA transcript expression levels of caspase 9 (A) and p53 (B) in female hPASMCs. BMPR2 mRNA (C) and protein expression (D) in female PASMCs. Data is shown as mean ± SEM. Statistical analysis determined by paired t-test. hPASMC, human pulmonary arterial smooth muscle cells.

Figure S4. Morphological changes in female hPASMCs associated with 48 hours 2ME2 treatment.



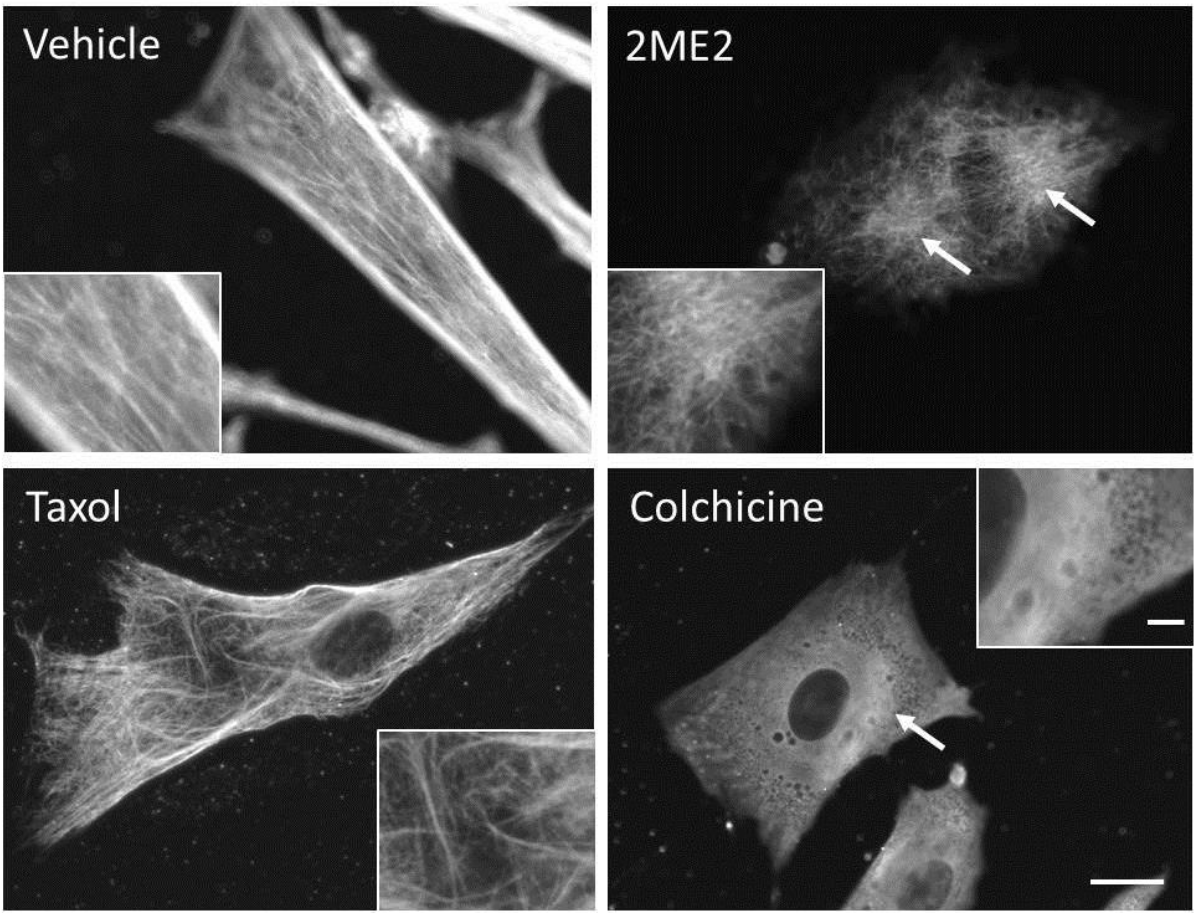
Bright field images after 1%CSS (A), 5% CSS (B), 100nM 2ME2 (C), 1µM 2ME2 (D), 10µM 2ME2 (E) and vehicle (F). Cellular perimeter measurements in female hPASMCs in presence of 10µM 2ME2 (G) (n=3). Scale bar indicates 70µm. Veh, vehicle; hPASMC, human pulmonary arterial smooth muscle cells.

Figure S5. Comparison of morphological changes in female hPASMCs after stimulation with various microtubule mediators.



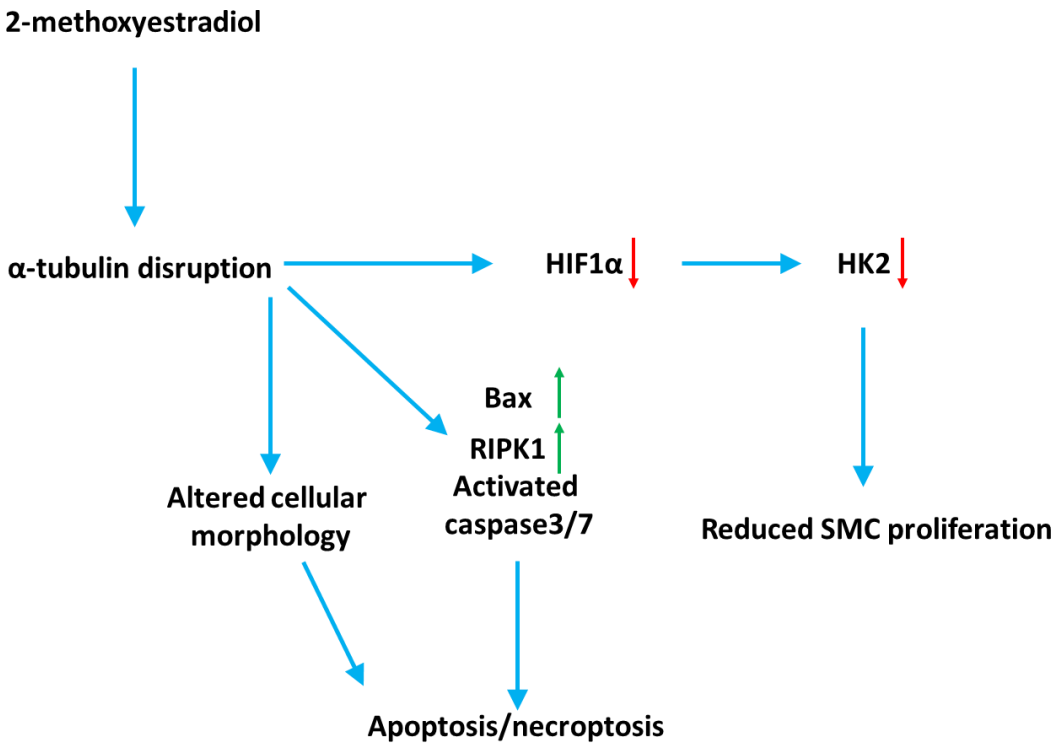
Bright field images after 24 hours treatment with Taxol (10 µM), Colchicine (10 µM) and 2ME2 (10 µM) (n=3). hPASMC, human pulmonary arterial smooth muscle cells. Scale bar indicates 70µm.

Figure S6. Effects of microtubule mediators on α-tubulin organization in female hPASMCs.



High resolution confocal microscopy images of α-tubulin after treatment with Taxol (10 μM), Colchicine (10 μM) and 2ME2 (10 μM) (n=3) with increased digital magnification (inset). hPASMC, human pulmonary arterial smooth muscle cells. Arrows indicate condensing/disruption to the microtubule network. Scale bar indicates 10μm and 5μm (inset).

Figure S7. Schematic representation of 2ME2 mechanisms of action in female hPASMCs.



2ME2 causes α-tubulin dysregulation which, in turn, can cause reduced HIF1α and HK2 protein expression. Disruption of α-tubulin may also lead to increases in apoptotic genes Bax and RIPK1 and activation of caspase3/7. 2ME2, 2-methoxyestradiol; HK2, hexokinase 2; Bax, Bcl-associated X; RIPK1, related receptor Interacting Serine/Threonine Kinase 1. Green arrow = upregulation. Red arrow = down regulation. Blue arrow = causes/leads to.

Supplemental References:

1. Tofovic SP, Jones T, Petrusevska G. Dose-dependent therapeutic effects of 2-Methoxyestradiol on Monocrotaline-Induced pulmonary hypertension and vascular remodelling. *Prilozi.* 2010;31:279-295.

2. Dean A, Gregorc T, Docherty CK, Harvey KY, Nilsen M, Morrell NW, MacLean MR. Role of the Aryl Hydrocarbon Receptor in Sugen 5416-induced Experimental Pulmonary Hypertension. *American journal of respiratory cell and molecular biology.* 2018;58:320-330.