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The influence of sex on the in vivo and in vitro effects of treprostinil in pulmonary arterial hypertension

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Submitted in fulfilment of the requirements of the degree of Doctor of Philosophy in the
Institute of Cardiovascular and Medical Sciences
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University of Glasgow

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Author’s Declaration

I declare that this thesis has been written entirely by myself and is a record of the work performed by myself except for the rat blood plasma treprostinil analysis, which was carried out by Tandem Labs/Covance (United States). This thesis has not been previously submitted for a higher degree. The research was carried out in the Institute of Cardiovascular and Medical Sciences, College of Medical, Veterinary and Life Sciences at the University of Glasgow under the supervision of Professor M.R. MacLean
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<tr>
<td>Cf.</td>
<td>Compared to</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-methyltransferase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>Co-Smad</td>
<td>Common mediator Smad</td>
</tr>
<tr>
<td>COX1/2</td>
<td>Cyclooxygenase 1/2</td>
</tr>
<tr>
<td>CT</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>CTEPH</td>
<td>Chronic thromboembolic pulmonary hypertension</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DCA</td>
<td>Dichloroacetate</td>
</tr>
<tr>
<td>DFEN</td>
<td>Dexfenfluramine</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s medium</td>
</tr>
<tr>
<td>DP₁</td>
<td>Prostaglandin D₂ receptor 1</td>
</tr>
<tr>
<td>DP₂</td>
<td>Prostaglandin D₂ receptor 2</td>
</tr>
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<td>dH₂O</td>
<td>Distilled water</td>
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<tr>
<td>dNTPs</td>
<td>Deoxyribonucleotide triphosphates</td>
</tr>
<tr>
<td>ECE-1</td>
<td>Endothelin converting enzyme-1</td>
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<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDCF</td>
<td>Endothelium derived constricting factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<tr>
<td>ENG</td>
<td>Endoglin</td>
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<td>EP₃</td>
<td>Prostaglandin E₂ receptor 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>-------------</td>
</tr>
<tr>
<td>EP₄</td>
<td>Prostaglandin E₂ receptor 4</td>
</tr>
<tr>
<td>EPAC</td>
<td>Exchange factor directly activated by cAMP</td>
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<tr>
<td>EPCs</td>
<td>Endothelial progenitor cells</td>
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<tr>
<td>ERα</td>
<td>Estrogen receptor α</td>
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<tr>
<td>ERβ</td>
<td>Estrogen receptor β</td>
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<td>Endothelin receptor antagonist</td>
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<td>Estrogen response elements</td>
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<td>Extracellular signal-regulated kinases</td>
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<td>Estrogen receptor 1</td>
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<td>ESR2</td>
<td>Estrogen receptor 2</td>
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<td>Endothelin-1</td>
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<td>ETₐ</td>
<td>Endothelin receptor A</td>
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<tr>
<td>ETₐ</td>
<td>Endothelin receptor B</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>FP</td>
<td>Prostaglandin F receptor</td>
</tr>
<tr>
<td>FPAH</td>
<td>Familial pulmonary arterial hypertension</td>
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<tr>
<td>GPCR</td>
<td>G protein coupled receptors</td>
</tr>
<tr>
<td>GPER</td>
<td>G protein coupled estrogen receptor</td>
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<tr>
<td>GRKs</td>
<td>G-protein coupled receptor kinases</td>
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<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
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<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
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<tr>
<td>HHT</td>
<td>Hemorrhagic telangiectasia type 2</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<td>HPAH</td>
<td>Heritable pulmonary arterial hypertension</td>
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<tr>
<td>hPASMCs</td>
<td>Human pulmonary artery smooth muscle cells</td>
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<tr>
<td>HPG</td>
<td>Hypothalamic-pituitary-gonadal axis</td>
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</table>
HPV  Hypoxic pulmonary vasoconstriction
HR   Heart Rate
HRT  Hormone replacement therapy
HSD  Hydroxysteroid dehydrogenase
Id   Inhibitors of DNA binding
IHC  immunohistochemistry
IP   Prostacyclin receptor
IP3  Inositol-1, 4, 5 triphosphate
IPAH Idiopathic pulmonary arterial hypertension
I-Smad Inhibitory Smad
IV   Intravenous
Jak  Janus kinase
K+   Potassium ions
K3-EDTA Tri-potassium ethylenediaminetetraacetic
KCNK3 Potassium channel subfamily K member 3
Kv   Voltage-gated K+ channel
LC/MS Liquid Chromotography/Mass Spectrometry
LDL  Low density lipoprotein
L-NAME L-N^3-nitroarginine methyl ester
L-NMMA L-N^3-monomethyl arginine
LV   Left ventricle
LVP  Left ventricular pressure
LVSP Left ventricular systolic pressure
MAPK Mitogen-activated protein kinase
MCT  Monocrotalene
MCTP Monocrotaline pyrrole
MDD  Matrix driven delivery
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>MID</td>
<td>Minimal important difference</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
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<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>mLVP</td>
<td>Mean left ventricle pressure</td>
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<tr>
<td>MMP</td>
<td>Metalloproteinases</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>mPAP</td>
<td>Mean pulmonary arterial pressure</td>
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<td>MSCs</td>
<td>Mesenchymal stromal cells</td>
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<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NBF</td>
<td>Neutral buffered formalin</td>
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<td>NO</td>
<td>Nitric oxide</td>
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<td>NPY</td>
<td>Neuropeptide-Y</td>
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<td>NSCLC</td>
<td>non-small cell lung cancer</td>
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<tr>
<td>NTC</td>
<td>No template control</td>
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<td>NYHA</td>
<td>New York Heart Association</td>
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<tr>
<td>SBE</td>
<td>Smad-binding elements</td>
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<tr>
<td>SHP</td>
<td>Short heterodimer partner</td>
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<td>Smad</td>
<td>SMA and Mothers against decapentaplegic</td>
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<td>SMURF1/2</td>
<td>SMAD ubiquitination and regulatory factors 1/2</td>
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<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<td>SU-Hx</td>
<td>Sugen-hypoxic</td>
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<td>SU-5416</td>
<td>Sugen 5416</td>
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<td>PAEC</td>
<td>Pulmonary artery endothelial cells</td>
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<td>PAH</td>
<td>Pulmonary arterial hypertension</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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PDE         Phosphodiesterase
PH          Pulmonary hypertension
PI3K        Phosphoinositide 3-kinase
PASMCs      Pulmonary artery smooth muscle cells
PAP         Pulmonary arterial pressure
PDK         Pyruvate dehydrogenase kinase
PGDS        Prostaglandin D synthase
PGD₂        Prostaglandin D₂
PGES        Prostaglandin E synthase
PGE₂        Prostaglandin E₂
PGFS        Prostaglandin F synthase
PGF₂α       Prostaglandin F₂α
PGH₂        Prostaglandin H₂
PGIS        Prostaglandin I synthase
PGI₂        Prostacyclin/Prostaglandin I₂
PI3K        Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKA         Protein kinase A
PKG         cGMP-dependent protein kinase
PLC         Phospholipase A
PPAR-β       Peroxisome proliferator-activated receptor beta
PPAR-γ       Peroxisome proliferator-activated receptor gamma
PPHTN       Portal hypertension
PPHN        Persistent pulmonary hypertension of the newborn
pSmad       Phosphorylated Smad
PVDF        Polyvinylidene difluoride
PVR         Pulmonary vascular resistance
qRT-PCR      Quantitative real time-polymerase chain reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>REVEAL</td>
<td>Registry to evaluate early and long-term PAH disease management</td>
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<td>RGS</td>
<td>Regulators of G-protein signalling</td>
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<td>RHC</td>
<td>Right heart catheterization</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>R-Smad</td>
<td>Regulatory Smad</td>
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<tr>
<td>RT</td>
<td>Reverse transcription</td>
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<tr>
<td>RV</td>
<td>Right ventricle</td>
</tr>
<tr>
<td>RVESP</td>
<td>Right ventricular end systolic pressure</td>
</tr>
<tr>
<td>RVEDP</td>
<td>Right ventricular end diastolic pressure</td>
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<td>RVH</td>
<td>Right ventricular hypertrophy</td>
</tr>
<tr>
<td>RVP</td>
<td>Right ventricular pressure</td>
</tr>
<tr>
<td>RVEF</td>
<td>Right ventricle ejection fraction</td>
</tr>
<tr>
<td>RVSP</td>
<td>Right ventricular systolic pressure</td>
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<tr>
<td>RV/LV+S</td>
<td>Right ventricle/ Left ventricle + septum</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio immuno precipitation assay</td>
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<tr>
<td>RQ</td>
<td>Relative quantification</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>S</td>
<td>Septum</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
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<td>sGC</td>
<td>Soluble guanylyl cyclase</td>
</tr>
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<td>SERT+</td>
<td>SERT over-expression</td>
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<td>SMURF</td>
<td>SMAD ubiquitination and regulatory factor</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<td>SU-HX</td>
<td>Sugen-Hypoxic</td>
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<td>Sulphotransferases</td>
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<td>TASK-1</td>
<td>TWK-related acid sensitive potassium channel</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TPH1</td>
<td>Tryptophan hydroxylase 1</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline tween</td>
</tr>
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<td>Trep</td>
<td>Treprostinil</td>
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<tr>
<td>TxAS</td>
<td>Thromboxane synthase</td>
</tr>
<tr>
<td>TXA₂</td>
<td>Thromboxane</td>
</tr>
<tr>
<td>TP</td>
<td>Thromboxane receptor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>Veh</td>
<td>Vehicle</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
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<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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Abstract

Pulmonary arterial hypertension (PAH) is a progressive and fatal vascular disease that is more prevalent in women than men. The underlying pathology of the disease involves various factors, including genetic risk (i.e. bone morphogenetic protein receptor type II (BMPR-II) mutations) as well as the influence of hormones such as estrogen. Among the frontline treatments for PAH is prostacyclin therapy; however, the short half-life and associated problems with the need for continuous intravenous administration of synthetic prostacyclin have led to the development of newer analogues such as treprostinil. These have the advantages of a longer half-life and the possibility of subcutaneous and inhaled administration. Mortality rates for PAH are still high despite advancements in treatment, with male survival rates remaining lower than females. The BMPR-II signalling pathway may underlie some of the sex disparity that exists in incidence of PAH. However, patient responses to treatments for PAH have also demonstrated sex-specific effects. A key aim of this study was to identify the influence that sex may have on the actions of treprostinil with in-vivo and in-vitro models of PH. The ability to target treatment to specific sub-cohorts of PH is important to maximise the therapeutic effect of treprostinil. A greater understanding of how the effects of treprostinil are mediated could assist this objective.

To examine any potential influence of sex on the effects of treprostinil, we examined the chronic hypoxic model of pulmonary hypertension (PH). Female and male rats were dosed with sub-cutaneously implanted pellets releasing treprostinil at 100ng/kg/min or 400ng/kg/min. Under hypoxic conditions both male and female rats had increases in right ventricular systolic pressure (RVSP), right ventricular hypertrophy (RVH) and pulmonary artery remodelling. 100ng/kg/min of treprostinil partially reversed RVSP, RVH and pulmonary artery remodelling in female hypoxic rats but not in male rats. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis revealed that a possible mechanism of treprostinil was increasing BMPR-II signalling, specifically Id1 and Id3 (inhibitor of DNA binding protein 1/3). This was only observed in female hypoxic rats. Despite no difference in terminal plasma levels of treprostinil, hypoxic males remained unaffected by treprostinil at 100ng/kg/min. However, 400ng/kg/min of treprostinil led to slightly greater decreases in RVSP, RVH and remodelling indices in female rats, also it partially reversed RVSP, RVH and remodelling in male rats. Taqman qRT-PCR of the prostaglandin receptors demonstrated an increase in prostaglandin E\(_2\) receptor 2 (EP\(_2\)) under hypoxic conditions with 100ng/kg/min and 400ng/kg/min treprostinil treatment, specific to female rats. Also under hypoxic conditions female and male rats had significant increases in mRNA expression of potassium two pore domain channel subfamily K (KCNK3).

To translate clinical relevance from the in-vivo findings, the influence of sex on treprostinil was investigated in human pulmonary artery smooth muscle cells (hPASMCs) taken from non-PAH (control) and PAH patients. In female control hPASMCs FBS (fetal bovine serum)
induced proliferation was partially ablated by treprostinil; this effect was only observed in male control hPASMCs at the highest treprostinil concentration (10µM). In patient hPASMCs, treprostinil had a similar effect in reducing FBS-induced proliferation in both female and males. The addition of a low dose (30nM) of endothelin-1 (ET-1) increased the anti-proliferative effect of treprostinil, specifically, in female control hPASMCs. The addition of a dual endothelin receptor antagonist (SB-217242) partially reduced the anti-proliferative effect of treprostinil in combination with ET-1. Taqman qRT-PCR and western blot analysis demonstrated no difference between sexes or hPASMC groups in the expression of the prostaglandin receptors. Using receptor specific antagonists, it was determined that the anti-proliferative actions of treprostinil in PAH patient hPASMCs were partially mediated via the EP$_2$ receptor. However, in female control hPASMCs, the IP receptor was primarily responsible for this effect. BMPR-II signalling was investigated to ascertain its role in the anti-proliferative effects of treprostinil. Taqman qRT-PCR indicated treprostinil (100nM and 1µM) induced increases in Id1 and Id3 mRNA in female control hPASMCs, this did not occur in male control hPASMCs. Treprostinil (100nM and 1µM) led to Id3 mRNA increases in female PAH patient hPASMCs, whereas in male PAH patient hPASMCs treprostinil (1µM) led to a significant Id3 mRNA increase. Western blots indicated that Id3 was upregulated by treprostinil (1µM) stimulation in female control and female PAH hPASMCs vs non-stimulated hPASMCs; this effect was not observed in males. The combination of ET-1 and treprostinil did not influence BMPR-II signalling. After 24 hours of treprostinil stimulation increased Id3 mRNA expression was observed in all hPASMCs groups. Treprostinil only increased Id1 mRNA in PAH patient hPASMCs. Although western blots confirmed treprostinil (100nM and 1µM) mediated increases in Id1 protein expression in female control hPASMCs. Treprostinil (100nM and 1µM) increased Id3 protein expression in female control and female PAH patient hPASMCs. Treprostinil (1µM) in combination with ET-1 led to a significant increase in Id3 protein expression in male control hPASMCs. As with 72-hour treprostinil stimulation, BMPR-II signalling was not influenced by the combination of ET-1 and treprostinil in the other hPASMC groups. The increased BMPR-II signalling in female control and female PAH patient hPASMCs at 24 hours led to the investigation of prostaglandin receptors role in activating BMPR-II signalling. After 24-hours of stimulation with treprostinil (100nM), Id protein induction was partially blocked by dual antagonism of the IP and EP$_2$ receptor in both female control and female PAH patient hPASMCs.

To summarise these findings, we have identified sex differences in the action of treprostinil in both in-vivo and in-vitro models of PH. Treatment with a low dose (100ng/kg/min) of treprostinil led to a significant reduction in chronic hypoxic induced PH in female rats but not in males. These differences are driven partially by increases in BMPR-II signalling. Treatment with a higher dose (400ng/kg/min) of treprostinil led to significant reductions in
chronic hypoxic PH in both female and male rats. In hPASMCs the results demonstrate that treprostinil can induce the Id proteins of the BMPR-II signalling pathway and that this may account for the greater anti-proliferative effect observed in female control hPASMCs. The induction of the Id proteins was found to be partially mediated by activation of the IP and EP\textsubscript{2} prostaglandin receptors. The results suggest that sex may influence the beneficial effects of treprostinil in an in-vivo model of PH and in hPASMCs.
Chapter 1

1 Introduction
1.1. The pulmonary circulation

1.1.1. Structural and functional organisation

The primary function of the pulmonary circulation is to enable for the exchange of gases between inspired air and red blood cells, especially oxygen and carbon dioxide. The right ventricle receives venous blood from the systemic circulation via the right atria. The pulmonary artery extends from the right ventricle and splits to become the left and right pulmonary artery. Each part of the artery extends into the hilum of the corresponding lungs.

The right lung can be sub-divided into three distinct lobes; superior, middle and inferior, which are separated by intra-lobar fissures, each lobe receiving a branch from the right pulmonary artery. The left lung is sub-divided into two lobes; superior and inferior, each of these lobes receives a branch from the left pulmonary artery. The lobes of the lungs are further sub-divided into distinct bronchopulmonary segments (Sealy et al., 1993). The pulmonary artery continues to branch into smaller arteries parallel to the bronchial tree until the capillary bed of the terminal alveoli. Each branch of the pulmonary artery was assigned an order diameter corresponding to the diameter of the vessel, as defined by the Strahler ordering system (Singhal et al., 1973). The Strahler ordering system was updated to the Diameter-Defined Strahler system due to the overlap in the diameter of successive-order vessels (Jiang et al., 1994). Based on the update, fifteen orders of pulmonary arteries were identified between the main pulmonary artery to capillary level (Huang et al., 1996).

The lung function is related to the changes in the intima and media of the pulmonary arteries. The most distal arteries before capillaries are known as order 1, the numbering increases with each branch back to the main pulmonary artery, which is order 15 (Huang et al., 1996). The most proximal arteries (order 13-15) have diameters ranging from 4mm to 14mm. These arteries are highly compliant demonstrating a developed elastic laminae, which facilitates compliance. Decreasing order number leads to a decreasing diameter of pulmonary arteries ranging from 1.75mm to 0.1mm. The branching of the pulmonary artery leads to an increase in smooth muscle cell population relative to other cells in the vasculature (Heath and Edwards, 1958). The final 4 orders of the pulmonary arteries (0.08mm to 0.02mm) have low numbers of smooth muscle cells (Meyrick and Reid, 1983, deMello et al., 1997). These vessels are primarily comprised of endothelial cells and undifferentiated smooth muscle cells (pericytes), which mediate blood gas exchange (Figure 1-1).

The pulmonary arteries eventually become the pulmonary capillary network. Capillaries have very small diameters of less than 10µm and are associated with alveoli. The combined capillaries have a large surface area, which helps facilitate the blood oxygenation. After oxygenation, the blood progresses to the left atria of the heart through the pulmonary venous circulation, which branches from venules to pulmonary veins until the formation of the right and left pulmonary veins that supply the left atria.
1.1.2. Development of pulmonary circulation

Development of the pulmonary artery is linked to the development of the airways. Smooth muscle cells are thought to have developed in pulmonary arteries from three different sources. Proximal smooth muscle cells are related to bronchial smooth muscle cells, other smooth muscle cells are thought to develop from the lung mesenchyme. The last source of smooth muscle is from endothelial cells. These endothelial cells can differentiate into smooth muscle cells (DeRuiter et al., 1997, Hall et al., 2000).
Figure 1-1 Pulmonary artery musculature
Decreasing levels of muscularisation occurs progressively as the size of pulmonary artery decreases. Last pre-capillary level arteries consist primarily of endothelial cells and pericytes. Taken from (MacLean et al., 2000).
1.1.3. Pulmonary vascular wall structure

The pulmonary vascular wall can be separated into three separate layers or tunics; the tunica intima, tunica media and tunica adventitia (Figure 1-2). The tunica adventitia, which is the outermost layer of the pulmonary vascular wall, consists of various contractile and extracellular matrix proteins, which combined with fibroblasts and progenitor cells form stromal cells (Stenmark et al., 2006a). These cells act to support the structural integrity of the vascular wall. They can also direct response to specific stimuli by interaction with other cells in the adventitia or in other tunics. The largest layer of the vascular wall is known as the tunica media. It contains an arrangement of a mixed population of immature/mature pulmonary artery smooth muscle cells (PASMCs). The importance of this layer (tunic) is determined by the ability of the PASMCs to produce a contractile response to stimulation, therefore they regulate the tone and blood pressure in the artery (Frid et al., 1997, Stenmark and Frid, 1998). The innermost tunic is the tunica intima, which consists of a connective tissue matrix with an attached monolayer of endothelial cells. Endothelial cells are the only cells that are in contact with the blood in the vessels and are believed to be important in the regulation of the lumen environment. Overall vascular tone can be modulated by the release of vasodilators and vasoconstrictors from endothelial cells (Aaronson et al., 2002).
Figure 1-2 Cell types and morphology of pulmonary vascular wall

Pulmonary vascular wall comprised of intimal, medial and adventitial layers. Endothelial cells predominately located in the intimal layer, smooth muscle cells in medial, which decrease in number as the artery reduces in size, fibroblasts are in the outermost adventitial layer of vascular wall.
1.1.4. Function of the pulmonary circulation
The primary function of the pulmonary circulation is to facilitate the exchange of oxygen and carbon dioxide between air and blood. Under normal conditions the pulmonary circulation is a high flow, low resistance, low pressure system that carries deoxygenated blood from the systemic circulation and facilitates its re-oxygenation (Comroe, 1966). Additionally, the pulmonary circulation can act as a filtration system, preventing foreign objects passing between respiratory and cardiovascular systems (Comroe, 1966). The capillaries within the pulmonary circulation can also act as a blood reservoir, which can be used to receive the cardiac output and supply the left ventricle of the heart (Comroe, 1966).

1.1.5. Regulation of pulmonary circulation and pulmonary vascular resistance
The pulmonary arteries contain less smooth muscle cells and elastin than arteries from the systemic circulation allowing greater distensibility (Kilner, 2004). Consequently, pulmonary arterial pressure is only one-fifth to one-tenth of total systemic resistance. The low vascular tone of the pulmonary arteries is dependent on the resting membrane potential of the PASMCs. This involves the activity of both potassium and calcium channels. Depolarisation of the cell membrane leads to the opening of voltage gated potassium (K\textsubscript{v}) channels, these allow the outflow of potassium ions (K\textsuperscript{+}) thereby restoring the membrane potential (Yuan, 1995). A blockade of the K\textsubscript{v} channels leads to an increase in intracellular calcium (Ca\textsuperscript{2+}) resulting in vasoconstriction of PASMCs. In PASMCs derived from pulmonary arterial hypertensive (PAH) patients, the K\textsubscript{v} channel were found to be dysfunctional leading to greater depolarisation of the cell membranes. This enhanced the vasoconstriction in PAH PASMCs compared to control PASMCs (Yuan et al., 1998). Some forms of heritable PAH have been found to have a loss of function of the alternative potassium channel KCNK3 (Ma et al., 2013, Girerd et al., 2014). Interestingly, the monolayer of pulmonary artery endothelial cells (PAEC) of the tunic intima are important regulators of the pulmonary vascular tone. PAEC are known to release both nitric oxide (NO) (Nong et al., 1997) and prostacyclin (PGI\textsubscript{2}) (Gryglewski et al., 1988). These vasodilators can lead to relaxation in the PASMCs of the tunica medial. PAEC can also release vasoconstrictors like endothelin-1 (Giaid et al., 1993) and thromboxane (Christman et al., 1992). Pulmonary vascular resistance (PVR) is the total peripheral resistance which is overcome to maintain constant blood flow through the pulmonary arteries. Small alterations in the lumen size can have a significant impact on PVR therefore can affect pulmonary arterial pressure (PAP) (Krenz et al., 1994). In pulmonary hypertension, increased proliferation of the smooth muscle cells of the tunica intima can lead to a narrower lumen leading to increased PVR and PAP. The autonomic nervous system can influence the pulmonary circulation via both the sympathetic and parasympathetic branches. In all species, sympathetic noradrenergic innervation density is higher in blood vessels outside the lung, this decreases as the vessel size decreases (Fisher, 1965). In humans,
sympathetic noradrenergic axons extend to 50µM sized small arteries, in rats and mice innervation stops after the lung hilus (Fisher, 1965, Čech, 1969). Increased sympathetic nerve signalling leads to increases in both PVR and PAP (Kadowitz et al., 1974). This is mediated by noradrenaline acting at the α-adrenoreceptors, which leads to vasoconstriction of smooth muscle cells (Hyman et al., 1986, Crystal, 1997). Noradrenergic sympathetic axons can release many transmitters (co-transmission), an example would ATP releasing with noradrenaline from sympathetic axons (Burnstock, 2009). However, different populations of axons have different combinations of transmitter. Additionally, sympathetic neurones can also synthesise and release neuropeptides like neuropeptide-Y (NPY) (Abe et al., 2010). NPY was found to cause contraction of isolated pig pulmonary arteries, however isolated rabbit pulmonary arteries were not dependent on NPY for contraction (Martling et al., 1990, Jackson et al., 2002). Interestingly, in isolated rabbit pulmonary arteries, electrically stimulated contraction was not altered by α-adrenoceptor blockade or mediated by endothelin-1, angiotensins or acetylcholine (Jackson et al., 2002). NPY was also found to be a mediator of ischemic angiogenesis via activation of Y2 and Y5 receptors as well as an inducer of smooth muscle proliferation (Abe et al., 2007, Movafagh et al., 2006). Alternatively, β1 and β2 adrenoreceptors mediate vasodilation of the smooth muscle cells of the pulmonary arteries (Hyman et al., 1981, Hyman et al., 1990). β-adrenoceptor obstruction led to increases in resting PAP in dogs, sheep and humans (Crystal, 1997). Interestingly, the parasympathetic branch of the autonomic nervous system is found to be less influential in altering pulmonary vascular tone (Downing and Lee, 1980, Murray et al., 1986). However, cholinergic fibres are distributed in a similar fashion to noradrenergic fibres, with less density (El-Bermani et al., 1982). The role of NO on the vasodilatory effects of acetylcholine on isolated rat pulmonary arteries was demonstrated by the blockade of nitric oxide synthase by either L-N^G^-nitroarginine methyl ester (L-NAME) or L-N^G^-monomethyl arginine (L-NMMA) (Yaghi et al., 1997). A NO-dependent relaxation was observed after vagal nerve stimulation in precontracted feline pulmonary arteries (McMahon et al., 1992). Vasoactive intestinal peptide (VIP) relaxes precontracted arteries partially dependent on NO action (Iwabuchi et al., 1997). VIP importance in relaxation is underlined by the observable decrease in VIP serum and lung expression in PAH patients. Also, VIP knock out mice suffer from moderate PH, decreased lumen of small arteries and upregulation of pro-proliferative genes like angiopoietin 1 and pro-collagen type V alpha1 (Petkov et al., 2003, Hamidi et al., 2008).

1.2. Pulmonary hypertension

Pulmonary hypertension is a general name for a group of progressive vasculopathies of the pulmonary vasculature. It is characterised by lower blood flow through the pulmonary circulations, this results in an increase PVR therefore increased PAP. Restrictive blood flow occurs due to significantly increased levels of remodelling, due to excessive
proliferation of both endothelial and smooth muscle cells. The increase pressure leads to a greater strain placed on the right ventricle of the heart. Hypertrophy of the right ventricle occurs as a compensatory mechanism; however, this eventually leads to right ventricle failure and the death of the patient. There are many different aetiologies of PH therefore the disease is grouped into subcategories to target the most appropriate medical intervention.

1.2.1. Diagnosis and classification

Clinically, PH is defined as a resting mean PAP (mPAP) greater than 25mmHg or a mPAP above 30mmHg during exercise (Badesch et al., 2009). This is approximately double the mPAP (14-20mmHg) that is observed in normal women and men. Patients with PH usually present with several symptoms, which may include shortness of breath, dizziness, fatigue and oedema (Rich et al., 1987). However, these symptoms may not be a direct sign of PH. It is therefore important to conduct further examinations to properly diagnose the disease. The application of an electrocardiogram (ECG) may suggest the presence of right ventricle hypertrophy, as 87% of PAH patients have RV hypertrophy confirmed by an ECG (Rich et al., 1987). Magnetic resonance imaging (MRI) can be used as a companion diagnostic test to ECG to discern RV size allowing for non-invasive measurements of cardiac and stroke volume (Marrone et al., 2010). Blood tests can also be used to identify the level of anti-nuclear antibodies, which are known to be elevated in a subset of PH patients (Rich et al., 1986). Right heart catheterization (RHC) is carried out to confirm the diagnosis of PH. Although it is an invasive procedure RHC is associated with low morbidity and mortality rates (Hoeper et al., 2006). During RHC, vasoreactivity of the pulmonary vasculature is tested to identify if patients would respond to calcium channel blocker (CCB) therapy. NO and PGI_{2} are predominately used to test vasodilatory response, a good response in patients is characterised by a drop in mPAP of more than 10mmHg (Sitbon et al., 1998, Rubin et al., 1982, Sitbon et al., 2005). However, this response does not always occur despite successful long term clinical outcomes (Sitbon et al., 2005). Despite different forms of PH having common features, patients are categorised into groups to aid understanding of disease aetiology and to assist in the therapeutic management of the disease (Table 1-1). Due to the severe effects that PH can have on the heart, patients are also classified according the New York Heart Association (NYHA) scale on heart failure (Table 1-2). The NYHA classifies PH patients as per the manifestations of the symptoms of the disease. Its range covers class I, at which no symptoms are present, to class IV, were symptoms are present even at rest.
# Classification of Pulmonary Hypertension

<table>
<thead>
<tr>
<th>Classification of Pulmonary Hypertension</th>
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<tbody>
<tr>
<td>1. Pulmonary Arterial Hypertension (PAH)</td>
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<tr>
<td>1.1. Idiopathic PAH (IPAH)</td>
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<tr>
<td>1.2. Heritable PAH (HPAH)</td>
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<tr>
<td>1.4.1. BMPR-II</td>
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<tr>
<td>1.4.2. ALK-1, ENG, SMAD9, CAV1, KCNK3</td>
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<tr>
<td>1.4.3. Unknown</td>
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<tr>
<td>1.3. Drug and toxin induced</td>
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<tr>
<td>1.4. Associated with</td>
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<tr>
<td>1.4.1. Connective tissue disease</td>
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<td>1.4.2. HIV infection</td>
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<td>1.4.3. Portal hypertension (PPHTN)</td>
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<td>1.4.4. Congenital heart disease</td>
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<td>1.4.5. Schistosomiasis</td>
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<td>1.4.6. Chronic haemolytic anaemia</td>
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<td>1.5. Persistent pulmonary hypertension of the newborn (PPHN)</td>
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<td>2. Pulmonary hypertension due to left heart disease</td>
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<tr>
<td>2.1 Left ventricular systolic dysfunction</td>
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<td>2.2 Left ventricular diastolic dysfunction</td>
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<tr>
<td>2.3 Valvular disease</td>
</tr>
<tr>
<td>2.4 Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies</td>
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<tr>
<td>3. Pulmonary hypertension due to lung diseases and/or hypoxia</td>
</tr>
<tr>
<td>3.1 Chronic obstructive pulmonary disease (COPD)</td>
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<td>3.2 Interstitial lung disease</td>
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<td>3.3 Other pulmonary diseases with mixed restrictive and obstructive patterns</td>
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<td>3.4 Sleep-related breathing behaviour</td>
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<td>3.5 Alveolar hypoventilation disorders</td>
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<td>3.6 Chronic exposure to high altitudes</td>
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<td>3.7 Developmental lung diseases</td>
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<td>4. Chronic thromboembolic pulmonary hypertension (CTEPH)</td>
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<tr>
<td>5. Pulmonary hypertension with unclear multifactorial mechanisms</td>
</tr>
<tr>
<td>5.1 Hematologic disorders: chronic haemolytic anaemia, splenectomy</td>
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<tr>
<td>5.2 Systemic disorders: sarcoidosis, pulmonary histiocytosis,</td>
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<tr>
<td>5.3 Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders</td>
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<tr>
<td>5.4 Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure</td>
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</tbody>
</table>

BMPR-II: Bone morphogenetic receptor type 2; ALK-1: Activin receptor like kinase-1 CAV1 = caveolin-1; ENG = endoglin; HIV = human immunodeficiency virus; PAH = pulmonary arterial hypertension.
### Table 1-2 New York Heart Association (NYHA)/WHO classification of functional assessment of patients with pulmonary hypertension (PH).

<table>
<thead>
<tr>
<th>Class 1/Class I</th>
<th>No symptoms with ordinary physical activity/Patients with PH but without resulting limitation on physical activity.</th>
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<tbody>
<tr>
<td>Class 2/Class II</td>
<td>Symptoms with ordinary activity/Patients with PH resulting in slight limitation of physical activity.</td>
</tr>
<tr>
<td>Class 3/Class III</td>
<td>Symptoms with less than ordinary activity/Patients with PH resulting in marked limitation of physical activity.</td>
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<tr>
<td>Class 4/Class IV</td>
<td>Symptoms with any activity or even at rest/Patients with PH with inability to carry out any physical activity without symptoms.</td>
</tr>
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</table>

**1.2.2. Epidemiology and prognosis**

There has been much revision and re-assessment of the epidemiology of PAH. There are several different registries of PAH constructed from clinical trials across different countries (McGoon et al., 2013). Recent analysis of the French and United Kingdom and Ireland registries concluded that the incidence of PAH was 2.4 and 1.1 cases per million respectively (Montani et al., 2010, Ling et al., 2012). The mean age of PAH diagnosis can vary between separate registries. A NIH cohort study from the 1980s found a lower mean age of 36 years with 62.5% of patients being female (Rich et al., 1987). More recent data suggests that the mean age has increased. The UK and Ireland registry found that the mean age was 50 years with 70% of patients being female (Ling et al., 2012). The French registry found similar results; 50 year mean age with 65% female (Montani et al., 2010). The registry to evaluate early and long-term PAH disease management (REVEAL) from the United States found that the mean age of the predominately (80%) female patients was 53 years (Badesch et al., 2010). At 1, 2 and 3 years’ post diagnosis PAH survival rates were found to be 83%, 67% and 58% respectively (Humbert et al., 2010a). Younger PAH patients have been found to have better survival rates compared to those over 50 years (Ling et al., 2012). Despite improvements in therapeutic intervention and diagnosis, survival rates for PAH remains stubbornly low, it remains a progressive and fatal disease. A major outcome from the registry studies is the confirmation of female predominance diagnosed with PAH; the underlying reasons for this sex difference are the subject of current experimental research. Despite the female predisposition for PAH, male sex is identified as a risk factor associated with poorer survival (Humbert et al., 2010a).
1.2.3. Pathobiology of PAH

PAH is a complex disease with many factors contributing to its development. Severe PAH is defined by excess proliferation and apoptosis of smooth muscle, endothelial and fibroblast cells. Localised hypoxia can occur in pulmonary arteries with decreasing lumens, these hypoxic conditions can lead to greater vasoconstriction and alterations in the Ca$^{2+}$ and K$^+$ balance in the local cells (Yuan et al., 1998).

1.2.3.1 Vasoconstriction in PAH

Much of the early research into the PAH pathobiology focused on vasoconstriction and its contribution to increased PVR and PAP. Increasing vasoconstriction has been demonstrated to increase PASMC hypertrophy and hyperplasia (Barst et al., 2004). Hypoxic vasoconstriction (HPV) of pulmonary arteries occurs in PH associated with cardiopulmonary diseases like Eisenmenger’s syndrome or chronic obstructive pulmonary disease (COPD). Pulmonary arteries denuded of their endothelium have increased vasoconstriction when exposed to hypoxia (Marshall and Marshall, 1992). Vasoconstriction also occurred in isolated bovine PASMCs exposed to hypoxia (Murray et al., 1990). To ensure maximum oxygenation of the blood, HPV redirects blood flow to areas of the lung that have the best ventilation. As mentioned previously both Ca$^{2+}$ and K$^+$ signalling is important in maintaining vessel homeostasis. Increased levels of cytosolic free Ca$^{2+}$ can trigger smooth muscle contraction. This occurs by the activation of myosin light chain (MLC) by a Ca$^{2+}$/calmodulin complex. Myosin cross-bridges then forms with actin filament leading to vasoconstriction of the smooth muscle cell (Somlyo and Somlyo, 1994). Several signalling pathways have been proposed to influence Ca$^{2+}$ increases in smooth muscle cells including the Rho-kinase and p38-mitogen activated (MAP) kinase. Studies have suggested that the endothelial cells can release an endothelium derived constricting factor (EDCF) (Giaid and Saleh, 1995, Robertson et al., 2001). In IPAH patient PASMCs, the activity and expression of K$_{v}$ channels are reduced, which can lead to increased intracellular Ca$^{2+}$ levels and vasoconstriction (Murray et al., 1986, Cumming et al., 1970).

In PAH, there is an imbalance in the productions of both vasodilator and vasoconstrictive agents. PGI$_2$ and NO are two key vasodilatory agents produced by the endothelium in the pulmonary circulation. Levels of PGI$_2$ are found to be significantly reduced in pulmonary hypertension patients (Christman et al., 1992). NO is locally produced in endothelial cells however, in PAH the bioavailability of NO is reduced (Klinger et al., 2013). This is possibly due to several factors, these include increased oxidative stress and reduced levels of NO synthase (Michelakis et al., 2002). Increased levels of asymmetric dimethylarginine (ADMA) exist in IPAH, ADMA is a competitive inhibitor of NO synthase (Kielstein et al., 2005). IPAH lung samples were also found to have reduced expression of dimethylarginine dimethylaminohydrolase (DDAH2), which is an important enzyme involved in the breakdown of ADMA (Zakrzewicz and Eickelberg, 2009). In PAH, cyclic guanosine
monophosphate (cGMP) expression levels are reduced due to the bioavailability of NO. The cGMP enzyme phosphodiesterase type 5 (PDE5) has been found at elevated levels in both lung and right ventricle of PAH patients (McLaughlin et al., 2009). The vasoconstrictor endothelin-1 (ET-1) is known to regulate vascular tone through both the $\text{ET}_A$ and $\text{ET}_B$ receptors. Across a range of PH patients; including IPAH, COPD and CTEPH, ET-1 circulating and local lung levels of ET-1 were increased (Behr and Ryu, 2008, Bauer et al., 2002, Giaid et al., 1993). ET-1 has also been implicated in fibrogenesis through association with matrix metalloproteinases (MMP) (Chelladurai et al., 2012, Jain et al., 2007). ET-1 and the reduced expression of the $\text{ET}_B$ receptor have been linked to increased expression of MMP9 in a MCT rat model of PAH (Ivy et al., 2005).

1.2.3.2 Hypoxic pulmonary vasoconstriction

Hypoxic pulmonary vasoconstriction (HPV) is a process by which in response to low levels of oxygen pulmonary blood flow to distributes to areas with high oxygen levels. This allows for the efficient gas exchange between blood and inspired air (Marshall et al., 1981). This was initially discovered by von Euler and Liljestrand (Euler and Liljestrand, 1946). In humans, HPV was eventually determine to be a 50% increase in pulmonary arterial resistance to an alveolar oxygen partial pressure of less than 50mmHg (Motley et al., 1947). In the pulmonary arteries PASMCs not endothelial cells are thought to monitor for changes in oxygen levels, this is supported by HPV continuing to occur in feline endothelium free arteries (Marshall and Marshall, 1992). However, in other animal models a pre-treatment of cells with ET-1 led to greater levels of hypoxic-induced vasoconstriction (Sham et al., 2000). Endothelin receptor antagonists were also found to block HPV, suggesting that an intact endothelium releasing ET-1 is required for PASMCs to respond to hypoxia (Oparil et al., 1995, Liu et al., 2001). Key components of the response to hypoxia in lung tissue is the action of both $\text{Ca}^{2+}$ and $\text{K}^+$ channels. Importantly, intracellular $\text{Ca}^{2+}$ is elevated in PASMCs exposed to hypoxic conditions (Wang et al., 2005a). Both L-type $\text{Ca}^{2+}$channels and nonspecific cation channels (NSCC) are believed to be involved, this was highlighted when blocking L-type $\text{Ca}^{2+}$ channels where only a partial elimination of HPV (Savineau et al., 1995, Ohe et al., 1989). Interestingly, a blockade of NSCCs led to a complete block on HPV (Weigand et al., 2005). The method by which hypoxic regulates L-type calcium channels has been shown to be at least partially dependent on membrane depolarisation through $\text{K}^+$ channels (Hasunuma et al., 1991, Yuan et al., 1993, Post et al., 1992).

Hypoxic mediated membrane depolarisation has been linked to several $\text{K}_v$ including $\text{Kv} 2.1$, 1.5 and 9.3 (Patel et al., 1997, Archer et al., 1998). However, research suggests that $\text{K}_v$ are not the only mechanism of mediating HPV, the removal of the $\text{K}_v 1.5$ from mice led to an incomplete inhibition of HPV (Archer et al., 2001). Both Redox changes and NSCCs have been shown to regulate $\text{K}_v$ channels (Post et al., 1992, Ward et al., 2005). NSCCs
are identified as transient receptor channels (TRP) that can be subdivided into store operated channels (SOCs) and receptor operated channels (ROCs) (Birnbaumer et al., 1996, Ward et al., 2005). Weissmann and colleagues have demonstrated that ROCs can be activated by protein kinases and diacylglycerol and that they are essential in the response to acute HPV (Weissmann et al., 2006). They demonstrated that removal of TPRC6 from mice led to the removal of a vasoconstrictive response to hypoxia, however non-hypoxic mediated vasoconstriction was maintained (Weissmann et al., 2006). Also, PASMCs isolated from TRPC6 knock out mice did not have a hypoxic mediated Ca\textsuperscript{2+} increase compared to normal PASMCs (Weissmann et al., 2006).

Reactive oxygen species (ROS) have also been implicated in HPV, ROS levels were found to increase in PASMCs exposed to hypoxia (Marshall et al., 1996, Killilea et al., 2000). ROS activates both L-type Ca\textsuperscript{2+} and intracellular Ca\textsuperscript{2+} channels and is thought by influenced by mitochondria (Wolin, 2000, Waypa et al., 2001). More recently HPV was confirmed to increase PASMCs Ca\textsuperscript{2+} by increasing ROS signalling from mitochondria (Waypa et al., 2006). ROS was generated from the electron transport chain (ETC) thereby increasing oxidative stress which led to an increase in cytosolic Ca\textsuperscript{2+} in PASMCs (Waypa et al., 2006). Latterly a further possible mechanism has linked hypoxic-induced Ca\textsuperscript{2+} increase with mitochondria. Liao and colleagues discovered that hypoxia led to ROS-mediated dissociation of FK506 binding protein 12.6 from ryanodine receptor 2 thereby inducing Ca\textsuperscript{2+} release (Liao et al., 2011).

1.2.3.3 Pulmonary vascular remodelling
A key component of PAH pathobiology is the imbalance in the proliferation and apoptosis of PASMCs, PAEC and fibroblasts. This imbalance is manifested in tunica intimal thickening and muscularisation of the distal pulmonary arteries, which previously had little or no smooth muscle presence (Figure 1-3). This results in a narrowing of the artery lumen and a reduction in blood flow therefore leading to less blood oxygenation (Figure 1-4).

Increased muscularisation occurs in distal and in more proximal pulmonary arteries, the primary difference between the two is in a healthy lung the distal arteries have little or no smooth muscle cell presence (Meyrick and Reid, 1983, deMello et al., 1997). Cells from the internal elastic laminae grow and differentiate into smooth muscle cells (Meyrick and Reid, 1978). However, in distal arteries lacking an elastic laminae, pericytes differentiate into cells with a similar phenotype to smooth muscle cells. Muscularisation of these distal arteries also involves the increased recruitment of fibroblasts from the surrounding lung tissue (Jones et al., 1999). The proximal pulmonary arteries also undergo increased muscularisation. There is an increase in collagen 1, which hardens the artery wall (Meyrick and Reid, 1980). Excessive muscularisation involves the activation of elastases, which disturbs the elastic laminae and contributes to increased proliferation and hypertrophy of
smooth muscle cells (Ilkiw et al., 1989). Severe pulmonary hypertension can be identified by the presence of neointima formations. Neointimas are formed from layers of extracellular matrix and various cells positioned between the endothelium and the internal elastic laminae (Yi et al., 2000, Botney et al., 1992). Neointima formation is also dependant on an increased blood flow. Okada and colleagues demonstrated that monocrotaline (MCT) rats that had undergone a left lung pneumonectomy developed neointimas in the intact right lung whereas rats without a pneumonectomy did not (Okada et al., 1997). Neointima are comprised of myofibroblasts, these cells express α-smooth muscle actin and vimentin (Maruyama et al., 1991). However, cells from the neointima do not express any endothelial cell markers (Maruyama et al., 1991). Increased dysfunctional proliferation of endothelial cells leads to the formation of plexiform lesions in severe PH. Within these lesions, the endothelial cells express vascular endothelial growth factor (VEGF), which is a marker of angiogenesis (Tuder et al., 2001, Hirose et al., 2000). A consequence of the formation plexiform lesions is the complete blockage of the pulmonary artery leading to drastic reduction in blood flow (Tuder et al., 1994). Peroxisome proliferator-activated receptor gamma (PPAR-γ) is an important anti-proliferative transcription factor, which has been shown to mediate beneficial effects in both experimental models of PH (Hansmann et al., 2008) and in PAH hPASMCs (Falcetti et al., 2007). Plexiform lesions were found to have decreased expression of PPAR-γ (Ameshima et al., 2003).
Pulmonary arterial hypertension is a progressive vascular disease that primarily affects the pulmonary arteries. During PAH, distal pulmonary arteries have increased smooth muscle infiltration and proliferation narrowing the lumen, this can lead to complete blockage of the distal artery. Muscularisation of distal arteries contributes to increased pulmonary vascular resistance and pulmonary arterial pressure.

Pulmonary arteriogram from a healthy adult lung (left) and from an idiopathic PAH patient lung (right). Loss of distal pulmonary arteries/vascular pruning is extensive in PAH lung. Taken from (Reid, 1986).
1.3. Genetic influence on PAH

A genetic influence on PAH has been suspected for several decades. Initially the role of genetics was suggested by the occurrence of PAH within a single family (Dresdale et al., 1954). An underlying genetic predisposition to PAH was originally termed familial PAH (FPAH) but is now identified as heritable PAH (HPAH). FPAH/HPAH was identified as having an autosomal dominant inheritance, leading to 50% chance of a child of a PAH sufferer developing the disease (Thompson and McRae, 1970). More recently, six families were identified as having an increased incidence of PAH with no obvious contributing secondary factors (Nichols et al., 1997). The gene associated with increased PAH incidence was identified on the long arm of chromosome 2. Latterly the gene was identified as the bone-morphogenetic protein receptor-2 (BMPR-II) (Deng et al., 2000, International et al., 2000). Other genetic risk factors have been identified in the activin receptor like kinase 1 (ALK-1), the potassium channel (KCNK3) (Harrison et al., 2003, Girerd et al., 2010a, Girerd et al., 2014) and the eukaryotic translation initiation factor 2α kinase 4 (EIF2AK4) (Eichstaedt et al., 2016).

1.3.1. BMPR-II gene mutations

BMPR-II gene mutations have been identified in 80% of cases of HPAH (Machado et al., 2006, Morrell, 2010). Interestingly, the penetrance of the mutation is only 20% in HPAH patients suggesting a secondary risk factor, this could be an additional genetic factor or environmental influences (Newman et al., 2004). The severity of HPAH has been shown to vary depending on the type of BMPR-II mutation that the carrier has. BMPR-II carriers with missense mutations were found to have greater PAH severity than those with a truncating mutation (Austin et al., 2009). In establishing inheritance in the children of HPAH patients, a higher number of females were identified as both carriers of mutation and more females had HPAH (Loyd et al., 1995).

1.3.1.1 Normal and dysfunctional BMPR-II signalling

BMPR-II is a serine-threonine receptor kinase member of the TGF-β superfamily of type II receptors, which have TGF-β, BMPs, activins and inhibins as some of its many ligands. The BMPR-II pathway is known to play important roles in early development of the pulmonary circulation, specifically in maintaining PAEC survival (Teichert-Kuliszewska et al., 2006), BMPR-II signalling also assists in the repair of lung tissue injury in adults (Sountoulidis et al., 2012). As stated previously BMPs (bone morphogenetic proteins) can activate BMPR-II signalling. BMPs can be divided into sub-groups including BMP-2/4, BMP-5/6/7/8, GDF-5/6/7 and BMP-9/10 (Kawabata et al., 1998). Subsequently, it was found that BMPs have many functions in regulating cell proliferation, apoptosis and differentiation (Massague and Chen, 2000, Miyazono et al., 2010). When a ligand binds to the BMPR-II receptor, it forms a heterodimer complex with a TGF-β type 1 receptor
(BMPR-I) and phosphorylates and activates the transmembrane region of the TGF-β type 1 receptor (Cai et al., 2012) (Figure 1-5). The activated type 1 receptor then activates Smad (small mothers against decapentaplegic) proteins (Massague et al., 2005). Smad proteins can be divided into three distinct groups; receptor-regulated Smads (R-Smads) including Smad 1/5/9, inhibitory Smads (I-Smads) such as Smad 6/7 and common mediator Smad (Co-Smad) like Smad4 (Heldin et al., 1997). Activation of the TGF--β type 1 receptor phosphorylates R-Smads, which then form heteromeric complexes with Co-Smads. These complexes then translocate to the nucleus of the cell, where they can influence and regulate gene expression by binding to Smad-binding elements (SBE). The inhibitors of DNA binding (Id) are among the key genes that the Smad complex influence. The Id proteins (1-4) inhibit DNA binding ability by blocking the action of the basic helix loop helix (BHLH) transcription factors. BHLH bind to promoter E boxes to form a heterodimer allowing the cell to progress to a proliferative state. Id proteins bind to E proteins to prevent their interaction with BHLHs thereby preventing proliferation of the cell (Russell et al., 2008). Id2, 3 and Id4 have a cyclin-dependent kinase 2 (CDK2) phosphorylation site that Id1 does not (Deed et al., 1997). Id4 is expressed at a much lower level in the vascular wall than Id1, Id2 and Id3 (Yang et al., 2014). BMP-4 and BMP-6 have been implicated in increasing expression of both Id1 and Id3 in PASMCs and different animal models of PH. (Yang et al., 2013b, Yang et al., 2013a, Yang et al., 2011). Smad signalling can be regulated via SMAD ubiquitination and regulatory factors (SMURF1/2) which can suppress SMAD activity (Zhang et al., 2001a).

Despite known association between BMPR-II mutations and PAH, the overarching mechanisms that lead to the development of the disease are not completely understood. Although the BMPR-II mutations are ubiquitous, the pulmonary vasculature appears to be the most affected system in the body. The different cells in the pulmonary vasculature have divergent responses to BMPR-II mutations. PAECs with BMPR-II mutations have been shown to lose mitochondrial function and undergo apoptosis (Diebold et al., 2015). BMPR-II also suppresses leukocyte transmigration by maintaining the PAEC barrier function in the vasculature (Burton et al., 2011). Interestingly, BMPR-II loss of function mutations lead to increased release of growth factors, which increase proliferation of PASMCs (Jen et al., 1996). HPAH patients with reduced BMPR-II expression in PAEC and PASMCs also demonstrate reduced Smad signalling (Atkinson et al., 2002, Yang et al., 2005). BMPR-II mutant PASMCs with reduced Smad dependent signalling have been shown to have an increase in Smad-independent signalling through the pro-proliferative extracellular signal regulated kinases (ERK1/2) (Yang et al., 2008). The reductions in BMPR-II signalling have also been demonstrated in the hypoxic, MCT and sugen hypoxic (SU-Hx) models of PH (Takahashi et al., 2006, Takahashi et al., 2007, Long et al., 2009, Mair et al., 2014). The expression levels of BMPR-II, SMAD1, Id1 and Id3 were found to be lower in female control
hPASMCs compared to male control hPASMCs, suggesting the influence of sex on BMPR-II signalling (Mair et al., 2015). In a heterozygous BMPR-II\(^{+/−}\) mouse model of PH, the addition of a second insult, serotonin, led to a more severe PH phenotype compared to their wildtype littermates (Long et al., 2006). The ability to reverse or mitigate reductions in BMPR-II signalling in PAH is now a focus of novel therapeutic strategies. Therapeutic agents, like iloprost and treprostinil can target specific aspects of the BMPR-II signalling pathway have been identified (Yang et al., 2010, Yang et al., 2013b). These therapies focus on increasing Smad dependent signalling leading to increases in the Id proteins expression.
BMPR-II is co-expressed with a TGF-β type 1 receptor (BMPR-I). Upon BMP binding BMPR-II forms heterodimer complex with BMPR-I, which then stimulates phosphorylation of Smad 1/5/9. Smad 1/5/9 forms a complex with Smad4 that then translocates to the nucleus where it can regulate gene transcription (primarily the Id genes). Activation of Id gene leads to an inhibition of cellular proliferation. Dysfunctional BMPR-II signalling can lead to greater activation of the MAPK pathway and therefore lead to cellular proliferation. SMAD= Sma and MAD (mothers against decapentaplegic), Id= inhibitor of DNA binding, MAPK= mitogen activated protein kinase, ERK= extracellular signal-regulated kinases. Adapted from (Clapp and Gurung, 2015).

Figure 1-5 BMPR-II activation and downstream signalling

BMPR-II is co-expressed with a TGF-β type 1 receptor (BMPR-I). Upon BMP binding BMPR-II forms heterodimer complex with BMPR-I, which then stimulates phosphorylation of Smad 1/5/9. Smad 1/5/9 forms a complex with Smad4 that then translocates to the nucleus where it can regulate gene transcription (primarily the Id genes). Activation of Id gene leads to an inhibition of cellular proliferation. Dysfunctional BMPR-II signalling can lead to greater activation of the MAPK pathway and therefore lead to cellular proliferation. SMAD= Sma and MAD (mothers against decapentaplegic), Id= inhibitor of DNA binding, MAPK= mitogen activated protein kinase, ERK= extracellular signal-regulated kinases. Adapted from (Clapp and Gurung, 2015).
1.3.2. ALK-1 mutations
ALK-1 is a similar serine-threonine receptor to BMPR-II and is a member of the TGF-β superfamily. ALK-1 mutations were found to be associated with HPAH patients and with hemorrhagic telangiectasia type 2 (HHT) (Harrison et al., 2003). These mutations were identified as missense, which led to a loss of receptor function. The detrimental impact of dysfunctional ALK-1 and BMPR-II signalling demonstrate the importance of a functional TGF-β signalling pathway in the pulmonary vasculature. PAH patients with ALK-1 mutations have been identified as having less severe haemodynamics but an overall worse prognosis than other PAH patients (Girerd et al., 2010a, Girerd et al., 2010b).

1.3.3. KCNK3 mutations
The gene KCNK3 encodes for the potassium channel subfamily K member 3, through whole exome sequencing this has been identified as an important gene in the development of PAH (Girerd et al., 2014). KCNK3, also known as TASK-1 (TWK-related acid sensitive potassium channel) is identified by its two pore domains per subunit and its ability to control K+ efflux (Reyes et al., 1998). The main roles of the KCNK3 is to control cellular resting membrane potential and to contribute to the relaxation of vessels (Patel et al., 1999). The mutation variant identified involved a loss of channel gate function, this would negatively impact ability of the KCNK3 to control K+ flow (Ma et al., 2013). KCNK3 expression was recently identified as downregulated in both IPAH and HPAH patients as well as in the MCT rat model of PH (Antigny et al., 2016). ET-1 has also been implicated in inhibiting KCNK3 through ET_A and ET_B receptors via Rho kinase in hPASMCs (Tang et al., 2009, Seyler et al., 2012). Also, hypoxia can downregulate KCNK3 in hPASMC (Olschewski et al., 2006b). The phospholipase A2 inhibitor ONO-RS-082 reversed MCT supressed expression of KCNK3 (Antigny et al., 2016). The prostacyclin analogue treprostinil activates KCNK3 via protein kinase A (PKA) dependent phosphorylation in hPASMCs (Olschewski et al., 2006a). An increasing understanding of the impact of KCNK3 mutations may lead to greater targeting by therapeutic intervention.

1.3.4. Serotonin transporter
Serotonin (5-hydroxytryptamine, 5-HT) was initially described and isolated from serum (Rapport et al., 1948). Serotonin can exert its effects through 14 distinct G protein coupled 5-HT receptors, subdivided into seven classes according the structure and downstream signalling mechanisms (5HT_1-7) (Alexander et al., 2007). The 5-HT_1B receptor has been implicated in both cerebral and pulmonary vasoconstriction, it has also been shown to be upregulated in experimental PAH (van den Broek et al., 2002, Morecroft et al., 1999, Rondelet et al., 2003). The 5-HT_1B was found to regulate calcium binding protein S100A4/Mts1 (MTS1) induced proliferation in cooperation with the serotonin transporter (SERT) (Lawrie et al., 2005). Increased 5-HT_1B expression in female PAH hPASMCs contributes to 5-HT-induced proliferation and the pathobiology of PAH, this has been linked
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to reduced expression of microRNA-96 (Wallace et al., 2015). The SERT is encoded by a single gene on chromosome 17q11.2 and is expressed in blood platelets, neurones, PAECs and PASMCs (Lesch et al., 1993, Ramamoorthy et al., 1993). SERT is sodium dependent transporter, which is like other Sodium/chloride (Na⁺/Cl⁻) transporters. Na⁺ binds to SERT followed by 5-HT and Cl⁻, this creates a conformational change which allows the release of 5-HT into the cell. To restore the original confirmation of SERT a K⁺ ion is released. Interestingly, SERT expression is higher in human lung tissue than in the brain (Ramamoorthy et al., 1993). In a small cohort of PAH patient, 65% presented homozygous for the LL allele SERT polymorphism compared to 8% and 27% for the SS and LS polymorphisms respectively. The LL allele corresponded with an increase in hPASMC proliferative response to 5-HT and serum suggesting a role for SERT in the pathological process of PAH (Eddahibi et al., 2001). However, latterly the LL allele polymorphism was found not to influence PAH survival. However, the LL polymorphism did occur at earlier age in HPAH patients possibly suggesting a link between SERT+ and BMPR-II mutations (Willers et al., 2006). Female mice overexpressing the human SERT (SERT+) leads to the development of severe PAH without additional insult (MacLean et al., 2004). Specific overexpression of SERT in the PASMCs of mice causes the development of PAH whereas mice with the SERT gene knocked out do not develop hypoxic induced PAH (Guignabert et al., 2006, Eddahibi et al., 2000).

1.4. Animal models of pulmonary hypertension

To gain a greater understanding of PAH, different models of the disease have been examined in animals. However, there are disputes as to whether animal models of PH accurately represent the human disease.

1.4.1. Hypoxic induced pulmonary hypertension

The chronic hypoxic model of PH can be used in a variety of animals, hypoxic conditions can be stimulated either via normobaric hypoxia or hypobaric hypoxia. Under hypoxic conditions muscularisation of small arteries occur, this is evidenced by the increase in α-smooth muscle actin (Stenmark et al., 2009). Indeed, early research into hypoxic induced PH in specific sets of animals linked the level of intrinsic vascular smooth muscle to severity of disease (Tucker et al., 1975). The ease of inducing hypoxic PH and the reproducibility of results led to popularity of this model of PH (Stenmark et al., 2009). Many types of cells involved in the vascular wall structure; endothelial cell, smooth muscle cells and fibroblasts all undergo changes that range from proliferation to changing expression of cytokines and receptors (Stenmark et al., 2006b). Additionally, hypoxia induces an inflammatory response within the artery which influences structural remodelling and vasoconstriction (Stenmark et al., 2006b). A stronger PH phenotype was found in hypoxic rats rather than hypoxic mice, with less vascular remodelling in mice (Hoshikawa et al., 2003). This believe to be due to changes in gene expression, in rats hypoxia induces
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Figures that promote; repression of endothelial cell apoptosis and endothelial cell proliferation (Hoshikawa et al., 2003). However, despite the simplicity of the chronic hypoxic model of PH, it does not fully represent the damage to the pulmonary vasculature observed in human PAH (Voelkel and Tuder, 2000, Campian et al., 2006).

1.4.2. Monocrotaline induced pulmonary hypertension

Monocrotaline (MCT) is a toxic pyrrolizidine alkaloid which exist within the plant Crotalaria spectabilis. Initial experimentation involving MCT required the oral administration of crotalaria spectabilis seeds to rats after-which they developed PH (Kay et al., 1967). More recently MCT is given as a single injection to rats to stimulate PH symptoms (Ghodsi and Will, 1981). MCT is metabolised in the liver to monocrotaline pyrrole (MCTP) via the action of cytochrome enzyme (CYP) 3A4 (Kasahara et al., 1997, Reid et al., 1998). The severity of MCT-induced PH is thought to originate from extensive endothelial cell damage, however other studies has demonstrated that a build-up of inflammatory cells in the adventitia of the small pulmonary arteries leads to increase in pulmonary pressure and vascular remodelling (Wilson et al., 1989). Interestingly, MCT can be combined with a pneumonectomy, this led to the formation of neointimal lesions and severe right ventricular hypertrophy (Okada et al., 1997). Unlike human PAH, the MCT PH model does not form obstructive intimal lesions in the pulmonary arteries. MCT induced PH has been shown to be improved by many different therapeutic agents, which is also different to how human PAH responds to therapy (Stenmark et al., 2009).

1.4.3. Sugen-5419-Hypoxic model

To create an animal model of PH that closely resembled human PAH, a study which compared rats under normoxic and hypoxic conditions dosed with the vascular endothelial growth factor (VEGF) inhibitor Sugen 5416 (SU-5416) (Taraseviciene-Stewart et al., 2001). Under normoxic conditions SU-5416 dosing led to mild PH symptoms, however in rats with SU-5416 combined with hypoxia developed a severe and irreversible PH, which was not reversible after removal from hypoxic conditions (Taraseviciene-Stewart et al., 2001). SU-5416 triggered endothelial apoptosis however under hypoxic conditions this led to increased endothelial cell proliferation, this was also accompanied by higher smooth muscle cell proliferation (Taraseviciene-Stewart et al., 2001). A comparison of changes in gene expression was made between lungs of human PAH patients and those from a SU-5416 under hypoxic conditions (SU-Hx). Interestingly, only four genes were found to be common between both (Moreno-Vinasco et al., 2008).

1.5. Established therapeutic strategies

Since the original identification of PAH, therapies have been developed to combat the progressive disease. Unfortunately, the multifactorial nature of PAH has hampered this development. Since many of the underlying molecular mechanisms in PAH are still being
clarified, specific therapeutic strategies are difficult to plan. This is further complicated by a lack of symptomatic manifestation of PAH until the disease is well progressed. Current therapies target pathways and molecular mechanisms identified 10-20 years ago. Despite this, patients currently treated have demonstrated better response to drug therapy. This is observed in the reduction in the 5-year mortality rate observed in the NIH registry in 1980s from 66% to 40% in the UK and Ireland registry from 2012 (Rich et al., 1987, Ling et al., 2012). Current therapeutic strategies for PAH revolve around targeting the increase PVR and PAP associated with increased proliferation and vasoconstriction in the pulmonary vasculature. PGI$_2$ analogues and endothelin receptor antagonists have been developed to target the divergent levels of PGI$_2$ and ET-1 found in PAH patients. As PAH remains an incurable disease, much greater strides should be made for therapeutic strategies to have serious impact on patient clinical outcomes.

1.5.1. G-protein coupled receptors
1.5.1.1 G-protein coupled receptors classification and signalling
G protein coupled receptors (GPCRs) are a useful target for therapeutic intervention, GPCRs are the largest superfamily of receptors and are expressed in many tissues (Lagerstrom and Schioth, 2008). It is thought about 30% of marketed therapeutics target GPCRs, however this only accounts for 10% of the available GPCRs suggesting many more targets to be identified (Vassilatis et al., 2003). GPCRs help regulate downstream cellular machinery like metabolic enzymes, ion channels, transcriptions, motility, contractility and secretion (Neves et al., 2002). GPCRs are subdivided into 6 classes based upon sequence homology and functional similarity, these are: Class A (Rhodopsin-like receptors) (Attwood and Findlay, 1994), Class B (Secretin receptor family) (Harmar, 2001), Class C (Metabotrophic glutamate receptor) (Hans et al., 2007), Class D (Fungal mating pheromone receptors) (Marsh and Herskowitz, 1988), Class E (Cyclic AMP receptors) (Klein et al., 1988) and Class F (Frizzled/Smoothened) (Malbon, 2004). Transmembrane nature of GPCRs made it difficult to understand structure, until the structure of rhodopsin was elucidated (Palczewski et al., 2000). Latterly $\beta_1$ and $\beta_2$ adrenergic structures identified via new crystallization techniques (Cherezov et al., 2007, Warne et al., 2008). Classical GPCR are thought to exist in either an active or inactive state. In the active state the receptor has a high affinity of G-proteins, which form a heterotrimer of GDP bound $G_{a\beta\gamma}$ when inactive. GPCR activation leads to the G-protein binding to the C-terminus of the receptor facilitating GDP to GTP exchange followed by detachment of the $G_a$ and $G_{\beta\gamma}$ subunits, which activated their respective downstream mediators like adenyl cyclase or phospholipase C (PLC) (Figure 1-6) (Bridges and Lindsley, 2008).
Figure 1-6 Schematic of G-protein coupled receptor signalling

After agonist binding to extracellular or transmembrane region of receptor, the receptor facilitates the exchange of GDP for GTP on the Gα subunit. The Gβγ subunit dissociates, which then can regulate ion channels. The activated Gα subunit has many subtypes including; Gαs, Gα12/13, Gαq and Gαi. These can activate different effectors like adenyl cyclase, RhoGEF and phospholipase C. Activation of the Gαi subunit leads to the inhibition of adenyl cyclase activity. GTP= Guanosine-5'-triphosphate, PKA/PKC= protein kinase A/C, PLC= phospholipase C, DAG= diacylglycerol, Ins(1,4,5)P= inositol 1,4,5 triphosphate, EPAC=, exchange factor directly activated by cAMP 1, Adapted from (Ritter and Hall, 2009).
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1.5.1.2 Regulation of GPCRs

A key aspect of GPCR signalling is the regulation of the receptors, this includes modulators that can increase GPCR signalling or those that reduce GPCR signalling. Homer proteins have been found to increase the signalling of β-adrenergic receptors through association with A-kinase anchoring protein (AKAP) thereby increasing receptor mediated ERK signalling (Fraser et al., 2000). Arrestins are well known modulators that can reduce the activity of GPCRs. Spinophilin interacts with the third intracellular loop of GPCRs like dopamine and adrenergic receptor families (Smith et al., 1999, Richman et al., 2001). Spinophilin is also known to interact with several regulators of G-protein signalling (RGS). Spinophilin can tether RGS proteins closely to GPCRs thereby inhibiting signalling (Wang et al., 2005b, Wang et al., 2007a). RGS can stimulate GTPase activity in G subunits (Neitzel and Hepler, 2006). RGS have also been postulated as targets for therapeutic intervention in cardiovascular disease (Cho et al., 2004). Another modulator of GPCR signalling is calmodulin, which is a calcium binding protein that is known to interact with several different GPCRs like serotonin receptors (Turner et al., 2004). Calmodulin can act as feedback inhibition to prevent specific GPCRs increasing intracellular Ca\(^{2+}\) levels (Turner et al., 2004, Turner and Raymond, 2005). Another important regulatory function in GPCRs is desensitisation, internalisation and either degradation or recycling of the receptor (Hanyaloglu and von Zastrow, 2008) (Figure 1-7). G-protein coupled receptor kinases (GRKs) and arrestins are key to receptor internalisation (Reiter and Lefkowitz, 2006). Both groups can be subdivided with GRKs consisting of GRK1 and GRK7 which expressed to retinal rods and cones, this also applies to arrestin 1 and arrestin 4. These are contrasted by the other GRKs, which are expressed more widely along with arrestin 2 and arrestin 3, these are also known as β-arrestin 1 and β-arrestin 2 (Pitcher et al., 1998, Shenoy and Lefkowitz, 2003). GRKs and β-arrestins act at three different stages to eventually internalise GPCRs, these stages include: homologous desensitisation in which the receptor is decoupled from its associated G-protein. The next stage is receptor internalisation, resensitisation and possibly degradation. The final stage is the inhibition of intracellular signalling pathways (Reiter and Lefkowitz, 2006). A more recent investigation in GPCRs signalling has identified the presence of biased agonists. These are agonists to GPCRs that selectively favour the activity of either G-protein or β-arrestin. A ‘balanced’ agonist can activate both G-protein downstream signalling pathways and can promote the recruitment of β-arrestins to the GPCR for desensitisation (Rankovic et al., 2016). Over the last few years biased ligands have been identified for many receptors including the ligand Sar1 favouring β-arrestin activation of angiotensin type 1 receptor (Aplin et al., 2007). The prostaglandin receptor EP\(_4\) has also been identified has having multiple biased agonists that activate either G-protein or β-arrestin (Leduc et al., 2009).
Figure 1-7 G-protein coupled receptor internalised by the action of G-protein coupled receptor kinases and β-arrestin

G-protein signalling can be desensitised via receptor phosphorylation by G-protein coupled receptor kinases and recruitment of β-arrestin. These interact with clathrin and the clathrin adaptor AP2, which assists in internalisation of the receptor into endosomes. Internalisation can lead to production of arrestin mediated signalling pathways, the receptor could also be degraded in a lysosome or recycled back to the cell surface in an endosome thereby undergoing resensitisation. GRK= G-protein coupled receptor kinases, GTP= Guanosine-5’-triphosphate, AP2= Clathrin adaptor AP2. Adapted from (Ritter and Hall, 2009).
1.5.2. Calcium channel blockers
Vasoconstriction of PASMCs is primarily influenced by the accessibility of intracellular calcium. An increased influx of calcium (Ca\(^{2+}\)) into PASMCs will eventually increase the PVR. The ability to block calcium flow into PASMCs presents an opportunity to promote vasodilation, thereby reduce PVR. The impact of Ca\(^{2+}\) blocker therapy on PAH is quite limited. Ca\(^{2+}\) blocker therapy is only recommended in patients who respond to a vasodilatory test, with approximately 5-10% of patients responding positively.

1.5.3. Endothelin receptors and antagonists
Endothelin-1 (ET-1) is a potent vasoconstrictor that can regulate vascular tone through both the ET\(_A\) and ET\(_B\) receptors. The endothelin receptors are G-protein coupled receptors that belong to the rhodopsin-type receptor superfamily. The ET\(_A\) receptor is found on vascular smooth muscle cells whereas the ET\(_B\) receptor is located on both endothelial and smooth muscle cells (Seo et al., 1994). Upon ET-1 binding both receptors activate phospholipase C (PLC), this leads to an increase in inositol triphosphate, diacylglycerol and intracellular Ca\(^{2+}\) (Pollock et al., 1995). These increases lead to increased protein kinase C (PKC) activity therefore leading to greater vasoconstriction of the cell (Ohlstein et al., 1992). The activation of endothelial ET\(_B\) receptors leads to increased release of both NO and PGI\(_2\) (Hirata et al., 1993). ET\(_B\) receptor activation was also implicated in increased prevention in apoptosis as well as a reduction in endothelin converting enzyme-1 (ECE-1) (Shichiri et al., 1997, Clozel et al., 1993). In healthy adults, the plasma levels of ET-1 are low (1-2pg/ml), this suggests that ET-1 is not a circulating hormone. However, ET-1 does play role in maintaining basal blood pressure and vasomotor tone (Haynes et al., 1996). Across a range of PH patients; including IPAH, COPD and CTEPH, ET-1 circulating and local lung levels are increased (Behr and Ryu, 2008, Bauer et al., 2002, Giaid et al., 1993). The increase in ET-1 levels in PAH is associated with right atrial pressure, pulmonary artery oxygen saturation and PVR (Cacoub et al., 1993, Cacoub et al., 1997, Nootens et al., 1995). In isolated PASMC and PAEC, ET-1 can cause vasoconstriction and proliferation via both ET receptors, this can lead to structural changes in the pulmonary vasculature leading to vessel remodelling (MacLean et al., 1994, Davie et al., 2002).

The endothelin system can be modulated by either reducing the level of ECE-1 or by antagonism of the ET receptors. By targeting ECE-1 the levels of ET-1 can be reduced, however several other pathways can influence the creation of ET-1 therefore targeting this enzyme would not be the ideal therapeutic strategy (Takahashi et al., 1998). Antagonism of both ET receptors may be the most effective method of treating the overactive endothelin system in PAH patients. In clinical studies, treatment of PAH patient with the dual ET receptor antagonist (ERA) bosentan leads to patients showing improvements in right atrial pressure, mean PAP, cardiac index, PVR, NYHA functional class and clinical worsening also improved with ET antagonist treatment (Sitbon et al., 2003). Dual
endothelin receptor antagonism reduces PAP and right ventricular hypertrophy (RVH) in a chronic hypoxic rat model of PAH, it also reverses ET-1 induced proliferation in hPASMCs (Davie et al., 2002). Bosentan and Macitentan are both dual ET receptor antagonists approved for use to treat human PAH and are both identified as improving clinical outcomes (Rubin et al., 2002, Pulido et al., 2013). As the ET\textsubscript{B} receptor is involved in ET-1 clearance and can stimulate the release of nitric oxide and PGI\textsubscript{2}, selective antagonism of the ET\textsubscript{A} receptor may be preferable than dual antagonism. Ambrisentan and sitaxsentan are potent ET\textsubscript{A} receptor antagonists, however sitaxsentan was withdrawn from clinical use due to hepatic toxicity. Ambrisentan was developed to counter the associated side effects of bosentan therapy, which include elevations of serum aminotransferase that could lead to liver toxicity (Molina Fernández-Posse et al., 2014). Clinical trials with Ambrisentan demonstrated lower incidences of liver toxicity and lower levels of aminotransferases (McGoon et al., 2009). The influence of sex on ERAs has been explored by Gabler and colleagues, it was demonstrated that women have greater improvements in 6-minute walk distances (6MWD) than men (Gabler et al., 2012). This suggests that targeting PAH therapies to sex could improve therapeutic outcomes.

1.5.4. Phosphodiesterase inhibitors

NO is an important regulator of vascular tone in the pulmonary circulation. As stated previously, NO is produced locally in endothelial cells from the conversion of L-arginine by endothelial nitric oxide synthase (eNOS) (Palmer et al., 1988, Stamler et al., 1994). NO stimulates soluble guanylyl cyclase (sGC), which increases the conversion of guanosine 5’ triphosphate to cyclic guanosine monophosphate (cGMP). The increased levels of cGMP lead to the activation of cGMP-dependent protein kinase (PKG) in PASMCs, which can contribute to vasodilation (Michelakis, 2003). The excessive vasoconstriction in PAH patient pulmonary arteries was linked to reduced expression levels of eNOS (Giaid and Saleh, 1995). Phosphodiesterases (PDEs) are a group of enzymes (PDE1-11) that inactivate both cyclic adenosine monophosphate (cAMP) and/or cGMP, PDE5 is known to degrade cGMP in the lung but also in the pulmonary arteries (Rybalkin et al., 2003, Rabe et al., 1994, Pauvert et al., 2002). PDE5 expression is increased in PAH patient lungs and in the right ventricle of the heart (Corbin et al., 2005, Nagendran et al., 2007). Both chronic hypoxic and MCT rodent models of PH also have elevated PDE5 expression (Murray et al., 2002, Schermuly et al., 2004). High expression of PDE5 in PAH led to the development of inhibitors of PDE5s actions, these inhibitors prevent the hydrolysis of cGMP. PDE5 inhibitor therapies currently used to treat PAH include; sildenafil, vardenafil and tadalafil, all inhibitors demonstrated improved clinical outcomes in placebo-controlled experiments.(Galiè et al., 2005, Jing et al., 2011, Galie et al., 2009a). Interestingly, clinical worsening did not improve with sildenafil or vardenafil, however tadalafil improved the incidence of clinical worsening (Galie et al., 2009a).
of PDE5 inhibitors. Male patients demonstrated greater achievement of a minimal important difference (MID) in the 6MWD compared to females (Mathai et al., 2009).

1.5.5. Soluble guanylyl cyclase stimulators
An alternative to targeting the increase levels of PDE5 in PAH patients is to increase the activation of soluble guanylyl cyclase (sGC) independently of NO, this can be achieved by direct drug stimulation of sGC. Both BAY 41-2272 and BAY 41-8543 were early sGC stimulators that reduced pulmonary vascular remodelling, right ventricular systolic pressure and right ventricle hypertrophy in both chronic hypoxic mouse and MCT rat models of PH (Dumitrascu et al., 2006, Schermuly et al., 2008). BAY 41-2272 and 41-8543 have poor drug metabolism and high clearance rate therefore riociguat (BAY 63-2521) was developed to counteract these problems (Mittendorf et al., 2009). Riociguat is the first sGC drug available for clinical use to treat PAH, clinical trials have demonstrated its effectiveness at reducing PVR, improving disease functional class, time to clinical worsening and increased 6MWD (Ghofrani et al., 2013, Rubin et al., 2015).

1.5.6. Prostaglandin receptors
Prostanoids are a subclass of eicosanoids derived from arachidonic acid that consist of both thromboxanes and prostaglandins (Bos et al., 2004). Classification of prostanoids is determined by the presence of a cyclopentane (prostaglandin) or cyclohexane (thromboxane) ring. Arachidonic acid is metabolised by cyclooxygenase 1 or 2 (COX1/2) to prostaglandin H2 (PGH2), which is the forerunner to the prostaglandins. The action of individual prostaglandin synthase enzymes determines the creation of each specific prostaglandin (Figure 1-8). Thromboxane synthase (TxAS), prostaglandin D synthase (PGDS), prostaglandin E synthase (PGES), prostaglandin I synthase (PGIS), and prostaglandin F synthase (PGFS) synthesise the creation of thromboxane (TXA2), prostaglandin D2 (PGD2), prostaglandin E2 (PGE2), prostaglandin I2/prostacyclin (PGI2) and prostaglandin F2α (PGF2α) respectively (Hata and Breyer, 2004). There are nine identified prostanoid receptors that exist on different cell types; thromboxane receptor (TP), prostaglandin D2 receptors 1 and 2 (DP1-2), prostaglandin E2 receptors 1-4 (EP1-4), prostacyclin receptor (IP) and the prostaglandin F receptor (FP) (Hata and Breyer, 2004). Prostanoid receptors are seven-transmembrane G-protein coupled receptors (GPCR), they can be sub-divided into different groups depending on the type of G-protein activated therefore the cell response generated. The IP, EP2, EP4 and DP receptors all associated with the G-protein Gs, this stimulates cAMP production by adenylyl cyclase leading to vasorelaxation in cells via PKA activation. The TP, EP1 and FP receptors activate the G-protein Ga, which increases intracellular Ca2+ levels by altering phosphatidylinositol production thereby leading to vasoconstriction of cells (Figure 1-9). TXA2 has many actions on the cardiovascular systems ranging from altering platelet aggregation, contributing to heart failure and vasoconstriction and proliferation of smooth muscle cells (Oates et al.,
1988, Castellani et al., 1997, Devillier and Bessard, 1997). The TP receptor can dimerise with the IP receptor to increase the production of cAMP (Wilson et al., 2004). As discussed previously, \( \text{TXA}_2 \) levels are elevated in urine samples from PAH patients whereas \( \text{PGI}_2 \) levels are reduced (Christman et al., 1992). \( \text{PGI}_2 \) is produced by endothelial cells and can reduce smooth muscle proliferation and migration (Moncada et al., 1976, Moncada and Vane, 1978, Clapp et al., 2002). In IPAH patient hPASMCs, IP receptor expression was found to be lower in comparison to control hPASMCs (Lai et al., 2008, Falcetti et al., 2010). PGE\(_2\) can cause both vasodilatation and vasoconstriction due to the different consequences of EP\(_{1-4}\) receptor activation (Hata and Breyer, 2004). PGD\(_2\) has been implicated in airway remodelling and bronchoconstriction, it also primarily associated with type 1 allergic response upon release from mast cells (Hata and Breyer, 2004, Lewis et al., 1982). PGF\(_{2\alpha}\) has been linked to encouraging cardiac hypertrophy in cardiac ventricular myocytes (Lai et al., 1996). Interestingly, both PGF\(_{2\alpha}\) and PGE\(_2\) can activate FP receptors (Abramovitz et al., 2000). As previously discussed prostaglandin receptors can be subdivided into two groups; relaxant receptors and contractile receptors depending on what G-protein they associate with (Alfranca et al., 2006). In pulmonary arteries, it was initially thought that the IP receptor was the only relaxant receptor expressed (Walch et al., 1999, Norel, 2007). However, more recently both EP\(_2\) and EP\(_4\) receptors have been confirmed in both proximal and distal pulmonary arteries in control and MCT rats (Lai et al., 2008). Both EP\(_2\) and EP\(_4\) have also been shown to play a role in blocking proliferation of hPASMCs (Jigisha et al., 2015, Lai et al., 2008). IP receptor expression has been found to be reduced in IPAH patients compared to non-PAH patients, however EP\(_4\) receptor expression was shown to remain stable in IPAH patients (Falcetti et al., 2010, Lai et al., 2008). The importance of the IP receptor to pulmonary artery homeostasis was highlighted by IP receptor knock-out mice (IP-KO). Hoshikawa and colleagues subjected IP-KO and wild type mice to high altitude (17,000 feet) for 3 weeks after-which IP-KO mice presented with a much greater pulmonary hypertensive phenotype (Hoshikawa et al., 2001). Both the TP and EP\(_3\) receptors have been implicated in vasoconstriction in pulmonary arteries (Walch et al., 1999, Norel, 2007). Blockade of the TP receptor was found to attenuate protamine-heparin induced pulmonary hypertension in sheep (Montalescot et al., 1990). EP\(_3\) expression would found to be elevated compared to controls in hPASMCs and pulmonary arteries exposed to hypoxia (Lu et al., 2015). Also, the study discovered that removing the EP\(_3\) in smooth muscle cells prevented increased pulmonary artery medial thickness (Lu et al., 2015).
Figure 1-8 Prostaglandin biosynthesis from arachidonic acid

COX 1 and/or 2 metabolise arachidonic acid to the unstable prostaglandin H₂. Individual prostaglandin synthase enzymes act on prostaglandin H₂ to create the five primary distinct prostaglandins. TXAS, PGDS, PGES, PGIS and PGFS respectively generate thromboxane A₂, prostaglandin D₂, E₂, I₂ and F₂α. COX1/2= cyclooxygenase 1/2, TXAS= thromboxane A synthase, PGDS= prostaglandin D synthase, PGES= prostaglandin E synthase, PGIS= prostaglandin I synthase and PGFS= prostaglandin F synthase. Adapted from (Mubarak, 2010).
1.5.6.1 Cyclic AMP signalling

Cyclic AMP (cAMP) is a key intracellular second mediator that is produced after specific GPCRs are activated. Adenylyl cyclase (AC) catalyses the conversion of adenosine triphosphate to cAMP, which can then act on several downstream effectors (Tasken and Aandahl, 2004). The three main targets of cAMP are protein kinase A (PKA), exchange factor directly activated by cAMP 1 (EPAC) and cyclic nucleotide gated ion channels (Fimia and Sassone-Corsi, 2001). PKA is constructed from a regulatory subunit dimer and two catalytic subunits, which together form an inactive complex known as a holoenzyme. A-kinase anchoring proteins (AKAPs) act as scaffolding proteins, which anchor the PKA holoenzyme and can help target the PKA to specific cellular locations (Wong and Scott, 2004). Four cAMP molecules bind to two distinct binding sites on the regulatory subunit, this releases the catalytic subunits. PKA directly phosphorylates cAMP responsive element binding protein (CREB), phosphorylating CREB at serine residue 133 (Gonzalez and Montminy, 1989). Phosphorylation at serine 133 leads to the recruitment of CREB binding protein (CBP) (Chrivia et al., 1993). CREB mediates the actions of cAMP responsive genes by interacting with conserved cAMP response elements (CRE) (Montminy et al., 1986). Gene transcription mediated by cAMP peaks approximately 30 minutes, however CREB activity continues for several hours and is dependent upon the dephosphorylation at the serine residue 133 by the serine/phosphatase PP-1 (Hagiwara et al., 1992). PKA can also phosphorylate adenylyl cyclase suggesting PKA functions as a feedback mechanism to prevent prolonged levels of cAMP (Iwami et al., 1995, Chen et al., 1997). The relationship between cAMP and EPAC was discovered after inhibition of PKA did not prevent the activation of the small GTPase Rap1 (de Rooij et al., 1998). Interestingly, EPAC contains a cAMP binding domain like that found on the PKA regulatory subunits. EPAC and PKA have been demonstrated as having differential actions in PC12 cells, specific cAMP mediated PKA activation led to cell proliferation via ERK1/2 activation. EPAC extended the period of PKA dependent ERK1/2 activation, however it promotes anti-proliferation of the PC12 cells (Kiermayer et al., 2005). Activation of EPAC is implicated in inducement of cell adhesion in ovarian tumour cell lines as well activation of protein kinase B (Rangarajan et al., 2003, Mei et al., 2002).

In pulmonary arteries mechanisms that elevate cAMP levels are crucial to maintain a relaxed state. Indeed, both cAMP and cGMP analogues have been shown to elicit smooth muscle cell vasodilation (Sakai and Voelkel, 1988, Francis et al., 1988). Forskolin, which is stimulator of AC and a cAMP analogue were shown to open the large-conductance, calcium- and voltage-activated potassium channel (BKCa) in PASMCs isolated from rats to induce vasodilation (Barman et al., 2003). In two studies β-adrenergic receptor mediation of cAMP led to the greatest relaxation, however the relaxations mediated by cAMP via the adenosine A2 receptor and the EP2 receptor closely matched that of the β-adrenergic
receptor (Fullerton et al., 1994, Fullerton et al., 1996). The overall effectiveness of cAMP in the pulmonary artery is strongly influenced by the action of PDEs. In IPAH patients the expression of several PDE isoforms was found to be increase, however PDE1C demonstrated the greatest impact on lowering cAMP levels and by association the increased proliferation of hPASMCs (Murray et al., 2007). Chronic hypoxic rats have shown to have decrease cAMP levels compared to their normoxic controls. (MacLean et al., 1996) The vasodilatory effects of cAMP on the pulmonary artery is a major reason why PDEs and cAMP elevating agents like prostaglandin analogues are used to treat PAH.

1.5.7. Prostaglandin analogues

1.5.7.1 Epoprostenol

Epoprostenol was the first PGI₂ therapy approved for the treatment of PAH (Chin and Rubin, 2008). However, it must be administered via continuous intravenous infusion due to its instability at a pH less than 10.5 and its very short half-life of 3-5 minutes (Vane and Corin, 2003). Despite its instability, epoprostenol therapy has been effective at improving exercise capacity, decreases in mean PAP and PVR in PAH patient vs control patients (Barst et al., 1996). A side effect common to epoprostenol treatment is the development of sepsis usually related to the method of drug administration (Barst et al., 1996, Humbert et al., 1999).

1.5.7.2 Iloprost

Iloprost is an alternative prostacyclin analogue used for the treatment of PAH. A major benefit of iloprost over epoprostenol is its stability at room temperature, the ability to vary the administration of the drug and its longer duration of vasodilation (Fitscha et al., 1987). The ability to inhale the drug allows for more direct treatment of the small peripheral pulmonary arteries (Olschewski et al., 2003). Both intravenous (IV) and inhaled forms of iloprost lead to significantly increased positive clinical outcomes in PAH patients. For inhaled iloprost this positive outcome was reached at a much lower dose than IV dosing (Olschewski et al., 2002). Interestingly, a more sustained haemodynamic improvement was observed in intravenous dosing with epoprostenol although there are less side effects and associated therapy complications with inhaled iloprost (McLaughlin et al., 1998). As a prostacyclin analogue, iloprost is known to mediate its vasodilatory and anti-proliferative effects via the IP receptor. However, recently iloprost has been identified as an agonist for the G₈ coupled EP₄ receptor as well as the G₉ coupled EP₁ receptor (Lai et al., 2008, Whittle et al., 2012). This suggests that iloprost can cause both vasoconstriction and vasodilation by increasing calcium influx levels and increasing cAMP levels. Iloprost has been shown to mediate its anti-proliferative effects by the activation of BMPR-II signalling in both non-mutant and mutant PAH hPASMCs (Yang et al., 2010).
1.5.7.3 Beraprost
Beraprost sodium was the first stable orally active prostacyclin analogue used in the treatment of PAH (Nishio and Kurumatani, 2001). In clinical studies, beraprost improved symptoms for NYHA class II and III PAH patients and increased exercise capacity as measured by 6WMD (Galie et al., 2002). However, the longer term clinical effectiveness of beraprost was questioned by Barst and colleagues. The clinical outcome improvements demonstrated after 3 months were not sustained at 9 or 12 months (Barst et al., 2003). Beraprost successfully prevented the development of PH in a MCT rat model primarily through anti-platelet aggregation and vasodilatory effects (Miyata et al., 1996). In isolated hPASMCs, beraprost reduced proliferation under normoxic and hypoxic conditions in a dose dependent manner through activation of cAMP (Kadowaki et al., 2007).

1.5.7.4 Selexipag/MRE-269
Common features of all prostacyclin analogue therapy are side effects associated with the vasodilation of the vasculature, these include flushing, headaches, nausea and jaw pain. This may be related to the variety of prostanoid receptors that specific prostaglandin analogues can activate. Selexipag/MRE-269 (active metabolite) is an orally active and highly selective IP receptor agonist that is not a prostacyclin analogue (Kuwano et al., 2007). Both beraprost and iloprost can activate the EP3 receptor leading to vasoconstriction that can detrimentally effect their IP receptor mediated vasodilation in rat pulmonary arteries. Also, the vasorelaxation induced by Selexipag/MRE-269 was no different between control and MCT rats whereas the vasodilation by beraprost and iloprost were attenuated (Kuwano et al., 2008). This sustained vasorelaxation of rat arteries was also demonstrated when compared to reduced treprostinil induced vasorelaxation in MCT rat arteries or rat arteries pre-constricted with ET-1 or phenylephrine (Morrison et al., 2012). In hPASMCs, MRE-269 reduces cellular proliferation through a IP receptor mediated increase in cAMP (Gatfield et al., 2014, Jigisha et al., 2015). A possible drawback of specific IP receptor targeting in PAH is the perceived reduction in the expression of the IP receptor in PAH patients (Falcetti et al., 2010).

1.5.7.5 Treprostinil
Treprostinil is a tricyclic benzindene analogue of PGI2 therefore like other PGI2 analogues it has similar vasodilatory and anti-platelet aggregation properties (Vachiery and Naeije, 2004). One of the largest clinical trials conducted examined the effectiveness of a constant subcutaneous infusion of treprostinil. Like alternative prostacyclin analogues, treprostinil improved exercise capacity via the 6MWD and improved symptoms, signs and haemodynamics of PH (Simonneau et al., 2002). Infusion site pain is the most common side effect of subcutaneous treprostinil. Previously, beraprost was shown not to cause longer-term improvements on exercise capacity and haemodynamics (Barst et al., 2003); similar follow-up studies were conducted with subcutaneous treprostinil. The early clinical
improvements were sustained and survival rates were 87% after 1 year and 68% over 4 years (Barst et al., 2006). As with inhaled iloprost therapy, inhaled treprostinil would reduce some of the associated side effects with prostacyclin analogue therapy, specifically infusion site pain. In smaller scale pilot studies, inhaled treprostinil produced a more sustained reduction on PVR and fewer systemic side effects than inhaled iloprost (Voswinckel et al., 2006). In symptomatic PAH patients on bosentan therapy, inhaled treprostinil significantly decreased mean PAP and PVR (Channick et al., 2006). In a larger clinical trial, PAH patients that were symptomatic on bosentan or sildenafil additional inhaled treprostinil therapy increased exercise capacity and quality of life measurements, however no improvements were detected in functional class or PAH signs and symptoms (McLaughlin et al., 2010). The development of an oral formulation of treprostinil has offered a potential advancement in the treatment of PAH. However, initial investigations demonstrated a lack of significantly positive clinical outcomes on patients with background therapies of ERAs and/or PDE5 inhibitors (Tapson et al., 2012, Tapson et al., 2013). Interestingly, oral treprostinil as a monotherapy was found to increase exercise capacity in NYHA functional class II or III PAH patients (Jing et al., 2013). A possible reason behind differences observed in therapeutic effectiveness of oral treprostinil has been the inability to identify a clinically effective dose (Chin et al., 2015). Treprostinil has been shown to partially reverse PH in different animal models; in chronic hypoxic mice treprostinil decreases RVSP, vascular remodelling and circulating fibrocytes (Nikam et al., 2010). Treprostinil also improved in pulmonary haemodynamics in the MCT rat model of PH (Yang et al., 2010). In initial studies in isolated hPASMCs, treprostinil induced greater increases in cAMP generation compared to alternative prostacyclin analogues; cAMP contributes to anti-proliferation mediated by treprostinil (Clapp et al., 2002). As a prostacyclin analogue, treprostinil was known to activate the IP receptor to mediate vasodilation and reduce proliferation. However, when the IP receptor was found to be downregulated in IPAH patients, the anti-proliferative effects of treprostinil were mediated through PPAR-γ (Falcetti et al., 2010). More recently treprostinil was found to only significantly reduce proliferation in IPAH hPASMCs in combination with the PDE2 inhibitor BAY 60-7550, this was also observed when PH indices were examined in a chronic hypoxic mouse model of PH (Bubb et al., 2014). In a comparative study examining the vasodilatory properties of both iloprost and treprostinil, human pulmonary arteries were found to vasodilate more effectively to iloprost than treprostinil (Benyahia et al., 2013). Recently both the EP_2 and DP_1 and to a lesser extent the EP_4 receptor have been confirmed as treprostinil targets (Table 1-3). As previously stated the EP_2, DP_1 and EP_4 receptors are all G_s protein coupled receptors that lead to cAMP generation therefore can contribute to vasodilation or anti-proliferation (Whittle et al., 2012). Unlike iloprost, treprostinil has a very low binding affinity for the vasoconstrictive EP_1 receptor, this highlights a possible difference in the vasorelaxation potential of the two prostacyclin
analalogues (Table 1-3). Activation of the EP2 receptors by treprostinil and subsequent increases in cAMP was also observed in alveolar macrophages isolated from rats (Aronoff et al., 2007). Treprostinil has been shown to activate KCNK3, which restores the K+ current and membrane potential in hPASMCs leading to vasodilation (Olschewski et al., 2006a). Research continues to be conducted into the mechanism of action of prostacyclin analogues and how they interact with other therapies. Interestingly, in hPASMCs, treprostinil mediated clearance of ET-1 was blocked by the addition of ERAs; this may have clinical relevance for those patients on similar combination therapies (Patel et al., 2014). Treprostinil metabolism has been identified to occur in the liver through the cytochrome enzyme 28C (CYP2C8) and through CYP2C9. A CYP2C8 inducer (rifampin) decreased the blood levels of treprostinil by 30% whereas the addition of an inhibitor of CYP2C8 (gemifibrozil) led to a doubling of treprostinil blood concentration (Skoro-Sajer et al., 2008, Rollins et al., 2009, Kumar et al., 2016). PAH patients that have hepatic dysfunction have reduced clearance of treprostinil by up 80% (Skoro-Sajer et al., 2008). In PAH patients with inoperable CTEPH, treprostinil treatment improved exercise capacity, haemodynamic and patient survival (Skoro-Sajer et al., 2007). These positive clinical outcomes were believed to due to the anti-proliferative, vasodilatory and anti-platelet aggregation effects (Skoro-Sajer et al., 2007). For many years anti-coagulation has been a recommended therapy in IPAH patients, more recently the effectiveness of the therapy was examined in other PAH groups with no conclusive results (Olsson et al., 2013). The anti-platelet aggregation effects of treprostinil led to the investigation of combination therapy of treprostinil with warfarin. Wade and colleagues found that no serious side effects or clinically important interactions occurred (Wade et al., 2003).
### Table 1-3 Treprostinil and Iloprost Ki values at prostaglandin receptors

<table>
<thead>
<tr>
<th>Prostaglandin Receptor</th>
<th>Treprostinil Ki (nM)</th>
<th>Iloprost Ki (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP</td>
<td>32.1 ± 0.2</td>
<td>3.9 ± 0.6</td>
</tr>
<tr>
<td>EP&lt;sub&gt;1&lt;/sub&gt;</td>
<td>212 ± 56</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>EP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>3.6 ± 0.3</td>
<td>1172 ± 159</td>
</tr>
<tr>
<td>EP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>2505 ± 263</td>
<td>208 ± 26</td>
</tr>
<tr>
<td>EP&lt;sub&gt;4&lt;/sub&gt;</td>
<td>826 ± 116</td>
<td>212 ± 27</td>
</tr>
<tr>
<td>DP&lt;sub&gt;1&lt;/sub&gt;</td>
<td>4.4 ± 0.4</td>
<td>1016 ± 63</td>
</tr>
<tr>
<td>FP</td>
<td>4680 ± 927</td>
<td>131 ± 17</td>
</tr>
<tr>
<td>TP</td>
<td>Not calculable</td>
<td>3778 ± 375</td>
</tr>
</tbody>
</table>

Treprostinil and Iloprost binding profile for prostanoid receptors. Data gather using displacement radioligand binding from cell membranes that expressed recombinant human prostanoid receptors. Table taken from (Whittle et al., 2012)
Figure 1-9 Overview of prostaglandin analogue activation of prostaglandin receptors

Each prostaglandin analogue has a different binding profile to prostaglandin receptors. Treprostinil binds with high affinity to the IP, EP2 and DP1 receptors (EP4 receptor not shown), Selexipag binds only the IP receptor, whereas Iloprost can activate both EP1 and IP receptors. IP, EP2 and DP1 receptors are Gs associated receptors, activation leads to adenylyl cyclase activating cAMP and PKA therefore vasodilation or anti-proliferation. The EP1 receptor associates with the Gq protein, activation leads to PLC mediated creation of IP3 and DAG, which leads to elevated levels of intracellular Ca2+. AC= adenylyl cyclase, cAMP, cyclic adenosine monophosphate; DAG= diacylglycerol, G= G protein, IP3= inositol triphosphate; PKA= protein kinase A, PLC= phospholipase C.
1.5.8. Developing therapies for PAH

As the understanding of PAH pathophysiology increases, novel pathways are being investigated as potential therapeutic targets.

1.5.8.1 Imatinib

Imatinib is an anti-proliferative drug originally developed to block the Bcr-abl tyrosine kinase in chronic myeloid leukaemia. Imatinib can also block the action of platelet-derived growth factor (PDGF) and c-KIT signalling, both of which are implicated in PH development and smooth muscle proliferation (Hoeper et al., 2013, Humbert et al., 1998). In two animal models of PH; chronic hypoxic mice and MCT rats, imatinib was found to almost restore both right ventricle pressure and hypertrophy to control levels (Schermuly et al., 2005). Imatinib also demonstrated anti-proliferative and pro-apoptotic actions on PASMCs isolated from IPAH patients (Nakamura et al., 2012). Long term safety and efficacy trial with imatinib resulted in a high discontinuation rate, with 31% due to adverse events like subdural haematomas (Frost et al., 2015).

1.5.8.2 Vasoactive intestinal peptide

Vasoactive intestinal peptide (VIP) can relax pulmonary arteries and diminish the actions of vasoconstrictors like ET-1 (Hamidi et al., 2011). The role of VIP in PAH has been suggested in studies using a VIP knock out mouse model, which had increased RV pressures and pulmonary vascular remodelling. The increased vascular remodelling was also reversed by treatment with VIP (Stenmark et al., 2009). In the MCT rat model of PH, VIP treatment partially reversed PAH indices more efficiently than the dual ERA bosentan (Hamidi et al., 2011).

1.5.8.3 Rho kinases

Both vasoconstriction of smooth muscle cells and endothelial dysfunction are key causative agents in the development of PAH. The rho-kinase pathway can increase calcium sensitisation leading to increased vasoconstriction and smooth muscle proliferation (Schwenke et al., 2011). Fasudil, a rho-kinase inhibitor, has mediated reductions in RV pressure, mean PAP, pulmonary arterial remodelling and RV hypertrophy in the MCT rats (Mouchaers et al., 2010). Combination with an ERA or a PDE5 inhibitor did not produce any enhanced therapeutic effect (Mouchaers et al., 2010).

1.5.8.4 Apelin

Another developing target for PAH therapy is the peptide Apelin. Apelin is known to regulate both proliferation and apoptosis of smooth muscle and endothelial cells. Goetze and colleagues have demonstrated that PAH patients have lower levels of apelin compared to control patients (Goetze et al., 2006). A link exists between BMPR-II signalling dysfunction and lower expression of apelin. This is mediated through disruption of the peroxisome proliferator-activated receptor (PPAR-γ)/β-catenin complex. This leads
to greater PASMC proliferation and a reduction in endothelial cell apoptosis (Alastalo et al., 2011). Currently, the short half-life, administration problems and the systemic effects of apelin prevents its use as a PAH therapy (Andersen et al., 2011).

1.5.8.5 PPAR-γ agonists
The importance of PPAR-γ in maintaining pulmonary vascular homeostasis was demonstrated in endothelial and/or smooth muscle PPAR-γ knock out mice, which develop PAH (Hansmann et al., 2008, Guignabert et al., 2009a). The PPAR-γ agonist rosiglitazone diminished PH in chronic hypoxic rats, rosiglitazone was also found to induce relaxation in human pulmonary arteries (Kim et al., 2010, Kozłowska et al., 2013). Prostacyclin analogues can mediate their anti-proliferative effects through activation of PPAR-γ, also rosiglitazone was demonstrated to increase the anti-proliferative effect of treprostinil in PASMCs from IPAH patients (Falcetti et al., 2007, Falcetti et al., 2010).

1.5.8.6 Dichloroacetate
The normal cycle of adenosine trisphosphate (ATP) creation by the Krebs cycle is disrupted in PAH patients when, ATP production is mediated through glycolysis activated by pyruvate dehydrogenase kinase (PDK) (Piao et al., 2010). This suggests that mitochondrial dysfunction occurs in PAH. In MCT mice and IPAH patients, the PDK inhibitor dichloroacetate (DCA) decreased proliferation and increased apoptosis of PASMCs (Guignabert et al., 2009b). DCA also reversed potassium channel (Kv1.5) downregulation in the pulmonary arteries of MCT rats (McMurtry et al., 2004).

1.5.8.7 MicroRNAs
MicroRNAs (miRNAs) have been identified as potential therapy targets for PAH. miR-204 expression was reduced in the MCT rat model of PH and in hPASMCs from PAH patients. Treatment with a synthetic miR-204 led to decrease RVSP, RV hypertrophy and vascular remodelling in rats, proliferation was also reduced in PAH hPASMCs (Courboulin et al., 2011). Depressed miR-96 levels were associated with increased 5-HT<sub>1B</sub> expression levels, contributing to serotonin (5-HT) induced proliferation. Increasing levels of miR-96 reduced 5-HT<sub>1B</sub> expression and 5-HT induced proliferation, it also reduced indices of hypoxic induced PH in mice (Wallace et al., 2015). Interestingly, reducing expression of both miR-17 and miR-20a by targeting with antagonirs lead to reductions in PH indices in hypoxic mice, reduced hPASMC proliferation and increased expression of important BMPR-II signalling mediators like Id1 and Id2 (Pullamsetti et al., 2012, Brock et al., 2014). The ability to target miRNAs involved in diseases like PAH is a growing area of research.

1.5.8.8 Endothelial progenitor cells and mesenchymal stromal cells
Endothelial progenitor cells, derived from bone marrow, are thought to assist in therapeutic angiogenesis (Asahara et al., 1997). In PAH patients, the circulating levels of EPC were found to be reduced (Diller et al., 2008, Junhui et al., 2008). Engrafted EPCs prevented
monocrotaline-induced RVSP increases in rats, interestingly EPCs transduced with eNOS demonstrated greater reversal of PH (Zhao et al., 2005). Administration of EPCs to PAH patients was found to be tolerated and led to improvements in both exercise capacity and short term haemodynamics (Wang et al., 2007b, Granton et al., 2015). Mesenchymal stromal cells (MSCs) like EPCs are derived from bone marrow. Interestingly, MCT rats treated with MSCs and MSCs overexpressing eNOS led to reductions in RVSP and RVH. However, the reductions in RVSP were much greater in rats treated with MSCs overexpressing eNOS (Kanki-Horimoto et al., 2006). Exosomes derived from MSCs inhibited the development of hypoxic pulmonary hypertension in mice, both the miR-17 superfamily and miR-204 were increased as well as inhibiting signal transducer and activator of transcription 3 (STAT 3) signalling in PAECs (Lee et al., 2012).

1.5.8.9 Anastrazole
Blocking the localised synthesis of estrogen in lung was demonstrated to attenuate PH in both female hypoxic mice and SU-HX (Mair et al., 2014). The application of the aromatase inhibitor anastrazole in human PAH has been initially examined in a small clinical trial. Anastrazole increased the 6MWD in treated patients compared to placebo, also levels of 17-β estradiol were decreased (Kawut et al., 2016). A large clinical trial is required to confirm the effectiveness of anastrazole in the treatment of PAH.

1.6. Sex in PAH
Registry data for PAH and other PH classifications demonstrate an increased female susceptibility to disease development. The ratio of disease development is 4 female patients to 1 male patient (Rich et al., 1987, Humbert et al., 2006, Ling et al., 2012). The increase female predisposition to PAH suggests that sex can influence the development of PAH. The increase frequency of females suffering PAH was first detected in the 1950s, however no obvious therapeutic strategy has been developed since to specifically target to females in PAH (Dresdale et al., 1951). Despite increased female susceptibility to PAH, females have greater survival than males, with less PAH severity in female patients (Humbert et al., 2010b, Humbert et al., 2010a). A critical difference between female and male patients is the presence of specific sex hormones. A key focus of ongoing PAH research is investigation of the survival difference and its potential link to sex hormones. Animal PH models can replicate the sex differences observed in clinical PAH patients, however, contradictions exist in these models, where the female sex hormone estrogen can have protective effects (Lahm et al., 2012). Other studies indicate that estrogen is a causative effect in PH development, mice overexpressing the serotonin transporter (SERT+) demonstrate female susceptibility to PH (White et al., 2011a). The conflicted role of estrogen in the development and susceptibility of PAH is commonly known as the ‘estrogen paradox’.
1.6.1. Biosynthesis of estrogen and metabolism

Female sex hormones are steroid hormones which include both progesterones and estrogens. Estrogens are primarily synthesised in the ovaries and corpus luteum in the female reproductive tract. Local estrogen synthesis can also take place in liver, adipose tissue and recently the estrogen-synthesising enzyme has been identified in endothelial and vascular smooth muscle cells (VSMC) (Tofovic, 2010). Synthesis of estrogen is controlled by the hypothalamic-pituitary-gonadal axis (HPG), which involves the action of endocrine hormones secreted from the hypothalamus and pituitary gland in the brain. Follicle stimulating hormone (FSH) can increase the transcription of aromatase, which is an important estrogen synthesising enzyme. The balance of estrogen synthesising hormones is controlled by positive and negative feedback, this forms the basis of the female reproductive cycle. The three primary naturally occurring isoforms of estrogen are: estrone, estradiol and estriol. In pre-menopausal women, estradiol is the highest circulating hormone whereas estrone is increased during menopause and estriol is highest post menopause. However, estriol also has role during pregnancy maintaining a healthy uterine lining (Khosla et al., 1997).

Estrogen biosynthesis begins with the conversion of cholesterol to pregnenolone by the cytochrome P450 (CYP) 11A1 enzyme (Figure 1-10). Cholesterol will only be converted under the presence of specific sex hormones control by the HPG axis. As stated previously, VSMC can produce estrogen locally, which may have role in maintaining vascular homeostasis (Tofovic, 2010, Harada et al., 1999). Locally produced estrogen acts in a paracrine manner at the site of synthesis whereas a majority of gonadal produced estrogen acts in an endocrine fashion (Labrie et al., 1998). Pregnenolone is rapidly converted to progesterone by the 3β hydroxysteroid dehydrogenase (HSD) which is subsequently hydroxylased to androstenedione by CYP17A1. Androstenedione can be converted into two distinct products; testosterone by 17-β HSD or estrone by CYP19A1 (aromatase). Both products can be converted to 17-β estradiol by aromatase or 17-β HSD respectively. Estrogens are metabolised by oxidation, this occurs primarily in the liver coordinated by CYP 450 enzymes. However, as with estrogen production, metabolism can take place in other areas of the body including brain, lung and breast tissue.

Estradiol can be oxidised at several difference carbon positions in its chemical structure. Oxidation at the carbon-17 (C17) position leads to estrone formation, whereas oxidation at C16, C4 and C2 produces alternative metabolites. The nicotinamide adenine dinucleotide phosphate (NADPH) dependent oxidation of estradiol to these metabolites is dependent on the action of CYP 450 enzymes. The formation of 2-hydroxyestradiol (2-OHE2) is mediated by CYP1A1, A2 and 3A4 whereas CYP1B1 is essential for the formation of 4-hydroxyestradiol (4-OHE2). The CYP enzymes have different expression levels in different tissues in the body. CYP1B1 is expressed at high levels in breast cancer
tissue, leading to a bias in estrogen metabolism in favour of 4-OHE2 (Wen et al., 2007). CYP1B1 lung levels were found increase when exposed to tobacco smoke, females were also identified as having greater expression of CYP1B1 than males (Peng et al., 2013). There is increasing interest in targeting CYP1B1 due its potential pathogenic properties. 17-β estradiol can engineer its own breakdown by increasing transcription of CYP1B1 through an estrogen response element in the promotor region (Tsuchiya et al., 2005). Also, polymorphisms identified in the CYP1B1 gene have contributed to the development of estrogen positive cancers (Hanna et al., 2000). Both hydroxyestradiol metabolites are broken down further to 2 and 4 methoxyestradiol metabolites by the catechol-O-methyltransferase (COMT) enzyme. Interestingly, the hydroxy or methoxy metabolites can undergo sulphation by sulphotransferases (SULTS) into water soluble molecules that are removed from the body via the kidneys in urine.

The aromatase gene is present in both gonadal and non-gonadal tissue; transcription is dependent on signalling pathways that act at different sites in its promotor region. Aromatase expression in adipose tissue is influenced by glucocorticoids, cytokines and the Janus kinase/signal transducers and activators of transcription (Jak/STAT) (Zhao et al., 1995). In adipose tissue, cortisol was found to increase aromatase activity specifically in females (McTernan et al., 2002). Aromatase has been implicated in estrogen positive diseases like breast cancer (Harada, 1997, Harada and Honda, 1998, Kazmi et al., 2012). The aromatase inhibitor anastrozole is used as a part of a combination therapy in breast cancer patients (Forbes et al., 2008, Masuda et al., 2012). However, current aromatase inhibitors will act on all aromatase in the body, this lack of specificity can lead to off target effects in bone formation maintenance or can disrupt estrogen levels in the human vena cava (Sasano et al., 1997, Sasano et al., 1999). Aromatase expression is increased within non-small cell lung cancer (NSCLC) cells and is also a predictor of survival in NSCLC (Márquez-Garbán et al., 2009, Mah et al., 2007).
Figure 1-10 Overview of estrogen synthesis and metabolism

Cholesterol is converted to androstenedione by enzymes CYP11A1, 3β HSD and CYP17A1. Androstenedione can be converted into either estrone or testosterone by CYP19A1 or 17β-HSD respectively. Both estrone and testosterone are converted to 17β estradiol through 17β-HSD or CYP19A1 respectively. 17β estradiol is converted to either 2, 4 or 16 hydroxyestradiol by the action of various CYPs (CYP1A1, CYP1B1). COMT can convert 2 and 4- hydroxyestradiol to 2 and 4 methoxyestradiol respectively. 

CYP = cytochrome P450, HSD = hydroxysteroid dehydrogenase, COMT = catechol-O-methyltransferase.
1.6.2. Estrogen receptors and signalling

Estrogen actions and influence was initially thought to be limited to reproduction. Greater research has highlighted the role that estrogens can play throughout the body. Local estrogen synthesis separate from the gonadal tissue highlights the role of paracrine estrogen signalling. Estrogen signalling can be mediated through either a genomic pathway or a non-genomic pathway (Mendelsohn, 2002). Genomic signalling involves estrogens acting on intracellular/membrane bound estrogen receptors leading to a conformational change and results in direct regulation of gene transcription. Non-genomic signalling involves estrogen activation of second messengers and signal transduction to mediate its effects. It was initially believed that estrogen mediated its effects through a single estrogen receptor. However, more recently, two types of estrogen receptor have been identified, estrogen receptor α (ERα) and estrogen receptor β (ERβ) (Green et al., 1986, Mosselman et al., 1996). Both ERα and ERβ are thought to act as membrane bound receptors as well as nuclear receptors however, an additional estrogen receptor known a G protein coupled estrogen receptor 1 (GPER/GPR30) has also been identified (Filardo et al., 2000). The ERα and ERβ are encoded by the ESR1 and ESR2 genes respectively and are identified as ligand activated transcription factors. Upon activation, the receptors translocate to the nucleus where they can influence gene transcription through estrogen response elements (EREs) found on target genes. Nuclear receptors like the estrogen receptors have five main domains; N-terminal regulatory domain, which contains the activation function 1 (AF-1) site. The DNA binding domain (DBD) region is highly conserved and binds nuclear response elements like EREs. The hinge region has an important function in receptor dimerization, the ligand binding region contains another activation function site (AF-2), this is required for activation by the specific estrogen ligand. The ligand binding domain can also bind chaperone proteins like heat shock protein, which aid in translocating the receptor to the nucleus. The ERs have different selectively regarding the AF regions, ERα facilitates transcription activation via AF-1, whereas ERβ acts through AF-2. A comparison of AF-1 regions on both ERs have identified that the receptor can have opposing effects on the cell under the same conditions (Nilsson et al., 2001). The actions of EREs on gene expression can vary dependent on the co-regulator they associated with. Recruitment of short heterodimer partner (SHP) leads to a block on ER mediated transcription, however when the regulator p300 is associated with the transcription complex of ERα gene transcription occurs (Johansson et al., 1999, Hanstein et al., 1996). The impact of different co-regulators on estrogen signalling can vary between cell types. As stated previously this leads to the two estrogens having different effects on the same cell type (Nilsson et al., 2001). As well as being expressed in the cytoplasm of the cell, the ERs are also expressed as membrane bound receptors. Upon activation, they can influence the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), cAMP and ERK/MAPK signalling pathways (Filardo et al., 2000). The GPER has been identified as
playing a role in rapid and transcriptional actions in response to estrogen stimulation (Filardo and Thomas, 2005, Prossnitz et al., 2008). GPER is a membrane bound receptor that can activate adenylyl cyclase, trans-activate epidermal growth factor receptors (EGFRs), mobilise intracellular calcium (Ca^{2+}), activate MAPK and phosphoinositide 3-kinase (PI3K) signalling pathways (Putti et al., 2005, Luo et al., 2012). There is conflicting evidence to what effect GPER can have on cells. Previously it has been shown that GPER activation led to moderate the growth of endometrial and breast cancer (Filigheddu et al., 2011, Ariazi et al., 2010). GPERs have also been confirmed as mediators of anti-proliferative effects in vascular endothelial and smooth muscle cells (Holm et al., 2011, Haas et al., 2009, Murata et al., 2013). Paradoxically, GPER has also been implicated in activating genes that are associated with proliferation observed in breast and endometrial cancer (Pandey et al., 2009, Du et al., 2012). Several studies suggest that activation of the GPER by the specific agonist G-1 leads to cell cycle arrest and inhibition of proliferation in endothelial cells, prostate cancer and ovarian cancer cells (Holm et al., 2011, Chan et al., 2010, Wang et al., 2013). Broselid and colleagues discovered that the GPER receptor was found to be localised both intracellularly and in the plasma membrane (Broselid et al., 2014). When activated the GPER, independently of the Gi subunit, creates a plasma membrane complex including guanylate kinase and protein kinase A anchoring protein (AKAP) 5, blocking cAMP production (Broselid et al., 2014). SiRNA knock down of GPER led to increased cAMP production, interestingly removal of the type 1 PDZ motif at the receptor C terminus led to increased receptor endocytosis and disrupted the blockage of cAMP production (Broselid et al., 2014).

1.6.1.1. Estrogen signalling in cardiovascular system
In cancer, estrogen and its associated receptors are implicated as pathogenic, however in the cardiovascular system they are thought to mediate protective effects. The level of cardiovascular disease is higher in men than that of pre-menopausal women. This difference is reduced post-menopause due to the loss of the protective estrogen effect (Meyer et al., 2006). Ovariectomised mice underwent transaortic constriction, after which mice were dosed with either estrogen or vehicle. The estrogen dosed group demonstrated much lower levels of ventricular hypertrophy compared to vehicle (van Eickels et al., 2001). A further study demonstrated that both ovariectomised mice and mice with low expression ERα showed a reduction in hypertrophy when treated with estrogen. However, removal or reduction of the ERβ receptor led to no reduction in hypertrophy suggest ERβ mediates the beneficial effects of estrogen (Babiker et al., 2006). Estrogen administration prior to ischemic injury has been demonstrated to reduce infarct size in isolated rat hearts, this has also been demonstrated in both male and female rabbits (Lagranha et al., 2010, Sbarouni et al., 2003, Hale et al., 1996). Estrogen also improved the contractile function in rat hearts after ischemia and reperfusion by reducing p38/MAPK signalling (Wang et al.,
2006). The mechanism by which estrogen has its cardioprotective effects is thought to involve both ERα and ERβ receptors increasing expression of nitric oxide synthase (Nuedling et al., 1999). However, the GPER receptor also has been shown to have protective effects in ischemia and reperfusion via inhibition of mitochondrial permeability (Bopassa et al., 2010). The ERα, ERβ and GPER are all expressed in cardiac fibroblasts and cardiac myocytes (Deschamps and Murphy, 2009). Apolipoprotein E (ApoE) deficient mice dosed with estrogen have reduced the development of atherosclerosis lesion formation, decreased levels of low density lipoprotein (LDL) and increased levels of high density lipoprotein (HDL) (Bourassa et al., 1996, White, 2002). It is possible that the protective effects of estrogen are dependent on the presence of endothelial progenitor cells (EPCs), these cells are involved in maintaining cardiovascular homeostasis (Doyle et al., 2006). In coronary artery disease, therapy with an angiotensin-converting enzyme inhibitor, ramipril increased the functional activity of EPCs (Min et al., 2004). 17-β estradiol was found to increase EPC proliferation and adhesion through ER activation. The expression and pro-angiogenic properties of EPCs were also found to be higher in females compared to males (Fadini et al., 2008). Interestingly, the prostacyclin analogue treprostinil can increase the angiogenic properties and expression of EPCs in child PAH patients, this may aid endothelial repair and function (Smadja et al., 2011). However, women undergoing hormone replacement therapy (HRT) display the potential pathogenic effects of estrogen. This can include elevations in prothrombotic factors/fragments and reduced plasma fibrinolytic inhibitory activity contributing to a greater risk of venous thrombosis (Teede et al., 2000, Wu, 2005). Despite observing positive outcomes for estrogen based therapy in certain cardiovascular disorders, the risks associated with estrogen in HRT and estrogen linked cancers depict a complex profile of estrogen that must be accounted for when examining estrogen signalling.

1.6.3. Estrogen pathway and PAH

The predisposition for PAH in females has led to the investigation of estrogen as a possible causative or risk factor in the development of the disease. The complexity of estrogen signalling is again highlighted in the pulmonary vasculature as it was in the systemic vasculature. HRT has been implicated in the development and prevention of PAH (Morse et al., 1999, Beretta et al., 2006). The importance of aromatase expression in estrogen metabolism has been highlighted previously. Patients with PPHTN have a single nucleotide polymorphism (SNP) in the aromatase gene that leads to greater plasma levels of estrogen (Roberts et al., 2009). The activity of aromatase has not been examined in PAH, however expression of aromatase was found not to change between female control hPASMCs and female PAH patient hPASMCs (Mair et al., 2014). Interestingly, aromatase expression was elevated in both hypoxic mice and SU-HX rats, both also exhibited increased estrogen levels. In SU-HX rats, the increased estrogen levels were associated
with increases in RVH and pulmonary vascular remodelling (Mair et al., 2014). When ovariectomies are undertaken in female susceptible models of PH (SERT+ and dexfenfluramine (DFEN) dosed mice) there is a prevention of PH phenotype development (White et al., 2011a, Dempsie et al., 2013). Due to the increased incidence of females developing PAH, the implications of the female specific PH models SERT+ and DFEN responding negatively to estrogen may have clinical significance. Estrogen also upregulated known mediators of PAH pathogenesis; tryptophan hydroxylase 1 (TPH1), SERT and 5-HT<sub>1B</sub> in hPASMCs (White et al., 2011a). This led to the investigation of directly blocking estrogen synthesis by inhibiting the activity of aromatase, this was achieved by anastrazole therapy. Anastrazole was found to reverse established PH phenotype in hypoxic mice and SU-HX rats, however this positive effect was only observed in females (Mair et al., 2014).

The conflicting role of estrogens in PAH development and pathogenesis was expressed in specific experimental models of PH, females repeatedly demonstrate a less severe PH phenotype than males. Rabinovitch and colleagues discovered that hypoxic male rats have greater pulmonary vascular remodelling and RVH compared to hypoxic female rats (Rabinovitch et al., 1981). Mice have also been shown to demonstrate similar characteristics (Stupfel et al., 1984). Female rats that had underwent an ovariectomy developed a more severe hypoxic induced PH phenotype (Resta et al., 2001). Treatment with 17-β estradiol reduced the PH phenotype in ovariectomised rats, demonstrating the protective effects of estrogen (Resta et al., 2001). Similar effects were observed in rats with MCT induced PH; an ovariectomy increased the severity of MCT induced PH in rats and estrogen improved haemodynamics in an established MCT phenotype (Ahn et al., 2003, Umar et al., 2011). In male rats, estrogen therapy was found to decrease RVSP, RVH and pulmonary arterial remodelling suggesting estrogen positive benefits also extends to males (Lahm et al., 2012). Estrogen also promotes both prostacyclin and nitric oxide mediated endothelium dependent vasodilation in pulmonary circulation (Sherman et al., 2002, Gonzales et al., 2001, Lahm et al., 2008). In PAEC, 17-β estradiol increased expression of endothelial nitric oxide synthase (eNOS) activity and mRNA expression (MacRitchie et al., 1997). Additionally, 17-β estradiol also blocks hypoxia induced endothelin-1 gene expression (Earley and Resta, 2002).

Female PAH patients were found to have increased expression of ESR1/ERα compared to non-PAH control patients (Rajkumar et al., 2010). Hypoxic female mice were also found to have increased expression of ERα compared to normoxic females, however males did not express any difference between groups (Mair et al., 2014). Dysfunctional BMPR-II signalling is an important factor in the development and pathogenesis of PAH. Estrogen has been implicated in reducing BMPR-II gene expression via the interaction of ERα with a estrogen receptor binding site present in the BMPR-II promoter (Austin et al., 2012).
Interestingly, the same study found that canonical BMPR-II signalling was increased with estrogen stimulation despite downregulation of the receptor (Austin et al., 2012). The negative implications of ERα in PAH were enhanced by the demonstration that inhibition of the receptor can prevent the development of a hypoxic induced PH phenotype in female mice (Mair et al., 2014). The ERβ was found to be decreased within pulmonary arteries of hypoxic female mice compared to normoxic controls, also loss of ERβ function was found to increase the development of RV hypertrophy in mice (Mair et al., 2014, Morani et al., 2006). Similar reductions in ERβ were found within the right ventricle and lung of MCT rat models of PH (Matori et al., 2012). Combining the published data presents a complicated picture for estrogen in the development of PAH. The ERα appears to be responsible for the negative effects of estrogen stimulation whereas the ERβ receptor mediates some beneficial or protective effects of estrogen. The distribution of the receptors will influence the effects of estrogen.

It would be simplistic to focus solely on estrogen effects in PAH and not consider the actions of its many metabolites. As discussed previously, the types of metabolites produced relies heavily on the action and expression of CYP enzymes. White and colleagues investigated the CYP1B1 enzyme and identified its elevated expression in hPASMCs from IPAH patients and in the pulmonary arteries of SERT+ mice, hypoxic mice and SU-HX rats (White et al., 2011b, White et al., 2012). Inhibition of CYP1B1 reduced and reversed PH indices in hypoxic and SU-HX PH models respectively (White et al., 2012). In addition, CYP1B1 knock out mice exposed to hypoxia expressed a reduced PH phenotype compared to wildtype controls (White et al., 2012). CYP1B1 is an important enzyme in metabolism of 17-β estradiol to 2-OHE2. MCT rats dosed with 2-OHE2 demonstrated decreases in RVSP and RVH (Tofovic et al., 2005). 2-OHE2 has been shown to demonstrate cell cycle arrest and reduce tumour growth and induce apoptosis in solid tumours (Schumacher and Neuhaus, 2001). CYP1B1 can also mediate the breakdown of 17-β estradiol to 16-alpha hydroxyestradiol (16αOHE1). 16αOHE1 is a known pro-proliferative mitogen that can affect the growth of hPASMCs, also in-vivo dosing with 16αOHE1 increased both RVSP, RVH and pulmonary artery remodelling (White et al., 2012). Recently 16αOHE1 has been implicated in increasing dinucleotide phosphate oxidase (NOX1) mediated reactive oxygen species (ROS) generation (Hood et al., 2016). Hood and colleagues found that 16αOHE1 increased ROS via NOX1 in both control and PAH patient hPASMCs (Hood et al., 2016). A NOX1 knock-out mouse model was also found to be protected from chronic hypoxic induced PH (Hood et al., 2016). 16αOHE1 was also found to promote the development of HPAH through upregulation of miR-29 (Chen et al., 2016). The treatment of BMPR-II female and male mutant mice with an antagonim (anti-miR-29) led to improved haemodynamics. Chen and colleagues believed that the increase in miR-29 can impair energy metabolism. This was demonstrated by an increase in
ceramide, which they believe suggested a dysfunctional fatty acid oxidation pathway (Chen et al., 2016). Interestingly, antagomir treatment also increased the size of PASMC mitochondria (Chen et al., 2016). The variety and diversity of estrogen metabolites produced will influence and modulate the severity and manifestation of PAH.
1.7. Aims
The incidence of PAH among women is much higher than found in males but males suffer worse clinical outcomes. For this study, the general hypothesis was that this disparity in clinical outcomes can be explained by a differential response to PAH treatment. The overall aim of this research was to investigate the influence of sex on the anti-proliferative and to ascertain how the therapeutic actions of treprostinil are mediated. This was investigated through the following experimental study hypotheses and aims for each chapter:

Chapter 3
Slow-release subcutaneously implanted pellets have been used previously to dose animals in the treatment of pulmonary hypertension (Wright et al., 2015). We hypothesise that the PGI$_2$ analogue treprostinil can be successfully delivered to chronic hypoxic rats using slow-release subcutaneously implanted pellets as an alternative to repetitive dosing. To investigate this hypothesis, the following aim was carried out.

1. To examine the haemodynamic effects of treprostinil, administered via a slow release subcutaneously implanted pellet in the chronic hypoxic model of pulmonary hypertension.

Females and males are known to differ in response to drug therapy for variety of reasons (Soldin and Mattison, 2009). To investigate this, our hypothesis is that sex can play a role in altering the effectiveness of treprostinil in an animal model of PH. The following aim was carried out:

2. Study to characterise the influence of sex on the actions of treprostinil in a chronic hypoxic rat model of PH and how these effects are mediated

Chapter 4
Sex has been shown to influence the clinical outcomes of patients receiving ERAs and PDE-5 inhibitors (Gabler et al., 2012, Mathai et al., 2015). We hypothesise that the PGI$_2$ analogue treprostinil can have sex specific effects in isolated hPASMCs. To investigate this hypothesis, the following aim was carried out.

1. Characterisation of the influence of sex on treprostinil in human pulmonary artery smooth muscle cells

BMPR-II signalling has been previously demonstrated to mediate the effect of prostacyclin analogues and other PAH therapies (Yang et al., 2010, Yang et al., 2013a). Also, sex differences have been identified in BMPR-II signalling in hPASMCs (Mair et al., 2015). The hypothesis is that increased BMPR-II signalling mediated by treprostinil accounted for the
differences in anti-proliferative properties of treprostinil. To investigate this hypothesis, the following aim was carried out.

2. Investigation into the role of canonical BMPR-II signalling in the effects of treprostinil in human pulmonary artery smooth muscle cells
Chapter Two

2 Materials and Methods
2.1 Chemicals and reagents
All regents and chemicals were supplied by Fisher Scientific (Loughborough, United Kingdom) and Sigma Aldrich (Dorset, United Kingdom) unless stated otherwise. Constituents of protein and RNA analysis were obtained from Life Technologies (Paisley, United Kingdom) and Qiagen (Manchester, United Kingdom). Experiments involving RNA were conducted using nuclease-free plastics and reagents from Ambion/Life Technologies (Paisley, United Kingdom). Plate plastics for cell culture experiments were supplied by Corning via Fisher Scientific (Loughborough, United Kingdom). Treprostinil (Tyvaso®) was supplied by United Therapeutics (Silver Springs, United States).

2.2 Ethical approval
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). All in vivo procedures were performed under project licence 60/4404 held by Professor Margaret R. Maclean, University of Glasgow and personal licence 1629F9947 held by Gerard Murphy, University of Glasgow. Ethical approval was granted by the University of Glasgow Ethics Committee. Experimental procedures using human lung issue and 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).

2.3 Animal study design
When appropriate, animals were randomised into respective groups and haemodynamic data from these groups were obtained blinded. The identities of the groups were disclosed after haemodynamic analysis (Figure 2-1).

2.3.1 Animal welfare conditions
All animals were maintained in a 12-hour light/dark cycle under the same environmental conditions with access to food and water ad libitum. Animals were housed at the licenced facility in the West Medical Building, University of Glasgow or at the Central Research Facility (CRF), University of Glasgow.
2.3.2 Sprague-Dawley rats

Male and female Sprague-Dawley rats aged 5-7 weeks were obtained from Harlan Laboratories/Envigo (Blackthorn (Oxfo), United Kingdom). Rats were housed at the CRF for a 1-week acclimatisation period, prior to experimental start.

2.3.2.1 Chronic hypobaric hypoxia

The chronic hypoxia model of pulmonary hypertension (PH) was studied. Rats were placed in a hypobaric hypoxic chamber for 28 days. On day 0, rats were placed into the chamber and the pressure was slowly reduced from an atmospheric (normoxic) 1013mbar to 750mbar, and left overnight for acclimatisation. On day 1, the pressure was further slowly reduced to 550mbar, at this pressure the equivalent level of oxygen has been reduced to 10%. This low level of oxygen stimulates vasoconstriction of the pulmonary arteries of the rat lungs which develops into a pulmonary hypertensive phenotype. Every 5 days, the chambers were slowly restored to atmospheric pressure to restock food and water and to change cage bedding and environment. The temperature and relative humidity were monitored and maintained at optimal levels.

2.3.2.2 Sub-cutaneous implanted pellet

Rats were anaesthetised in an anaesthetic chamber containing 3-3.5% (v/v) isoflurane (Abbot Laboratories, Berkshire, United Kingdom) supplemented with oxygen. After anaesthetic induction rats were weighed and transferred to a suitable facemask constantly administering 2.5-3% (v/v) isoflurane supplemented with oxygen flow (1L/min). In order to maintain a steady body temperature, rats were placed on a heat mat for the procedure and their temperature was monitored via a temperature probe. Pellets containing either treprostinil, (releasing 100ng/kg/min and 400ng/kg/min) or a vehicle (Innovative Research of America, Florida, United States) were implanted. These were implanted subcutaneously into the dorsal neck using a sterile 10-gauge trochar. These pellets were implanted in rats after 14-days in hypoxia or in normoxic conditions. The Matrix Driven Delivery (MDD) pellets continuously release treprostinil over a 14-day timespan. Vehicle pellets are designed to act in the same way as drug containing pellets but will not release any active product. The rats were allowed to recover in clean bedding cages before being transferred back to their original cage. This was the first study to examine the effects of treprostinil released in pellet form, however previous studies have demonstrated the effectiveness of this dosing method.
Figure 2-1 In-vivo experimental timeline

(A) At day 0 male and female sprague-dawley rats were subjected to 14-day hypoxic or normoxic period. At day 14, rats were dosed with either 100ng/kg/min treprostinil or vehicle (containing no treprostinil). After dosing rats were returned to hypoxic conditions until day 28 at which time haemodynamics were assessed and blood was taken via the carotid artery and centrifuged at 8000xg to isolate plasma. (B) At day 0 male and female sprague-dawley rats were subjected to 14-day normoxic period after dosing with 100ng/kg/min treprostinil. Blood plasma was taken via the carotid artery and rats sacrificed at day 4, 7, 10 and 14. (C) At day 0, male and female sprague-dawley rats were subjected to 14-day hypoxic or normoxic period. At day 14, rats were dosed with either 400ng/kg/min treprostinil or vehicle (containing no treprostinil). Rats were returned to hypoxic conditions until day 28 at which time haemodynamics were assessed and blood plasma taken via the carotid artery.
2.4 Haemodynamic assessment of pulmonary hypertension

2.4.1. Anaesthesia
Rats were anaesthetised in an anaesthetic chamber containing 3-4% (v/v) isoflurane supplemented with oxygen flow (1L/min). Confirmation of acceptable anaesthesia was achieved by the lack of hind-limb or tail reflex. Over the course of the procedure the level of anaesthesia was constantly measured via testing of the previously described reflexes, heart rate and breathing rate. After anaesthetic induction, rats were weighed and transferred to a suitable facemask constantly administering 2.5-3% (v/v) isoflurane supplemented with oxygen flow (1L/min). To maintain a steady body temperature, rats were placed on a heat mat for the entirety of the procedure and their temperature was monitored via temperature probe.

2.4.2. Measurement of right ventricular systolic pressure
To achieve an indirect measurement of pulmonary arterial pressure (PAP), right ventricular pressure (RVP) was measured by the cannulation of the right external jugular vein by the Mikro-Tip® Pressure Volume System Ultra Foundation System (Millar, Texas, United States). This was coupled to a PowerLab 16/36 data acquisition system (AD Instruments, Oxfordshire, United Kingdom) (Lawrie et al., 2011). After successful induction of anaesthetic, an incision was made into the ventral neck area of the rat to reveal the layer of muscle underneath. This layer was blunt dissected through until exposition of the right external jugular vein. The vein was isolated from the surrounding tissue, surgical non-sterile nylon black monofilament suture size 3-0 (Harvard Apparatus, Massachusetts, USA) was placed underneath the vein and knotted at the proximal end (towards head) of the vein. A second suture was place at the distal end (towards heart) of the vein and taped down to expose a greater area of the jugular vein. Using a 25mm gauge needle a small incision was made into the jugular vein. A Mikro-tip catheter SPR-869 (Millar, Texas, United States) was inserted into the vein and advanced slowly until the catheter reached the right ventricle of the heart. At this point a typical pressure trace of RVP was obtained (Figure 2-2). Measurements of right ventricular systolic pressure (RVSP) and heart rate (HR) were taken at this point and analysed using Labchart 7 (AD Instruments, Oxfordshire, United Kingdom). After pressure measurement had been taken, the catheter was withdrawn from the jugular vein and the vessel was tied off using surgical suture.
2.4.3. Measurement of left ventricular systolic pressure

Post measurement of RVP, the right internal carotid artery was cannulated in-order to measure left ventricular pressure (LVP). In a similar manner to the isolation of the jugular vein, the carotid artery was exposed by blunt dissection of the overlapping muscle tissue. Once identified the artery was cautiously separated from the vagus nerve and any other surrounding tissue. As with the jugular vein, surgical suture was used to tie off the proximal end of the exposed artery while the distal end was exposed further. To prevent the flow of blood a microsurgical clip (Fine Science Tools, Heidelberg, Germany, FST#18055-04), was clamped at the distal end of the artery. A small incision was made in the vessel just below where the proximal suture was tied. This allowed the insertion of a Mikro-tip catheter SPR-838 (Millar, Texas, United States) into the artery, this was then advanced as the microsurgical clip was released, at this point the distal suture was tied around the catheter and the incision point on the artery to prevent an outflow of blood. The catheter was slowly advanced until it entered the left ventricle and the typical pressure trace was achieved (Figure 2-3). After receiving a suitable measurement, the catheter was withdrawn from the carotid artery, at this point blood was collected from the artery to calculate cardiac output.
and to analyse for treprostinil levels in the plasma. The rats were then killed by an anaesthetic overdose.

Figure 2-3 Left ventricular pressure measurement
(A) Diagram to show of placement of catheter to attain LVP measurement. (B) A five second representative trace of LVP from a normoxic rat. Y axis in mmHg, LVP=left ventricular pressure.

2.4.4. Cardiac output measurement
To measure cardiac output from the left ventricle a cuvette calibration was used. Blood collected from carotid artery was supplemented with heparin sodium to prevent clotting and kept at 37°C. Heparinised blood was then added to a volume calibration cuvette (AD Instruments, Oxfordshire, United Kingdom), each well in the cuvette corresponds to a known volume. The Mikro-tip catheter SPR-869/838 was then inserted into each well of the cuvette to obtain volume measurements that resemble a standard curve (Figure 2-4). Labchart 7 software extrapolated the cardiac volume in the left ventricle from this standard curve when establishing the LVP measurements, thus calculating the cardiac output.
2.4.5. Right ventricular hypertrophy

Following the sacrifice of the rat, the heart and lungs were flushed with ice cold Dulbecco’s phosphate buffered saline (DPBS) solution (Life Technologies Paisley, United Kingdom). The heart was then detached from the lungs and cleared of any excess connective tissue, vessels and the atria were removed. Right ventricular hypertrophy is defined by the use of Fulton’s index, which is the ratio of the free right ventricle (RV) wall weight to the left ventricle (LV) wall plus septum (S) weight (RV/LV+S) (Fulton et al., 1952). The right ventricle was dissected free of the heart, blotted dry and weighed. This was then carried out for the left ventricle and septum and the Fulton’s index was calculated.

2.4.6. Tissue harvest

To analyse underlying molecular mechanisms, the lobes of the right lung were flash frozen in liquid nitrogen and stored at -80°C. For immunohistochemistry analysis, the left lung was slowly infused with 5-10ml 10% (v/v) neutral buffered formalin (NBF) (90% (v/v) distilled water, 10% (v/v) formalin, 33mmol/L monosodium phosphate (NaH2PO4), 45mmol/L disodium phosphate (Na2HPO4)) and dissected free of the cadaver. The inflated left lung was then submerged in 10% NBF on a gentle shaker for minimum of 24 hours prior to embedding.

2.4.7. Blood plasma isolation

Blood not required for cardiac output measurement was collected in tri-potassium ethylenediaminetetraacetic acid (K3-EDTA) tubes (Teklab, Durham, United Kingdom) and mixed gently. The blood was stored on ice for a maximum of 1 hour, then the tubes were centrifuged at 8000xg at 4°C for 15 minutes. The plasma supernatant was then pipetted into chilled eppendorf tubes, snap frozen in liquid nitrogen then stored at -20°C. Treprostinil plasma analysis was carried out by Liquid Chromotography/Mass Spectrometry (LC/MS) by Tandem Labs/Covance (Durham, North Carolina, United States). Briefly, treprostinil
levels in plasma samples were assessed by extrapolation to a standard curve of known
treprostinil concentrations for all in-vivo studies.

2.5 Histology

2.5.1. Lung preparation, paraffin embedding
After a minimum of 24 hours on a gentle shaker the fixed left lung lobe underwent paraffin
embedding. Using a Citadel 1000 tissue processor (Thermo Fisher Scientific, United
Kingdom) the lung went through water-alcohol-histoclear dehydration steps before
embedding in paraffin wax (Table 2-1).

2.5.1.1. Sectioning
The embedded lung blocks were kept at room temperature for long term storage. Prior to
sectioning they were placed at -20°C to harden the wax which assisted with the cutting
process. Frontal plane lung sections were cut at 5µm thickness using a microtome (Leica
RM2125, Lecia Microsystems, Milton Keynes, United Kingdom). Cut sections were placed
in water at 37°C and then mounted onto polylysine coated slides, these were stored at
room temperature.
Table 2-1 Paraffin embedding process for rat lung samples

<table>
<thead>
<tr>
<th>Step condition</th>
<th>Time for step condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>Instant</td>
</tr>
<tr>
<td>70% Ethanol in water</td>
<td>2.5 hours</td>
</tr>
<tr>
<td>80% Ethanol in water</td>
<td>2.5 hours</td>
</tr>
<tr>
<td>95% Ethanol in water</td>
<td>2.5 hours</td>
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<tr>
<td>95% Ethanol in water</td>
<td>2.5 hours</td>
</tr>
<tr>
<td>100% Ethanol in water</td>
<td>2 hours</td>
</tr>
<tr>
<td>100% Ethanol in water</td>
<td>2 hours</td>
</tr>
<tr>
<td>100% Ethanol in water</td>
<td>2 hours</td>
</tr>
<tr>
<td>Histoclear</td>
<td>1.5 hours</td>
</tr>
<tr>
<td>Histoclear</td>
<td>1.5 hours</td>
</tr>
<tr>
<td>Paraffin wax</td>
<td>1 hour</td>
</tr>
<tr>
<td>Paraffin wax</td>
<td>1 hour</td>
</tr>
</tbody>
</table>

Lung tissue was washed in running water to remove excess NBF before placement in processor.

2.5.2. Immunohistochemistry

Paraffin wax was removed from lung sections by incubation in xylene for 30 minutes, the sections were rehydrated through an alcohol-water gradient that consisted of; 100% ethanol for 10 minutes, 100% ethanol for a further 5 minutes, 90% ethanol for 2 minutes and 70% ethanol for 2 minutes. The sections were washed in running water for 10 minutes. Fixing of tissues in formalin can lead to the masking of antigen sites by protein cross-linking, this was overcome by incubating the sections in 10mM citric acid buffer (pH 6.0 2.1g/L) (Sigma Aldrich, Dorset, United Kingdom). Antigen retrieval was achieved by boiling the buffer containing the sections in a microwave for 4x 5 minutes, the sections were then left to cool in the buffer at room temperature for 20 minutes. Sections were washed in running water for 10 minutes before being immersed for 30 minutes in 3% hydrogen peroxide (v/v) in methanol solution. This solution blocks the action of endogenous peroxidases present in the tissue to prevent excess non-specific staining. The sections were washed in running water for 10 minutes before a further 10-minute wash in tris-buffered saline (TBS) pH 8.4 (50mmol/L Tris, 150mmol/L NaCl). The sections were blocked for 1 hour with 2.5% (v/v) horse serum (ImmPRESS kit, Vector Labs, Peterborough, United Kingdom) in a humidified chamber. The primary antibody (Table 2-3) was diluted in 1% (w/v) bovine serum albumin (BSA) in 10mM phosphate buffered saline (PBS) (Sigma Aldrich, Dorset, United Kingdom). The sections were incubated with the
primary antibody overnight at 4°C in a humidified chamber. The primary antibody was removed by 3x 10 minute washes in TBS and sections were incubated with anti-rabbit IgG peroxidase polymer secondary antibody (ImmPRESS kit, Vector Labs, Peterborough, United Kingdom) for 1 hour at room temperature in a humidified chamber. The sections were washed twice for 10 minutes in TBS before visualisation of the bound primary antibody. This was achieved by a 3-5 minute incubation of the sections with 3,3'-diaminobenzidine (DAB) substrate kit (Vector Labs, Peterborough, United Kingdom). A positive visualisation was indicated by dark brown staining. To prevent excessive staining, sections were placed in running water for 10 minutes to terminate the reaction. To stain nuclei and cytoplasm the sections was counterstained with haematoxylin and eosin for 5 minutes before a final wash in running water for 5 minutes to remove excess stain. The lung sections were rapidly dehydrated by an alcohol-water gradient that consisted of 70% ethanol for 2 minutes, 90% ethanol of 2 minutes, 100% ethanol for 5 minutes and 100% ethanol for 10 minutes. Sections were immersed twice in histoclear for 10 minutes each. Tissue-tek (Sakura Finetek, Netherlands) was used to mount cover slides onto lung sections and the antibody staining was identified and photographed on a Zeiss Image M.1 AX10 and axiovision Rel 4.8 (Carl Zeiss Microscopy Ltd, Cambridge, United Kingdom).

2.5.2.1. Elastin pico-sirius red staining

To measure pulmonary vascular remodelling an adapted Millar’s Elastin protocol was used (Miller, 1971). Wax was removed from lung sections by immersion in histoclear for 20 minutes, the sections were rehydrated through an alcohol-water gradient. This consisted of 100% ethanol for 5 minutes, 90% ethanol for 5 minutes and 70% ethanol for 5 minutes, sections were place in running water for 5 minutes. To enhance staining sections were placed in potassium permanganate (KMNO₄) for 5 minutes, followed by rinsing in running water. To remove excess KMNO₄ staining sections were immersed in 1% oxalic acid followed by a further rinse in running water. Lung sections were submerged in Millers Elastin stain (Thermo Fisher Scientific, United Kingdom) for 2 hours. Excess elastin stain was removed firstly by a short rinse in 95% ethanol followed by a rinse in running water. The sections were counter-staining with picro-sirius red for 2-3 minutes and rinsed in running water. Sections were rapidly dehydrated through an alcohol-water gradient consisting of 70% ethanol for 1 minutes, 90% ethanol for 1 minute, 2 100% ethanol incubations for 5 minutes each and 2 final immersions in histoclear for 5 minutes. As with immunohistochemistry, the sections were mounted by cover slips and staining was identified and analysed on a light microscope. The resultant staining visualised elastic fibres having a dark/black appearance and collagen as red.
2.5.2.2. Pulmonary vascular remodelling

Pulmonary vascular remodelling was assessed in 5µm lung sections stained with Miller’s elastin and picro-sirius red as per section 2.5.2.1. Pulmonary arteries <80µm external diameter were microscopically assessed for a change in the elastic lamina. Pulmonary arteries were classified as non-remodelled vessels due to the presence of a single elastic lamina. Remodelled pulmonary arteries were identified by the presence of a double elastic laminae. The level of remodelling was expressed as a percentage of remodelled and non-remodelled pulmonary arteries to the total number of pulmonary arteries. This assessment was carried out in a blinded fashion.

2.6 Western Blotting

2.6.1. Protein extraction

Protein was isolated from human pulmonary artery smooth muscle cells (hPASMCs) grown in 6-well cell culture plates. Briefly, upon experimental conclusion the plates were placed on ice, the media was immediately removed and the cells were wash twice with 2ml of ice-cold PBS. Following the removal of PBS, 120µl of ice-cold radio immuno precipitation assay (RIPA, Thermo Fisher Scientific, United Kingdom) buffer was added to each well. The buffer was supplemented with protease inhibitors; 0.1mmol/l PMSF (phenylmethylsulfonyl fluoride), 1µg/ml soybean trypsin inhibitor and 1µg/ml benzamidine. RIPA buffer with protease inhibitors lyse whole cells allowing solubilisation of protein while preventing denaturing. While on ice the cells were scraped using a sterile plastic scraper (#3010, Corning, United Kingdom) and collected in a 1.5ml eppendorf also on ice. The samples were left on ice for 30 minutes to allow for the dissociation of protein complexes. To isolate proteins from whole lung tissue, a portion of the frozen right lung lobe was placed in a 2ml eppendorf tube supplemented with 600µl of RIPA buffer with added protease inhibitors at 4°C. To homogenise the lung, a 5mm stainless steel ball was added to each eppendorf and they were placed in a Tissue Lyser II (Qiagen, United Kingdom). At a frequency of 25Hz the samples were shaken at high speed for 4x 30 second intervals with 30 seconds pauses in between, to prevent excess heat build-up. The samples were left for 30 minutes on ice after-which they were sonicated on ice for 2 minutes to aid dissociation of protein complexes. Samples were then centrifuged at 8,500xg for 10 minutes at 4°C. The supernatant from the sample was transferred into a fresh ice-cold eppendorf and stored at -80°C, the remaining sample pellet was discarded.

2.6.2. Bicinchoninic acid assay

Protein concentrations were determined using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, United Kingdom). The reaction involves the reduction of Cu$^{2+}$ to Cu$^{+}$ by protein, the bicinchoninic acid binds to the reduced Cu$^{+}$ to form a complex that is purple in colour and absorbs light at a 562nm wavelength (Smith et al., 1985). With hPASMCs and lung, protein concentration was evaluated against a standard curve of BSA (0.2mg/ml
diluted in RIPA buffer). Briefly, concentrations were determined by an average of experimental samples and BSA standards pipetted in duplicate into a 96-well plate. BCA assay solution was formulated by supplementing 9.8ml of reagent A with 200µl of reagent B and thoroughly mixed. 200ul of the BCA solution was added to each well and the plate was placed on a micro-plate shaker for 15-20 minutes until a colour change was evident. A POLARstar OPTIMA microplate reader (BMG Labtech, Germany) read the plate at a 562nm wavelength. A standard curve of BSA standards was obtained and the experimental protein sample concentrations were determined.

2.6.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Protein from hPASMCs and lung tissue was separated according to their molecular weight using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). NuPAGE reducing agent and sample buffer (Life Technologies, United Kingdom) was added to protein samples for loading visualisation. Prior to loading, protein samples were heated for 10 minutes at 70°C to ensure denaturing and protein reduction, this enabled greater antibody recognition. Protein samples were loaded into Noves NuPAGE 4-12% Bis-Tris mini gels, the concentration of protein examined was 10µg for hPASMCs and 25µg for lung tissue. SeeBlue Plus2 (Life Technologies, United Kingdom) was used as pre-stained molecular weight marker, this allowed the electrophoresis progression to be examined and to check the molecular weight of proteins. The loaded gel was immersed in 5% NuPAGE MOPS running buffer in distilled water (dH_{2}O) and a constant current of 150 volts was applied thereby the proteins were slowly separated due to molecular weight.

2.6.4. Protein transfer
Once electrophoresis was completed the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Massachusetts, United States). The PVDF membrane was pre-soaked in 100% methanol to activate it. The protein transfer solution contained 5% NuPAGE transfer buffer, 20% (v/v) methanol in dH_{2}O. The transfer was run at a constant 30 volts for 2-3 hours, this allow for the proteins to migrate from gel to PVDF membrane. To ensure successful transfer, Ponceau S in a 5% (v/v) acetic acid solution was added to visualise protein bands on the PVDF membrane. Ponceau was washed from the blots with TBS-tween (TBS-T) (0.1% (v/v) Tween 20 (Fisher Scientific, United Kingdom)). PVDF membranes were then placed in 5% (w/v) non-fat milk (Marvel) solution in TBS-T for 1 hour to block non-specific background binding of antibodies. Before incubation with primary antibody the membrane was washed in TBS-T for 3x 10 minutes.

2.6.5. Immunoblotting
Specific protein analysis was carried out by incubating the membrane with a primary antibody (Table 2-3) diluted in 5% (w/v) marvel or 5% (w/v) BSA in TBS-T. Membranes were stored overnight on a shaker at 4°C. After incubation, the membranes were washed
for 3x 10 minutes in TBS-T, this removed any unbound excess primary antibody. Suitable secondary antibody, conjugated with HRP was added to membranes for 1 hour at room temperature, the membranes were placed on a shaker for the duration of incubation. The HRP allows detection by an enhanced chemiluminescence (ECL) solution, due to the presence of luminol. Luminol when oxidised by HRP which emits light which can be visualised by exposure to x-ray film. The two ECL solutions used were Immobilon western chemiluminescence HRP substrate (Merck Millipore, Massachusetts, United States) and Pierce ECL (Thermo Scientific, United Kingdom). The human IP receptor has been previously identified as having two sites for N-linked glycosylation (Zhang et al., 2001b). The choice of primary antibody for the IP receptor highlighted that due to receptor glycosylation visualisation of the receptor may occur at 67kDa or 40kDa. However, no band at 67kDa was identified therefore the protein band at 40kDa was used for analysis. After protein visualisation, membranes were washed in TBS-T for 10-15 minutes before being immersed in Restore western blot stripping buffer (Thermo Scientific, United Kingdom) for 15 minutes on a shaker. Consistent protein loading across the membrane was ensured by membranes being re-probed with a loading control antibody as above. For hPASMCs α-tubulin was used, whereas β-actin was used for lung tissue. Protein expression was quantified by densitometry analysis using TotalLab 1D gel analysis (TotalLab, United Kingdom). Expression was defined as protein: loading control ratio.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host animal</th>
<th>Source</th>
<th>Primary Dilution</th>
<th>Secondary Dilution</th>
<th>Molecular Weight</th>
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</thead>
<tbody>
<tr>
<td>BMPR-II</td>
<td>Mouse</td>
<td>BD Biosystems (612292)</td>
<td>1:500</td>
<td>1:10000</td>
<td>130/115kDa</td>
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<tr>
<td>pSmad 1/5/9</td>
<td>Rabbit</td>
<td>Cell Signalling (9511)</td>
<td>1:1000</td>
<td>1:10000</td>
<td>55-60kDa</td>
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<td>Cal Bioreagents (M085)</td>
<td>1:1000</td>
<td>1:10000</td>
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</tr>
<tr>
<td>Id3</td>
<td>Rabbit</td>
<td>Cal Bioreagents (M100)</td>
<td>1:1000</td>
<td>1:10000</td>
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<tr>
<td>IP Receptor</td>
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<td>1:5000</td>
<td>40kDa (can be glycosylated at 67kDa)</td>
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<tr>
<td>EP₂ Receptor</td>
<td>Rabbit</td>
<td>Abcam (ab167171)</td>
<td>1:1000</td>
<td>1:5000</td>
<td>50kDa</td>
</tr>
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<td>DP₁ receptor</td>
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<td>Abcam (ab99446)</td>
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<td>1:5000</td>
<td>40kDa</td>
</tr>
<tr>
<td>ETₐ Receptor</td>
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<td>Abcam (ab117521)</td>
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<td>1:5000</td>
<td>49kDa</td>
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<td>ETₑ Receptor</td>
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<td>Santa Cruz (sc-33537)</td>
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<td>α-tubulin</td>
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<td>Abcam (ab80779)</td>
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<td>50kDa</td>
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<tr>
<td>β-Actin</td>
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<td>Sigma Aldrich (A5441)</td>
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<td>α-smooth muscle actin</td>
<td>Rabbit</td>
<td>Abcam (ab5694)</td>
<td>1:200</td>
<td></td>
<td>Immuno-histochemistry stain</td>
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</table>

BMPR-II = bone morphogenetic protein receptor-II; pSmad = phosphorylated smad; Id = inhibitor of DNA binding; IP = prostaglandin I2 receptor (prostacyclin); EP₂ = prostaglandin E2 receptor 2; DP₁ = prostaglandin D2 receptor 1; ET = Endothelin.
2.7 RNA analysis

Ribonucleic acid (RNA) was isolated from human pulmonary artery smooth muscle cells (hPASMCs) grown in 6-well cell culture plates. Briefly, upon experimental conclusion the plates were placed on ice, the media was immediately removed and the cells were washed twice with 2ml of ice-cold PBS. Following the removal of PBS, 700µl of Qiazol (Qiagen, United Kingdom) was added to each well. Qiazol is a lysis reagent that facilitates the lysis of cells and/or tissue, while inhibiting RNases. While on ice the cells were scraped using a sterile plastic scraper (#3010, Corning, United Kingdom) and collected in a 1.5ml eppendorf, which was also on ice. To isolate RNA from whole lung tissue, a portion of the frozen right lung lobe was placed in a 2ml eppendorf tube, at 4°C, supplemented with 700µl of Qiazol. To homogenise the lung, a 5mm stainless steel ball was added to each eppendorf and they were placed in a Tissue Lyser II (Qiagen, United Kingdom). At a frequency of 25Hz the samples were shaken at high speed for 4x 30 second intervals with 30 seconds pauses in between to prevent excess heat build-up. Purification and extraction of RNA was carried out using the miRNEASY extraction kit (Qiagen, United Kingdom). Briefly, after homogenisation samples were left at room temperature for 5 minutes, this helps promote dissociation of nucleoprotein complexes. 140µl of chloroform was added to the samples and shaken vigorously for 15 seconds. The samples were left for 3 minutes at room temperature before being centrifuged at 4°C at 12,000xg for 15 minutes. The samples separate into 3 distinct layers; an upper aqueous layer, a white interphase and a low red organic phase. The aqueous phase was carefully transferred to a new RNAse free 1.5ml eppendorf. To precipitate the RNA, 525µl of ethanol was added to the separated aqueous phase and mixed thoroughly. 700µl of the mixed sample was transferred to a RNeasy mini column in a 2ml collection tube and centrifuged at 8,000xg for 15 seconds at room temperature. The flow through in the collection tube was discarded before the remaining mixed sample was added to the mini column and centrifuged as before. To prevent deoxyribonucleic acid (DNA) contamination of the RNA, a DNase digest was carried out. Firstly, the mini column was washed with 350µl of RWT buffer and centrifuged at 8,000xg at room temperature for 15 seconds, then the flow through was discarded. 80µl of DNase digestion solution (1:7 ratio DNAse1/RDD buffer) was added to each mini column to ensure complete digestion of DNA. After a room temperature incubation of 15 minutes the columns were washed with 350µl of RWT buffer and centrifuged at 8,000xg for 15 seconds. Subsequently the flow through was discarded and 500µl of RPE buffer was added to the column. The column was centrifuged at 8,000xg for 15 seconds with the flow through discarded, the previous step was repeated with RPE buffer and the column centrifuged for 2 minutes. The mini columns were placed in a new 2ml collection tube and were centrifuged at 12,000xg for one minute to remove any excess RPE buffer. The mini columns were placed in 1.5ml eppendorf tubes and 33ul of RNase free water was added directly to the column membranes. The columns were centrifuged for 1 minute at 8,000xg,
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this isolated the RNA from the column membrane. The collected RNA was immediately placed on ice or transferred to -80°C freezer.

2.7.1. RNA quantification and integrity

The concentration and integrity of RNA was assessed by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, United Kingdom). The absorbance of RNA was quantified by measuring the absorbance of the sample at 260nm and 280nm. RNA purity was determined by a 2.0 or greater ratio of 260nm/280nm measurements. The concentration of RNA was determined by the Beer-Lambert equation, the extinction coefficient for RNA is 40 ng-cm/µl:

$$\text{RNA (ng/µl)} = \frac{\text{Absorbance (260nm)} \times \text{wavelength-dependant extinction coefficient}}{\text{Path length in cm}}$$

2.7.2. Reverse transcription

To measure messenger RNA (mRNA) expression in RNA isolated from hPASMCs or lung tissue, they were first reverse transcribed to single stranded complementary DNA (cDNA). This was achieved using Taqman® reverse transcription reagents (Life Technologies, United Kingdom). For each 20µl reaction 500ng of RNA from hPASMCs and 1µg of RNA from lung samples were reverse transcribed. Each reaction mix consisted of: 10x RT buffer, MgCl₂ (25mM), deoxyribonucleotide triphosphates (dNTPs) (10mM), random hexamers (50µM), RNase inhibitor (20U/µl) and Multiscribe™ (50u/µl). Each cDNA preparation was carried out in 96-well plates, which were centrifuged after addition of reaction mix and RNA. Reverse transcription (RT) was carried out using the Veriti® Thermal Cycler (Life Technologies, United Kingdom) with the temperature cycling sequence: 25°C for 10 minutes, 48°C for 30 minutes and 95°C for 5 minutes. The sequence allows for RNA template annealing, reverse transcription and transcription inactivation. The 96-well cDNA plate was then stored at -20°C.

2.7.3. Quantitative real time-polymerase chain reaction

mRNA expression was validated by quantitative real time-polymerase chain reaction (qRT-PCR). This was achieved using Taqman® universal master mix and fluorescently tagged Taqman® primers (Life Technologies, United Kingdom) (Figure 2-4). Each Taqman® primer had a FAM® dye label on the 5’ end and nonfluorescent quencher on the 3’ end. Samples were loaded in triplicate using a 384-well plate format. For every Taqman® primer used a no template control (NTC) was added to ensure no contamination occurred. The ViiA7™ Real-Time PCR system (Life Technologies) was used under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes then 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Comparative cycle threshold (CT) was used to quantify the expression results. The CT value was inversely proportional to the level of mRNA expression in the sample. A ΔCT is obtained from difference between CT of the mRNA of
interest and the CT of the housekeeper RNA. A $\delta\delta$CT value is obtained from the difference in $\delta$CT from test sample to the $\delta$CT of a control sample. Data is represented as the relative quantification (RQ: $2^{-\delta\delta\text{CT}}$) or fold change compared to chosen control group.
## Table 2-3 Taqman gene expression assay IDs

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<tr>
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<th>Species</th>
<th>Assay ID</th>
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<tbody>
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</tr>
<tr>
<td></td>
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<td>DP1 Receptor</td>
<td>Human</td>
<td>Hs00235003_m1</td>
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IP = prostaglandin I2 receptor (prostacyclin); EP1 = prostaglandin E2 receptor 1; EP2 = prostaglandin E2 receptor 2; DP1 = prostaglandin D2 receptor 1; BMPR-II = bone morphogenetic protein receptor-II; Id = inhibitor of DNA binding; EDNRA/B = endothelin receptor A/B; EDN1 = prepro-endothelin-1; SMURF1/2 = SMAD Specific E3 Ubiquitin Protein Ligase 1/2; KCNK3 = Potassium Two Pore Domain Channel Subfamily K Member 3.
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2.8 Cell culture

All cell culture procedures were carried out under sterile conditions using a Biological Safety Class II vertical laminar flow cabinet. All cells were maintained and stored in a humidified incubator (Eppendorf, United Kingdom) at 37° with a continuous supply of 5% CO₂ and 95% air.

2.8.1. Human pulmonary artery smooth muscle cells

Human pulmonary artery smooth muscle cells (hPASMCs) were provided by Professor Nicholas W. Morrell (University of Cambridge, Cambridge, United Kingdom). Briefly, both male and female hPASMCs were explanted from the distal pulmonary arteries (~1mm external diameter) of macroscopically lung tissue as described previously (Wharton et al., 2000). Human PAH PASMCs were explanted from patients with either idiopathic, heritable or associated PAH. These PASMCs were studied collectively as PAH patient cells (Table 2-5). Non-PAH/control PASMCs were explanted from patients with no evidence of PAH, these PASMCs were studied as controls (Table 2-5). The smooth muscle phenotype was confirmed by the general cell morphology (Figure 2-5) and by alpha smooth muscle staining in Cambridge. HPASMCs were grown in 75cm³ (T75) culture cell flasks in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies, United Kingdom). This was supplemented with 10% (v/v) fetal bovine serum (FBS, Life Technologies, United Kingdom), 1% (v/v) antibiotic antimycotic solution (10,000 units penicillin, 10mg streptomycin and 25µg amphotericin B, Sigma Aldrich, Dorset, United Kingdom). HPASMCs were grown to 90-95% confluency in T75 flasks to avoid growth arrest by cell-cell contact inhibition. For passaging, the reagents used were warmed to 37°C before DMEM was removed from the flask and hPASMCs were washed twice in 5ml sterile DPBS. The cells were removed from the flask using 2ml of trypsin/EDTA solution (0.1% (w/v) EDTA in PBS, Life Technologies, United Kingdom) and incubation at 37°C for two minutes, to aid trypsinisation. Trypsin is a proteolytic enzyme that can detach the hPASMCs from the flask however it must be deactivated quickly to ensure cell viability. This was achieved by adding 8ml of 10% (v/v) FBS DMEM to the flask. The hPASMCs were then further sub-cultured into 96, 24, 6-well plates and T75 flasks depending on experimental protocol. HPASMCs were used for experiments in between passages 3-8.

2.8.2. Proliferation assay protocols

For proliferation assays, hPASMCs were seeded in 24-well plates at a density of 10,000 cells per well and grown in 10% (v/v) FBS DMEM to 50-60% confluency. HPASMCs were subsequently quiesced in 0.5% (v/v) FBS phenyl-red free DMEM (Life Technologies, United Kingdom) for 24 hours, this allows the synchronisation of hPASMCs’ cell cycles before experimental start. For experiments involving treprostinil with/without endothelin and prostaglandin receptor agonists, hPASMCs proliferation was carried out over 72 hours in presence 7% (v/v) FBS phenyl-red free DMEM. The concentrations of treprostinil used
over the course of hPASMCs study allowed the examination of all the prostaglandin receptors that treprostinil activates (Table 2-5). Previously published research involving treprostinil also influenced the range of concentrations chosen (Falcetti et al., 2007, Falcetti et al., 2010). Drugs and media were replaced after 48-hours in all 72-hour experiments. For collection of protein and RNA, hPASMCs were seeded in 6-well plates and grown to 60-70% confluency in 10% (v/v) FBS DMEM. When appropriate, antagonists were added 30 minutes prior to the addition of agonists and drugs and media were replaced after 48-hours. Concentrations of antagonists used in experiments were influenced by previous published research carried out in hPASMCs (Falcetti et al., 2010, Bubb et al., 2014, Patel et al., 2014).

2.8.3. Haemocytometer cell counting

To evaluate proliferation, hPASMCs were manually counted using a haemocytometer (Camlab, United Kingdom). At the end of experiments, DMEM was aspirated off and hPASMCs were washed in 1ml of 37°C sterile DPBS. After removing DPBS, 150µl of trypsin/EDTA solution (Life Technologies, United Kingdom) was added to each well. The plate was placed at 37°C to aid the detachment of the hPASMCs from the wells. The detached hPASMCs were re-suspended in 500µl of 10% (v/v) FBS DMEM per well and mixed. The suspended cells and media were transferred to a 1.5ml eppendorf tube and the suspension was centrifuged at 3,500xg at 4°C for 10 minutes, this produced a cell pellet. The media was carefully removed to ensure the pellet remained intact. The pellet was then re-suspended in a known volume of 10% (v/v) FBS DMEM and vortexed to ensure complete re-suspension. Using the haemocytometer, 10µl of cell suspension was pipette onto the grid. Via a hand tally counter, the cells in each 16-squared corner were counted. The haemocytometer is designed so that every 16-squared corner is equal to the number of cells $10^4$/ml (Figure 2-6). An average of the 4 corners was used to calculate the mean number of cells $10^4$/ml. These experiments were carried out in a blinded manner.
Figure 2-5 Morphology of human pulmonary artery smooth muscle cells.
Photograph of sub-confluent human pulmonary artery smooth muscle cells grown in a T75 culture flask. Scale bar 100μM.
Figure 2-6 Haemocytometer layout

Layout representation of haemocytometer grid. Pulmonary artery smooth muscle cells counted in 16-square corners to produce an average cell number x10⁴ per ml.
Table 2-4 Patient information for human pulmonary arterial smooth muscle cells (hPASMCs)

<table>
<thead>
<tr>
<th>Patient ID number</th>
<th>Sex</th>
<th>Age</th>
<th>Disease state</th>
</tr>
</thead>
<tbody>
<tr>
<td>34MP</td>
<td>Male</td>
<td>62</td>
<td>Control (Emphysema)</td>
</tr>
<tr>
<td>103MP</td>
<td>Male</td>
<td>52</td>
<td>Control (Adenocarcinoma)</td>
</tr>
<tr>
<td>79MP</td>
<td>Male</td>
<td>60</td>
<td>Control (Squamous cell carcinoma)</td>
</tr>
<tr>
<td>75MP</td>
<td>Male</td>
<td>78</td>
<td>Control (Metastatic disease)</td>
</tr>
<tr>
<td>9MP</td>
<td>Male</td>
<td>72</td>
<td>Control (N/A)</td>
</tr>
<tr>
<td>105MP</td>
<td>Female</td>
<td>57</td>
<td>Control (Emphysema)</td>
</tr>
<tr>
<td>77MP</td>
<td>Female</td>
<td>64</td>
<td>Control (Mild emphysema)</td>
</tr>
<tr>
<td>106MP</td>
<td>Female</td>
<td>70</td>
<td>Control (Lung carcinoma)</td>
</tr>
<tr>
<td>32MP</td>
<td>Female</td>
<td>58</td>
<td>Control (Mild emphysema)</td>
</tr>
<tr>
<td>84MP</td>
<td>Female</td>
<td>59</td>
<td>Control (Squamous cell carcinoma)</td>
</tr>
<tr>
<td>82MP</td>
<td>Female</td>
<td>71</td>
<td>Control (Adenocarcinoma)</td>
</tr>
<tr>
<td>56MP</td>
<td>Male</td>
<td>N/A</td>
<td>HPAH (C347R)</td>
</tr>
<tr>
<td>67MP</td>
<td>Male</td>
<td>22</td>
<td>HPAH (W9X)</td>
</tr>
<tr>
<td>23MP</td>
<td>Male</td>
<td>43</td>
<td>APAH (Eisenmengers Syndrome)</td>
</tr>
<tr>
<td>74MP</td>
<td>Male</td>
<td>N/A</td>
<td>PAH</td>
</tr>
<tr>
<td>37MP</td>
<td>Female</td>
<td>24</td>
<td>IPAH</td>
</tr>
<tr>
<td>115MP</td>
<td>Female</td>
<td>N/A</td>
<td>APAH</td>
</tr>
<tr>
<td>35MP</td>
<td>Female</td>
<td>N/A</td>
<td>HPAH (N903S)</td>
</tr>
<tr>
<td>38MP</td>
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<td>N/A</td>
<td>PAH</td>
</tr>
<tr>
<td>73MP</td>
<td>Female</td>
<td>30</td>
<td>HPAH (R899X)</td>
</tr>
</tbody>
</table>

IPAH = Idiopathic pulmonary arterial hypertension; APAH = associated pulmonary arterial hypertension; HPAH = Heritable pulmonary arterial hypertension.
# Table 2-5 Treprostinil Ki values at prostaglandin receptors

<table>
<thead>
<tr>
<th>Prostaglandin Receptor</th>
<th>Treprostinil Ki (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP</td>
<td>32.1 ± 0.2</td>
</tr>
<tr>
<td>EP&lt;sub&gt;1&lt;/sub&gt;</td>
<td>212 ± 56</td>
</tr>
<tr>
<td>EP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>EP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>2505 ± 263</td>
</tr>
<tr>
<td>EP&lt;sub&gt;4&lt;/sub&gt;</td>
<td>826 ± 116</td>
</tr>
<tr>
<td>DP&lt;sub&gt;1&lt;/sub&gt;</td>
<td>4.4 ± 0.4</td>
</tr>
<tr>
<td>FP</td>
<td>4680 ± 927</td>
</tr>
<tr>
<td>TP</td>
<td>Not calculable</td>
</tr>
</tbody>
</table>

Treprostinil binding profile for prostanoid receptors. Data gathered using displacement radioligand binding from cell membranes that expressed recombinant human prostanoid receptors. Table taken from (Whittle et al., 2012)
2.9 Statistical Analysis

All statistical analysis was carried out using GraphPad Prism 6.0© (GraphPad Software Inc, United States). All values are expressed as mean ± standard error of the mean (SEM). Unpaired student’s t-tests were used for comparing two treatment groups. One or two-way ANOVA followed by Bonferroni’s post-hoc test was performed to evaluate the statistical significance between three or more groups where appropriate. A probability level of <0.05 was defined as being statistically significant.
Chapter 3

3 The effect of treprostinil in the chronic hypoxic model of pulmonary hypertension
3.1. Introduction

Registry and epidemiological studies have demonstrated the increased incidence of females over males in developing pulmonary arterial hypertension (PAH) (McGoon et al., 2013, Badesch et al., 2010). However, the estimated 5-year survival rate for males is lower than females, at 52% and 62% respectively (Shapiro et al., 2012). Studies have found that females with PAH have better compensation of the right ventricle of the heart in response to increased pulmonary arterial pressure than males. This leads to greater right ventricle function in PAH and this may contribute to greater survival (Humbert et al., 2010a, Benza et al., 2012).

Much of the research into sex differences in PAH has focused on the female predisposition to the disease. Indeed, estrogen has been identified to be a possible risk factor in PAH. In serotonin-dependent animal models of pulmonary hypertension (PH) there is a female susceptibility related to circulating estrogen (Dempsie et al., 2011, Dempsie et al., 2013, White et al., 2011a). Recent studies from the Maclean lab have shown that aromatase, the enzyme synthesising estrogen, was found to have greater expression in female human, rat and muscle pulmonary artery smooth muscle (Mair et al., 2014). Inhibiting aromatase by anastrozole reversed PH in hypoxic and sugen-hypoxia (SU-HX) models of PH but only in females (Mair et al., 2014). In contrast, there is also some evidence to suggest that estrogen can have beneficial effects in PH models. 17-β estradiol has been shown to attenuate PH development in male hypoxic (Xu et al., 2010, Lahm et al., 2012) and monocrotaline animal models of PH (Farhat et al., 1993). Similar beneficial effects of estrogen were latterly discovered in female rats (Resta et al., 2001, Nadadur et al., 2012, Yuan et al., 2013).

Mutations of the BMPR-II encoding gene are found in up to 75% of heritable PAH (HPAH) and 20% of idiopathic patients (IPAH) (Hamid et al., 2009, Atkinson et al., 2002). Dysregulated BMPR-II receptor can lead to a reduction in the function of its downstream signalling pathway. (Yang et al., 2005). In pulmonary arteries, reduced BMPR-II receptor expression has been associated with increased proliferation of smooth muscle cells (Yang et al., 2005) and increased apoptosis of pulmonary endothelial cells (Teichert-Kuliszewska et al., 2006). BMPR-II has been previously identified as a target for prostacyclin analogues such as treprostinil (Yang et al., 2010). The Maclean lab has detected sex differences in BMPR-II signalling expression, as female control hPASMCs have lower expression than in males (Mair et al., 2015).

This was the first study to examine the impact of sex on the therapeutic actions of treprostinil, however the impact of sex on other drug therapies has been previously explored. Greater positive clinical outcomes for tadalafil, a phosphodiesterase 5 inhibitor, were associated with males over females (Mathai et al., 2015). However, females with
PAH had greater therapeutic responses to endothelin receptor antagonists than males (Gabler et al., 2012). Both studies suggest that sex can influence the response to drug therapy, however the molecular mechanisms associated with these sex differences were not identified.

It is unclear if sex differences exist in the prostaglandin receptors (IP, EP$_2$ and DP$_1$), which are known as major targets of treprostinil (Clapp and Gurung, 2015). Although not examined in this current study, treprostinil metabolism occurs primarily through the action of CYP2C8 and to a lesser extent through CYP2C9. The addition of a CYP2C8 inducer (rifampin) was found to decrease treprostinil blood concentration by 30% whereas a CYP2C8 inhibitor (gemfibrozil) doubled treprostinil concentration (Rollins et al., 2009, Kumar et al., 2016).

Clinically, prescribed doses of treprostinil range from an initial dose of 1.25ng/kg/min, this can be increased incrementally to 40ng/kg/min, in some cases this can increase to 125ng/kg/min (Lang et al., 2006, McSwain et al., 2008). In the current study, the initial dose of treprostinil was 100ng/kg/min, which is closest to highest dose for clinical use. The higher dose (400ng/kg/min) was examined to explore if the female specific response to treprostinil was dose dependant.

This data demonstrates that a slow release of treprostinil in a pellet that was subcutaneously implanted was effective method of drug delivery. Novel sex specific effects were also observed with a 100ng/kg/min treprostinil, partially reversing an established hypoxic PH phenotype in female rats, not in male rats. It demonstrates that the therapeutic effects of treprostinil may be partially mediated through increased signalling of the BMPR-II pathway mRNA. At a higher dose of 400ng/kg/min treprostinil led to a therapeutic response in hypoxic male as well as an increased therapeutic response in female rats.

Hypotheses and aims of this chapter:

Slow-release subcutaneously implanted pellets have been used previously to dose animals in the treatment of pulmonary hypertension (Wright et al., 2015). We hypothesise that the PGI$_2$ analogue treprostinil can be successfully delivered to chronic hypoxic rats using slow-release subcutaneously implanted pellets as an alternative to repetitive dosing. To investigate this hypothesis, the following aim was carried out.

1. To examine the haemodynamic effects of treprostinil, administered via a slow release subcutaneously implanted pellet in the chronic hypoxic model of pulmonary hypertension.

Females and males are known to differ in response to drug therapy for variety of reasons (Soldin and Mattison, 2009). To investigate this our hypothesis is that sex can play a role
in altering the effectiveness of treprostinil in an animal model of PH. The following aim was carried out:

2. Study to characterise the influence of sex on the actions of treprostinil in a chronic hypoxic rat model of PH and how these effects are mediated.
3.2. Results

3.2.1. Rat blood plasma levels of treprostinil administered via a slow release subcutaneous pellet

Treprostinil is a prostacyclin analogue that is used clinically for the treatment of PAH. We wished to investigate if treprostinil administered via a slow release subcutaneously implanted pellet could be an effective method of dosing in rats. Briefly, female and male rats were implanted with pellets releasing treprostinil at 100ng/kg/min for 14 days. After 4 days of dosing female and male rats had similar levels of treprostinil in isolated plasma (Figure 3-1). After 10 days, we found a significant decrease in treprostinil when compared to 4 days in female and male rats. However, this significant reduction persists in male rats at 14 days (Figure 3-1).
Figure 3-1 Blood plasma concentration of subcutaneously implanted prostacyclin analogue treprostinil

Female and male sprague-dawley rats were implanted with sub-cutaneous pellet containing treprostinil (100ng/kg/min). Blood taken after carotid artery catheterisation, treprostinil levels assessed by LC/MS. N=3, *P<0.05 cf. (Female) Day 4, †† P<0.01 cf. (Male) Day 4, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM.
3.2.2. Treprostinil (100ng/kg/min) attenuates development of hypoxia induced PH in female rats

To examine the effectiveness of treprostinil administered via slow release pellet on the chronic hypoxic PH model we examined right ventricular systolic pressure (RVSP), right ventricular end systolic pressure (RVESP), right ventricular end diastolic pressure (RVEDP), right ventricular hypertrophy (RVH) and the level of pulmonary artery remodelling. Chronic hypoxia significantly increased RVSP, RVESP, RVH and pulmonary remodelling but not RVEDP (Figure 3-2-3-5). Under normoxic conditions, treprostinil had no effect on any haemodynamic measurement. However, RVSP was significantly reduced by treprostinil in female hypoxic rats but no significant reduction was observed in male rats (Figure 3-2). Treprostinil also reduced RVESP (Figure 3-3) and RVH (Figure 3-4). In addition, treprostinil reversed pulmonary artery remodelling in females under hypoxic conditions, whereas no significant reduction was identified in male rats (Figure 3-5-Figure 3-6). Right ventricular ejection fraction (RVEF) was significantly decreased in female and male hypoxic rats. RVEF was unchanged in female rats treated with treprostinil whereas RVEF was decreased by treprostinil in males (Table 3-1).
Figure 3-2 Chronic hypoxia increases right ventricular systolic pressure and prostacyclin analogue treprostinil attenuates increase in female chronic hypoxic rats

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (100ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Right ventricular systolic pressures (RVSP) were obtained by right heart catheterisation through jugular vein. N=5-6, ***P<0.001 c.f (Female) Normoxic Vehicle, φφφ P<0.001 c.f (Female) Normoxic Treprostinil, ζ P<0.05 cf. (Female) Hypoxic Vehicle, ₢₃₃₃ P<0.001 cf. (Male) Normoxic Vehicle, ₢₃₃₃ P<0.001 c.f (Male) Normoxic Treprostinil. Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM.
Figure 3-3 Chronic hypoxia increases right ventricular systolic end pressure and prostacyclin analogue treprostinil attenuates increase in female chronic hypoxic rats

Female and male Sprague-Dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (100ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. (A) Right ventricular systolic end pressure (RVSEP) and (B) right ventricular diastolic end pressure (RVDEP) were obtained by right heart catheterisation through jugular vein. N=4-6, ***P<0.001 c.f (Female) Normoxic Vehicle, φφφ P<0.001 c.f (Female) Normoxic Treprostinil, ζζζ P<0.01 c.f. (Female) Hypoxic Vehicle, ♂♂♂ P<0.001 c.f. (Male) Normoxic Vehicle, +++ P<0.001 c.f (Male) Normoxic Treprostinil, Two-way ANOVA with Bonferroni's post-hoc test. Data expressed as mean ± SEM.
Figure 3-4 Chronic hypoxia increases right ventricular hypertrophy and prostacyclin analogue treprostinil attenuates increase in female chronic hypoxic rats

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (100ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Right ventricular hypertrophy (RV Hypertrophy) was assessed by the ratio of the dry weight of the right ventricle over the left ventricle plus septum (RV/LV+S; Fulton’s index). N=5-6, ***P<0.001 c.f (Female) Normoxic Vehicle, ♂♂♂ P<0.001 c.f (Female) Normoxic Treprostinil, ξ P<0.05 cf. (Female) Hypoxic Vehicle, ΤΤΤ P<0.001 cf. (Male) Normoxic Vehicle, +++ P<0.001 c.f (Male) Normoxic Treprostinil, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM.
Figure 3-5 Chronic hypoxia increases pulmonary vessel remodelling and prostacyclin analogue treprostinil attenuates increase in female chronic hypoxic rats

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (100ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. The level of pulmonary vessel remodelling was assessed by the ratio of the remodelled and non-remodelled arteries and expressed as % of the remodelled vessels over the total number of vessels. N=5-6, ***P<0.001 c.f (Female) Normoxic Vehicle, φφφ P<0.001 c.f (Female) Normoxic Treprostinil, ζ P<0.05 cf. (Female) Hypoxic Vehicle, ₯₯₮ P<0.001 cf. (Male) Normoxic Vehicle, ₯₮₮ P<0.001 c.f (Male) Normoxic Treprostinil, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM.
Figure 3-6 Representative Miller’s elastin and α-smooth muscle actin staining in chronic hypoxic rats treated with treprostinil or vehicle

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (100ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Vessel remodelling was determined in (A) female and (C) male rats by Miller’s elastin stain, remodelled vessels determined by presence of double elastic laminae. Smooth muscle proliferation was visualised in (B) female and (D) male rats by α smooth muscle actin (α-SMA) staining. α-SMA visualised by DAB staining (dark brown/orange stain). Scale bar = 20μm.
3.2.3. Treprostinil (100ng/kg/min) does not change systemic haemodynamics

As treprostinil is a vasodilator we wanted to examine any changes in systemic haemodynamic measures. Neither hypoxia nor treprostinil affected LVSP (Figure 3-7), cardiac output (CO) (Figure 3-8) or heart rate (HR) (Figure 3-9) (Table 3-1).
Figure 3-7 Chronic hypoxia and prostacyclin analogue treprostinil do not alter left ventricular systemic pressure

Female and male Sprague-Dawley rats were subjected to normoxic/hypoxic conditions (1000/550 mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (100 ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Left ventricular systolic pressures (LVSP) were obtained by left heart catheterisation through carotid artery. N=4-6, Two-way ANOVA with Bonferroni's post-hoc test. Data expressed as mean ± SEM.
Figure 3-8 Chronic hypoxia and prostacyclin analogue treprostinil do not alter cardiac output

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (100ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Cardiac output taken from left heart catheterisation through carotid artery. N=4-6, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM.
Figure 3-9 Chronic hypoxia and prostacyclin analogue treprostinil do not alter heart rate

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (100ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Heart rate was measured by heart beats per minute (BPM). N=5-6. Two-way ANOVA with Bonferroni's post-hoc test. Data expressed as mean ± SEM.
Table 3-1 Effect of prostacyclin analogue treprostinil (100ng/kg/min) in normoxic or hypoxic rats on haemodynamics and phenotypes

<table>
<thead>
<tr>
<th>Group</th>
<th>mRVP (mmHg)</th>
<th>RVEF (%)</th>
<th>mLVP (mmHg)</th>
<th>Body Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n)</td>
<td>(n)</td>
<td>(n)</td>
<td>(n)</td>
</tr>
<tr>
<td>Female Normoxic (Veh)</td>
<td>13.1 ± 1.89 (5)</td>
<td>20.74 ± 1.69 (4)</td>
<td>42.19 ± 1.35 (4)</td>
<td>216.4 ± 5.14 (5)</td>
</tr>
<tr>
<td>Female Normoxic (Trep)</td>
<td>14.22 ± 1.19 (6)</td>
<td>30.3 ± 3.74 (6)</td>
<td>44.39 ± 2.36 (5)</td>
<td>212.6 ± 5.69 (6)</td>
</tr>
<tr>
<td>Female Hypoxic (Veh)</td>
<td>24.02 ± 1.19 (6) ***</td>
<td>10.83 ± 1.79 (5) ⧨⧨⧨</td>
<td>44.42 ± 4.47 (5)</td>
<td>228.8 ± 3.07 (6)</td>
</tr>
<tr>
<td>Female Hypoxic (Trep)</td>
<td>19.32 ± 1.93 (5)</td>
<td>12.83 ± 2.73 (5) ⧨</td>
<td>41 ± 3.42 (5)</td>
<td>229.9 ± 3.25 (6)</td>
</tr>
<tr>
<td>Male Normoxic (Veh)</td>
<td>17.94 ± 1.18 (6)</td>
<td>22.14 ± 2.10 (6)</td>
<td>47.3 ± 5.69 (6)</td>
<td>276.8 ± 7.91 (6)</td>
</tr>
<tr>
<td>Male Normoxic (Trep)</td>
<td>15.2 ± 0.95 (6)</td>
<td>28.62 ± 4.18 (6)</td>
<td>46.44 ± 2.21 (5)</td>
<td>276.1 ± 11.1 (6)</td>
</tr>
<tr>
<td>Male Hypoxic (Veh)</td>
<td>30.2 ± 1.05 (6) ⧨⧨⧨</td>
<td>11.69 ± 2.28 (6) ⧨</td>
<td>46.32 ± 6.15 (5)</td>
<td>262.9 ± 5.33 (6)</td>
</tr>
<tr>
<td>Male Hypoxic (Trep)</td>
<td>27.95 ± 2.29 (6) ⧨⧨⧨</td>
<td>9.69 ± 0.96 (6) ⧨</td>
<td>41.36 ± 2.70 (6)</td>
<td>251.9 ± 3.49 (6)</td>
</tr>
</tbody>
</table>

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (100ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Mean right ventricle pressure (mRVP) and right ventricle ejection fraction (RVEF) was measured by cannulation of right external jugular vein. Mean left ventricle pressure (mLVP) was measured by cannulation of right internal carotid artery. N numbers indicated in brackets. *P<0.05 c.f normoxic vehicle, **P<0.01 c.f female normoxic (Veh), ⧨⧨ P<0.01, ⧨⧨⧨ P<0.001 c.f. female normoxic (Trep), ⧨⧨⧨ P<0.001 c.f male normoxic (Veh), ⧨ P<0.05, ⧨⧨⧨ P<0.001 c.f. male normoxic (Trep). Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Veh = vehicle, Trep = treprostinil
3.2.4. No sex difference in terminal plasma treprostinil levels

Having demonstrated a sex difference in response to treprostinil, it was important to ascertain the level of treprostinil from the blood plasma of rats collected at the termination of the study. Despite the low levels of treprostinil found in the plasma there was no difference between rats dosed under normoxic or hypoxic conditions (Figure 3-10).
Figure 3-10 Terminal measurements of prostacyclin analogue treprostinil from rat blood plasma
Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (100ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Blood taken after carotid artery catheterisation, treprostinil levels assessed by LC/MS. n=3-4, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM.
3.2.5. Treprostinil (100ng/kg/min) influence on prostaglandin receptors: increased expression of EP$_2$ mRNA in hypoxic treprostinil treated female rats.

As treprostinil is known to act through the activation of specific prostaglandin receptors, these were examined in the lung tissue of the rats. For this we examined the mRNA levels of the prostacyclin (IP), prostaglandin E$_2$ receptor 2 (EP$_2$) and the prostaglandin D$_2$ receptor 1 (DP$_1$) by Taqman® analysis. Results showed that treprostinil, hypoxia and sex had no influence on the expression of any of the IP or DP$_1$ prostaglandin receptors (Figure 3-11A&C). Treprostinil increased the mRNA expression of the EP$_2$ receptor under hypoxic conditions (Figure 3-11B). Interestingly, mRNA expression of the IP receptor was significantly higher than both EP$_2$ and DP$_1$ receptors in both female and male rats (Figure 3-12A-B). Also, in untreated male normoxic rats EP$_2$ mRNA expression was found to be significantly higher than DP$_1$ mRNA expression (Figure 3-12B).
Figure 3-11 Lung expression of the EP₂ receptor was increased by the addition of prostacyclin analogue treprostinil

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (100ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Expression of the (A) IP, (B) EP₂ and (D) DP₁ receptors was assessed from whole lung homogenates by Taqman® quantitative Real Time-PCR. Results were normalised to β₂-microglobulin. N=2-6 per group in triplicate (N=2 for Female Normoxic (Veh) DP₁), ¥¥ P<0.01 cf. (Female) Hypoxic (Veh), Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ\text{max}/RQ\text{min}. RQ= relative quantification, Veh= vehicle, Trep= treprostinil.
Figure 3-12 IP receptor mRNA expression in significantly higher in both female and male rats than the EP$\textsubscript{2}$ and DP$\textsubscript{1}$ receptors

(A) Female and (B) male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (100ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Expression of the IP, EP$\textsubscript{2}$ and DP$\textsubscript{1}$ receptors was assessed from whole lung homogenates by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=2-6 per group in triplicate (N=2 for Female Normoxic (Veh) DP$\textsubscript{1}$). ***P<0.001 cf. respective IP group, ⧼P<0.01 cf. respective EP$\textsubscript{2}$ group, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ$\textsubscript{max}$/RQ$\textsubscript{min}$. RQ= relative quantification, Veh= vehicle, Trep= treprostinil.
3.2.6. Treprostinil (100ng/kg/min) increases expression of Id mRNA in lungs of chronic hypoxic female rats

As BMPR-II dysregulation occurs in PAH we investigated the effects of treprostinil on BMPR-II signalling within lung tissue. The mRNA expression of BMPR-II, Id1 and Id3 was examined by Taqman® analysis (Figure 3-13). Hypoxia had no impact on the expression of BMPR-II, Id1 or Id3. Interestingly we found that, in female hypoxic rats treated with treprostinil, there was a significant increase in the mRNA expression of Id1 compared to female hypoxic vehicle treated rats. (Figure 3-13B). Id3 mRNA expression was also significantly increased in female hypoxic rats treated with treprostinil compared to both normoxic treated and untreated female rats (Figure 3-13C). Id3 mRNA expression in treprostinil treated hypoxic rats was found to be significantly higher than BMPR-II mRNA levels in the equivalently treated female rats (Figure 3-14A). Also, Id3 mRNA levels were significantly higher than Id1 in hypoxic vehicle female rats (Figure 3-14A). A comparison of BMPR-II, Id1 and Id3 found no significant difference in mRNA expression in male rats (Figure 3-14B). mRNA expression of SMAD specific E3 ubiquitin protein ligase 1 and 2 (SMURF1/2) was not altered by either hypoxic conditions or treprostinil treatment (Figure 3-15A&B). Expression of the potassium two pore domain channel subfamily K member 3 (KCNK3) was elevated in female chronic hypoxic rats treated with treprostinil (Figure 3-15C). The protein levels of BMPR-II, pSmad 1/5/9, Id1 and Id3 were examined in lung tissue (Figure 3-16–Figure 3-17). BMPR-II, pSmad 1/5/9, Id1 and Id3 protein expressions were not affected by hypoxia (Figure 3-16A-D). Treatment with treprostinil did not significantly alter the protein expression of BMPR-II, pSmad 1/5/9, Id1 or Id3 (Figure 3-17A-D).
Figure 3-13 Lung expression of BMPR-2 signalling was unchanged by chronic hypoxia however Id1 and Id3 mRNA expression was up-regulated with the addition of prostacyclin analogue treprostinil

Female and male Sprague-Dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (100ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Expression of (A) BMPR-II, (B) Id1 and (C) Id3 mRNA were assessed from whole lung homogenates by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=3-6 per group in triplicate, *P<0.05, cf. (Female) Normoxic (Veh), φ P<0.05, ΦΦ P<0.01 cf. (Female) Normoxic (Trep), ζζ P<0.01 cf. (Female) Hypoxic (Veh), Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ_{max}/RQ_{min}. RQ= relative quantification. Veh= vehicle, Trep= treprostinil.
Figure 3-14 Id3 mRNA expression was higher than BMPR-II expression under hypoxic conditions upon treprostinil treatment in female rats

(A) Female and (B) male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (100ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Expression of BMPR-II, Id1 and Id3 mRNA were assessed from whole lung homogenates by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=3-6 per group in triplicate, *P<0.05, cf. respective BMPR-II group, f P<0.05 cf. respective Id1 group, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ\textsuperscript{max}/RQ\textsuperscript{min}. RQ= relative quantification. Veh= vehicle, Trep= treprostinil.
Figure 3-15 Lung expression of SMURF1, SMURF2 were unchanged by chronic hypoxia; KCNK3 was increased by treprostinil in combination with hypoxia

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (100ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Expression of (A) SMURF1, (B) SMURF2 and (C) KCNK3 mRNA were assessed from whole lung homogenates by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=3-5 per group in triplicate, $\phi$ P<0.05, cf. (Female) Normoxic (Trep), Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ^{max}/RQ^{min}. RQ= relative quantification. Veh= vehicle, Trep= treprostinil.
Figure 3-16 Lung protein expression of BMPR-II signalling was not altered by chronic hypoxia

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (100ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Expression of (A) BMPR-II, (B) pSmad 1/5/9, (C) Id1 and (D) Id3 were assessed from whole lung homogenates by western blot. (E) Representative immunoblot, β-actin was used as the internal loading control. N=3-6 per group in duplicate (n=3 for Female Normoxic (Veh)), Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Veh= vehicle, Trep= treprostinil.
Figure 3-17 BMPR-II, pSmad 1/5/9, Id1 and Id3 protein expression was unchanged by treprostinil in female and male rats.

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (100ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Expression of (A) BMPR-II, (B) pSmad 1/5/9, (C) Id1 and (D) Id3 were assessed from whole lung homogenates by western blot. (E) Representative immunoblot, β-actin was used as the internal loading control. N=5-6 per group in duplicate, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Veh= vehicle, Trep= treprostinil.
3.2.7. Higher treprostinil dose (400ng/kg/min) attenuates PH in female and male hypoxic rats

Having demonstrated that the slow release pellet was an effective dosing method for reversing a PH phenotype in females we studied the effects of a higher dose of treprostinil (400ng/kg/min) in both female and male rats. This was to determine if the sex differences in response to treprostinil would still exist with a higher dose of treprostinil. As before, hypoxia caused increases in RVSP, RVSEP, RVH and remodelling in female and male rats. Treprostinil decreased RVSP and RVSEP in female and male rats under hypoxic conditions (Figure 3-18-Figure 3-19A). Treprostinil reduced RVH and remodelling in both female and male hypoxic rats (Figure 3-20-Figure 3-21). Reductions in female RVSP, RVH and remodelling were more significant than those observed in the 100ng/kg/min study, although the level of reductions were only a small improvement on the early study. As before, RVEF was reduced under hypoxic conditions and treprostinil treatment did not significantly alter RVEF (Table 3-2). The higher dose of treprostinil did not completely reverse the increased pulmonary haemodynamics in either female or male rats.

The higher dose of treprostinil had no effect on the systemic haemodynamics including LVSP, CO, HR or body weight of both rats (Figure 3-23-Figure 3-25), Table 3-2). As observed previously, there was no difference in plasma levels of treprostinil between normoxic or hypoxic conditions in female and male rats (Figure 3-26).
Figure 3-18 Chronic hypoxia increases right ventricular systolic pressure and prostacyclin analogue treprostinil attenuates increase in female and male chronic hypoxic rats

Female and male Sprague-Dawley rats were subjected to normoxic/hypoxic conditions (1000/550 mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Right ventricular systolic pressures (RVSP) were obtained by right heart catheterisation through jugular vein. N=4-6, ***P<0.001 c.f (Female) Normoxic Vehicle, ᵃʰᵃʰᵃ P<0.001 c.f (Female) Normoxic Treprostinil, ξξξ P<0.001 cf. (Female) Hypoxic Vehicle, ŦŦŦ P<0.001 cf. (Male) Normoxic Vehicle, +++ P<0.001 c.f (Male) Normoxic Treprostinil, ǂǂǂ P<0.001 c.f (Male) Hypoxic Vehicle, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM.
Figure 3-19 Chronic hypoxia increases right ventricular systolic end pressure and prostacyclin analogue treprostinil attenuates increase in female and male chronic hypoxic rats

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. (A) Right ventricular systolic end pressure (RVSEP) and (B) right ventricular diastolic end pressures (RVDEP) were obtained by right heart catheterisation through jugular vein. N=4-6, ***P<0.001 c.f (Female) Normoxic Vehicle, ♂♂♂ P<0.001 c.f (Female) Normoxic Treprostinil, ξξξ P<0.001 c.f. (Female) Hypoxic Vehicle, ₊ moduleId P<0.001 cf. (Male) Normoxic Vehicle, $$$ P<0.001 c.f (Male) Normoxic Treprostinil, I P<0.05 c.f (Male) Hypoxic Vehicle, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM.
Figure 3-20 Chronic hypoxia increases right ventricular hypertrophy and prostacyclin analogue treprostinil attenuates increase in female and male chronic hypoxic rats

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Right ventricular hypertrophy (RV Hypertrophy) was assessed by the ratio of the dry weight of the right ventricle over the left ventricle plus septum (RV/LV+S; Fulton’s index). N=5, ***P<0.001 c.f (Female) Normoxic Vehicle, φ P<0.05 c.f (Female) Normoxic Treprostinil, ζζ P<0.01 cf. (Female) Hypoxic Vehicle, ††† P<0.001 cf. (Male) Normoxic Vehicle, ++ P<0.01 c.f (Male) Normoxic Treprostinil, ¶ P<0.05 c.f (Male) Hypoxic Vehicle, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM.
Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. The level of pulmonary vessel remodelling was assessed by the ratio of the remodelled and non-remodelled arteries and expressed as % of the remodelled vessels over the total number of vessels. N=5-6, ***P<0.001 c.f (Female) Normoxic Vehicle, ɸɸɸ P<0.001 c.f (Female) Normoxic Treprostinil, ξξ Π<0.01 cf. (Female) Hypoxic Vehicle, ⠼⠢⠢⠢ P<0.001 cf. (Male) Normoxic Vehicle, ☱☱☱ P<0.001 c.f (Male) Normoxic Treprostinil, ǁ P<0.05 c.f (Male) Hypoxic Vehicle, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM.
Figure 3-22 Representative Miller’s elastin and α-smooth muscle actin staining in chronic hypoxic rats treated with treprostinil or vehicle.

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Vessel remodelling was determined in (A) female and (C) male rats by Miller's elastin stain, remodelled vessels determined by presence of double elastic laminae. Smooth muscle proliferation was visualised in (B) female and (D) male rats by α smooth muscle actin (α-SMA) staining. α-SMA visualised by DAB staining (dark brown/orange stain). Scale bar = 20µm.
Figure 3-23 Chronic hypoxia and prostacyclin analogue treprostinil do not alter left ventricular systemic pressure.
Female and male Sprague-Dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Left ventricular systolic pressures (LVSP) were obtained by left heart catheterisation through carotid artery. N=4-6, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM.
Figure 3-24 Chronic hypoxia and prostacyclin analogue treprostinil do not alter cardiac output

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Cardiac output taken from left heart catheterisation through carotid artery. N=4-6, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM.
Figure 3-25 Chronic hypoxia and prostacyclin analogue treprostinil do not alter heart rate
Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Heart rate was measured by heart beats per minute (BPM). N=5-6, Two-way ANOVA with Bonferroni's post-hoc test. Data expressed as mean ± SEM.
Table 3-2 Effect of prostacyclin analogue treprostinil (400ng/kg/min) in normoxic or hypoxic rats on haemodynamics and phenotypes

<table>
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<tr>
<th>Group</th>
<th>mRVP (mmHg)</th>
<th>RVEF (%)</th>
<th>mLVP (mmHg)</th>
<th>Body Weight (g)</th>
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<td>Female Normoxic (Veh)</td>
<td>12.7 ± 0.59</td>
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<td>48.42 ± 2.35</td>
<td>260.6 ± 8.49</td>
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<td>252.2 ± 5.04</td>
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<tr>
<td>Female Hypoxic (Veh)</td>
<td>26.76 ± 2.06</td>
<td>8.304 ± 1.43</td>
<td>38.25 ± 6.58</td>
<td>251 ± 5.45</td>
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<tr>
<td>Female Hypoxic (Trep)</td>
<td>17.26 ± 1.61</td>
<td>7.729 ± 0.93</td>
<td>45.27 ± 4.24</td>
<td>254.4 ± 3.24</td>
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<tr>
<td>Male Normoxic (Veh)</td>
<td>10.9 ± 1.25</td>
<td>16.8 ± 1.44</td>
<td>49.34 ± 1.84</td>
<td>321.9 ± 3.99</td>
</tr>
<tr>
<td>Male Normoxic (Trep)</td>
<td>14.4 ± 1.64</td>
<td>19.77 ± 2.69</td>
<td>40.25 ± 3.32</td>
<td>321.9 ± 1.68</td>
</tr>
<tr>
<td>Male Hypoxic (Veh)</td>
<td>28.05 ± 0.82</td>
<td>8.872 ± 0.71</td>
<td>47.33 ± 3.11</td>
<td>301.1 ± 8.24</td>
</tr>
<tr>
<td>Male Hypoxic (Trep)</td>
<td>18.13 ± 1.45</td>
<td>9.28 ± 2.18</td>
<td>50.91 ± 1.79</td>
<td>312.1 ± 6.79</td>
</tr>
</tbody>
</table>

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Mean right ventricle pressure (mRVP) and right ventricle ejection fraction (RVEF) was measured by cannulation of right external jugular vein. Mean left ventricle pressure (mLVP) was measured by cannulation of right internal carotid artery. N numbers indicated in brackets, ***P<0.001 c.f female normoxic (Veh), ϕϕ P<0.01, ϕϕϕ P<0.001 cf. female normoxic (Trep), ξξ P<0.01 c.f female hypoxic vehicle, ΤΤΤΤ P<0.001 c.f male normoxic (Veh), † P<0.05, ††† P<0.001 cf. male normoxic (Veh), *P<0.05 c.f male normoxic (Veh), || P<0.01 cf. male hypoxic (Veh), Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Veh = vehicle, Trep = treprostinil
Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Blood taken after carotid artery catheterisation, treprostinil levels assessed by LC/MS. n=2-3, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM.
3.2.8. Higher treprostinil dose (400ng/kg/min) increases mRNA expression of EP$_2$ receptor in female rats under hypoxic conditions

MRNA levels of the prostaglandin receptors; IP, EP$_2$ and DP$_1$ were assessed in the whole lung of the rats. Consistent with the previous results, hypoxia caused no change in any receptors (Figure 3-27A, B&C). However, the higher dose of treprostinil, under hypoxic conditions, led to a significant increase in the mRNA levels of the EP$_2$ receptor in female rats compared to normoxic treated and untreated female rats (Figure 3-27C). IP mRNA expression was found to be significantly higher than both EP$_2$ and DP$_1$ receptors in both female and male rats (Figure 3-28A&B). Interestingly, DP$_1$ mRNA expression was found to be significantly lower than EP$_2$ mRNA levels (Figure 3-28A&B).
Figure 3-27 The addition of prostacyclin analogue treprostinil in combination with chronic hypoxia increases EP$_2$ receptor expression in female rats

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Expression of the (A) IP, (B) EP$_2$ and (C) DP$_1$ receptors was assessed from whole lung homogenates by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=5-6 per group in triplicate. **P<0.01 cf. (Female) Normoxic (Veh), φφφ P<0.001 cf. (Female) Hypoxic (Trep), Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ$^{max}$/RQ$^{min}$. RQ= relative quantification, Veh= vehicle, Trep= treprostinil.
Figure 3-28 IP and EP\textsubscript{2} mRNA expression is significantly higher than DP\textsubscript{1} receptor mRNA expression in both female and male rats.

(A) Female and (B) male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Expression of the IP, EP\textsubscript{2} and DP\textsubscript{1} receptors was assessed from whole lung homogenates by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=5-6 per group in triplicate. ***P<0.001 cf. respective BMPR-II group, ⧫⧫⧫P<0.001 cf. respective EP\textsubscript{2} group, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ\textsuperscript{max}/RQ\textsuperscript{min}. RQ= relative quantification, Veh= vehicle, Trep= treprostinil.
3.2.9. Treprostinil (400ng/kg/min) did not change whole lung expression of Id3 in chronic hypoxic female and male rats

Although sex differences in the effect of treprostinil on BMPR-II signalling were identified in the first dosing study it was important to also investigate the effects of the higher dose of treprostinil on BMPR-II signalling. As before there was no significant effect of treprostinil or hypoxia on BMPR-II mRNA levels (Figure 3-29A). Interestingly, there was significant increases in Id1 and Id3 mRNA levels in male rats treated with treprostinil under hypoxic conditions when compared to normoxic treprostinil dosed male rats (Figure 3-29B&C). Despite the higher dose, treprostinil did not increase Id1 or Id3 mRNA in female hypoxic rats (Figure 3-29B&C) as seen in the first study. BMPR-II mRNA expression was found to be higher than both Id1 and Id3 mRNA expression in female rats (Figure 3-30A). Normoxic female rats treated with treprostinil were found to have higher Id3 mRNA levels compared to Id1 mRNA expression (Figure 3-30A). Both treated and untreated normoxic male rats had higher BMPR-II mRNA expression than Id1 mRNA expression (Figure 3-30B). Interestingly, there was no difference in BMPR-II and Id1 mRNA levels in treated and untreated hypoxic male rats (Figure 3-30B). However, BMPR-II mRNA expression was found to be significantly higher than Id3 mRNA in all male rat groups (Figure 3-30B). Treprostinil treatment combined with hypoxia increased SMURF1 mRNA expression solely in female rats (Figure 3-31A). There was no significant change in SMURF2 mRNA expression in either female or male rats (Figure 3-31B). KCNK3 mRNA expression was significantly elevated in females under hypoxic conditions compared to normoxic female rats (Figure 3-31C). Protein levels of BMPR-II, pSmad 1/5/9, Id1 and Id3 were examined. Treprostinil at the higher dose did not change the protein expression of the BMPR-II signalling pathway under normoxic conditions (Figure 3-32). Interestingly, the protein expression of pSmad 1/5/9 protein expression was found to increase in female hypoxic rats (Figure 3-33B). However, Id1 protein expression was increased in both female and male rats under hypoxic conditions (Figure 3-33C). Under hypoxic conditions the higher dose of treprostinil did not influence the expression of BMPR-II or pSmad 1/5/9 (Figure 3-34A&B). The higher dose of treprostinil did not significantly change the expression of both Id1 and Id3 (Figure 3-34C&D).
Figure 3-29 Id1 and Id3 mRNA expression increased in chronic hypoxic male rats treated with prostacyclin analogue treprostinil

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Expression of (A) BMPR-II, (B) Id1 and (C) Id3 mRNA were assessed from whole lung homogenates by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=5-6 per group in triplicate. † P<0.05 cf. (Male) Normoxic (Trep), Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ^{max}/RQ^{min}. RQ= relative quantification. Veh= vehicle, Trep= treprostinil.
Figure 3-30 Id1 and Id3 mRNA expression increased in chronic hypoxic male rats treated with prostacyclin analogue treprostinil

(A) Female and (B) male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Expression of BMPR-II, Id1 and Id3 mRNA were assessed from whole lung homogenates by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=5-6 per group in triplicate. *P<0.05, **P<0.01, ***P<0.001 cf. respective BMPR-II group, ϕ P<0.05 cf. respective Id1 group, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ^max/RQ^min. RQ= relative quantification. Veh= vehicle, Trep= treprostinil.
Figure 3-31 Lung expression of SMURF1 and KCNK3 mRNA were increased by chronic hypoxia and treprostinil

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Expression of (A) SMURF1, (B) SMURF2 and (C) KCNK3 mRNA were assessed from whole lung homogenates by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=5-6 per group in triplicate, *P<0.05, ***P<0.001 cf. (Female) Normoxic (Veh), φ P<0.05, φφ P<0.01, φφφ P<0.001 cf. (Female) Normoxic (Trep), Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ_{max}/RQ_{min}. RQ= relative quantification. Veh= vehicle, Trep= treprostinil.
Figure 3-32 Lung protein expression of BMPR-II signalling was not altered under normoxic conditions in female and male rats

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Expression of (A) BMPR-II, (B) pSmad 1/5/9, (C) Id1 and (D) Id3 were assessed from whole lung homogenates by western blot. (E) Representative immunoblot, β-actin was used as the internal loading control. N=5-6 per group in duplicate, Two-way ANOVA with Bonferroni's post-hoc test. Data expressed as mean ± SEM. Veh= vehicle, Trep= treprostinil.
Figure 3-33 Lung protein expression of BMPR-II signalling was altered by chronic hypoxia: Id1 expression increased in chronic hypoxic female and male rats

Female and male sprague-dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Expression of (A) BMPR-II, (B) pSmad 1/5/9, (C) Id1 and (D) Id3 were assessed from whole lung homogenates by western blot. (E) Representative immunoblot, β-actin was used as the internal loading control. N=5-6 per group in duplicate. *P<0.05, **P<0.01 cf. (Female) Normoxic (Veh), † P<0.05 cf. (Male) Normoxic (Veh), Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Veh= vehicle, Trep= treprostinil.
Chapter Three

Figure 3-34 Lung protein expression of BMPR-II signalling was unchanged with treatment with prostacyclin analogue treprostinil

Female and male Sprague-Dawley rats were subjected to normoxic/hypoxic conditions (1000/550mbar) for two weeks, a sub-cutaneous pellet containing treprostinil (400ng/kg/min) or vehicle was implanted and rats returned to normoxic/hypoxic conditions for a further two weeks. Expression of (A) BMPR-II, (B) pSmad 1/5/9, (C) Id1 and (D) Id3 were assessed from whole lung homogenates by western blot. (E) Representative immunoblot, β-actin was used as the internal loading control. N=3-6 per group in duplicate, (BMPR-II blot n=3 per group). Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Veh= vehicle, Trep= treprostinil.
3.3. Discussion

A primary focus of PAH research has been to understand the gender disparity that exists in the incidence of the disease. Female gender has been identified as a risk factor for PAH, studies demonstrate a female to male ratio of 4.3:1 with total PAH (Walker et al., 2006) and 4.1:1 in IPAH patients (Badesch et al., 2010). Despite an increased incidence of PAH in females, males have poorer survival despite treatment (Humbert et al., 2010b, Benza et al., 2010). This was the first study to examine the role of sex on the therapeutic action of treprostinil in the chronic hypoxic rat model of PH.

The findings suggest that sex may influence the actions of treprostinil. This study demonstrated that female chronic hypoxic rats are more responsive to 100ng/kg/min of treprostinil than chronic hypoxic male rats. The sex-specific therapeutic response appeared to be partially mediated through an increase in Id1 and Id3 mRNA expression. A higher treprostinil dose of 400ng/kg/min led to a therapeutic response in male hypoxic rats, but also an increased response in chronic hypoxic female rats. The higher dose of treprostinil did not alter BMPR-II signalling in female or male rats. However, in females exposed to hypoxia it triggered an increase in the expression of prostaglandin E2 receptor 2 (EP2) mRNA.

Initially, the viability of treprostinil administered through a subcutaneously implanted slow-release pellet as a method for dosing a chronic hypoxic model of PH was investigated. Initially we investigated the level of treprostinil in the blood plasma of female and male rats under normoxic conditions. Both females and males had small but significant decreases in treprostinil plasma levels after 10 days compared 4 days. Male rats maintained a significant lower treprostinil plasma levels after 14 days whereas females did not. The very low levels of treprostinil may explain the significant changes observed between different days. Previous studies have found that treprostinil is not at detectable levels in rat plasma after 4 hours, suggesting a rapid clearance rate (Malinin et al., 2014). The effectiveness of subcutaneously implanted pellets has been previously demonstrated in two different PH models and cardiovascular knock-out models (Wright et al., 2015, Karas et al., 2001, Frump et al., 2015b).

The chronic hypoxic rat model of PH demonstrates increased PH indices, for example; increased RVSP, remodelling including smooth muscle proliferation and RV hypertrophy (Meyrick and Reid, 1980, Meyrick and Perkett, 1989). These changes were evident in the present study. Treprostinil is a prostacyclin analogue that has similar vasodilatory properties to prostacyclin (Vachiery and Naeije, 2004) as well as anti-proliferative actions (Clapp et al., 2002). Previously, treprostinil was found to decrease both RVSP and RVH in the monocrotaline rat model of PH (van Albada et al., 2006, Yang et al., 2010). Therapeutic effects of treprostinil have also been examined in the chronic hypoxic mouse
model of PH (Nikam et al., 2010, Liu et al., 2015). The present study used a reversal protocol, rats were dosed with treprostinil after developing hypoxic induced PH, then were restored to hypoxic conditions. This approach was deemed more relevant to the clinical disease; however, remodelling is not as severe. However, previous studies used either a preventative protocol, in which animals were dosed before being placed in hypoxic conditions (Nikam et al., 2010) or did not restore mice to a hypoxic environment after dosing with treprostinil (Liu et al., 2015). All the treprostinil studies involve the use of male rodents exclusively, therefore offer little insight into PAH therapy in a disease that predominately effects women.

This study is the first to demonstrate a female-specific reduction in PH indices by treprostinil. Several previous studies have demonstrated female-specific therapies in animal models of PH; including targeting estrogen (Wright et al., 2015, Mair et al., 2014) and serotonin pathways (White et al., 2012, Dempsie et al., 2013, Wallace et al., 2015). As treprostinil is a prostacyclin analogue, it can act through the G-protein (G_s) coupled prostaglandin receptors (GPCRs); prostacyclin (IP) receptor, prostaglandin E2 receptor 2 (EP_2) and prostaglandin D2 receptor 1 (DP_1) (Falcetti et al., 2010, Li et al., 2012, Whittle et al., 2012). Other prostacyclin analogues have been shown to act through more than one receptor; iloprost can mediate its therapeutic effects through the prostaglandin E2 receptor 4 (EP_4) (Lai et al., 2008, Whittle et al., 2012). IP receptor knock-out studies have demonstrated that mice with IP receptor knock-out have a much more severe PH phenotype after chronic hypoxic exposure than wildtype animals (Hoshikawa et al., 2001). Previously, has been shown that the IP receptor is down-regulated in the monocrotaline model of PH (Lai et al., 2008). Although a beraprost dosing study carried out in chronic hypoxic rats demonstrated no downregulation of IP mRNA expression in the lungs (Abe et al., 2001). In the present study, hypoxic conditions did not change the expression of the prostaglandin receptors suggesting that different models of PH can have differing effects on the expression of the prostaglandin receptors. Interestingly, the lower treprostinil dose led to an increase in female EP_2 mRNA expression compared to the untreated female hypoxic rats. Also, the higher dose of treprostinil combined with hypoxia increased the mRNA expression of the EP_2 receptor exclusively in female hypoxic rats. This may offer a possible explanation for the greater response to treprostinil in females.

Despite highlighting increases in the expression of the EP_2 receptor, this does not offer a complete picture as to why female rats responded better to treprostinil. The mRNA expression of the prostaglandin receptors was obtained from whole lung lysates from the rats, however the specific distribution of the receptors was not investigated. Endothelin receptors have been shown to have different distribution in different sections of the rat pulmonary artery; ET_A receptors were primarily found in the media of the pulmonary artery. Whereas the ET_B receptor was found in the intima and media of pulmonary arteries (Soma
et al., 1999). The distribution of the receptors influences the vasoconstrictive response to ET-1, where the ET\textsubscript{A} was responsible for vasoconstriction in the proximal artery, however both ET\textsubscript{A} and ET\textsubscript{B} mediate this response in distal arteries (Soma et al., 1999). This highlights that receptor expression and function can vary depending on location in the pulmonary artery. A possible explanation of the weaker response to treprostinil may involve receptor desensitization. Morrell and colleagues discovered that in rat hPASMCs, stimulation of the IP receptor by a PGI\textsubscript{2} analogue, cicaprost, led to desensitization of the receptor (Sobolewski et al., 2004). The desensitization of the receptor was dependant on protein kinase A (PKA) inhibiting the enzyme adenyl cyclase (AC) 5/6 (Sobolewski et al., 2004). AC is the key enzyme in converting ATP to cAMP therefore any changes in functionality or expression could impact the effectiveness of a cAMP increasing drug like treprostinil. In isolated rat PASMCs, AC2, AC3, AC5 and AC6 were the predominate isoforms of AC detected by RT-PCR and western blots (Jourdan et al., 2001). Sex differences in AC expression have been investigated in isolated mice hearts, where a forskolin, an inducer of cAMP, had a greater contractile effect in male mice than in females (McIntosh et al., 2011). Exchange factor directly activated by cAMP 1/2 (EPAC) are downstream mediators of cAMP that have been shown to influence the effectiveness of treprostinil in fibroblasts (Nikam et al., 2011). EPAC-1 mRNA and protein expression were also found to be downregulated in MCT-rats (Murray et al., 2009). Although it was not examined in the current study it may be possible that any reduced EPAC expression in the current study may influence the effectiveness of treprostinil in male rats. Another potential mechanism that could account for the weaker response to treprostinil in the male rats could be changes in phosphodiesterases (PDEs), which break down cAMP. Previously, Wang and colleagues discovered that sex plays a role in the expression of PDEs in rat vascular endothelial cells. mRNA levels of PDE1A were found to be higher in males than females (Wang et al., 2010a). Interestingly, they also discovered that females had higher mRNA expression of PDE3B (Wang et al., 2010a).

Dysfunctional BMPR-II signalling plays a key role in endothelial/smooth muscle cell proliferation and apoptosis (Machado et al., 2009a). BMPR-II dysfunction mediates proliferation by lowering the induction of Id proteins, which are regulators of cell cycle. (Yang et al., 2005). BMPR-II expression in the lungs of female and male rats have been found to be decreased in 3-week hypoxic, monocrotaline and SU-HX rat models of PH (Takahashi et al., 2006, Long et al., 2009, Mair et al., 2014). In this present study, there was no observable decrease in mRNA or protein expression of BMPR-II under 4-week hypoxic conditions. This may suggest an eventual recovery of BMPR-II expression under hypoxic conditions. Presently, pSmad 1/5/9 was found to increase in the second hypoxic study, this may also suggest a recovery of BMPR-II signalling. Sex has been proven to influence BMPR-II signalling both in animal models of PH and human patients. BMPR-II
signalling expression was recently found to be lower in females compared to males in rodent lungs and in human pulmonary artery smooth muscle cells (hPASMCs) (Mair et al., 2014, Mair et al., 2015). Treprostinil can activate BMPR-II signalling, specifically Id1, in human and animals PH models (Yang et al., 2010). This is the first study to demonstrate that treprostinil can induce expression of Id3 mRNA. The female-specific therapeutic effect of 100ng/kg/min treprostinil was partially mediated through increases in both Id1 and Id3 mRNA. Interestingly, the present study does not correlate with other research which suggests Id down-regulation under hypoxic conditions (Xiao-Hui et al., 2012). The effect of estrogen in animal models of PH has been disputed; studies suggest that the addition of exogenous estrogens can reverse established PH in animal models (Resta et al., 2001, Lahm et al., 2012, Farhat et al., 1993). However, others find that endogenous estrogen is a risk factor contributing to greater incidence of PH in female animal models (White et al., 2011a, Dempsie et al., 2011, Wright et al., 2015, White et al., 2012). In human breast cancer studies, 17-β estradiol increased the levels of cAMP in a dose dependent manner via the estrogen alpha (ER-α) receptor, suggesting a negative feedback effect (Aronica et al., 1994, Zivadinovic et al., 2005). This was also observed in rat pulmonary vascular smooth muscle cells (Farhat et al., 1996). Estrogen was also found to attenuate the activation of MAPK signalling via stimulation of adenyl cyclase and cAMP through ER-α and GPR30 receptors in MCF-7 breast cancer cells (Filardo et al., 2002). Treprostinil and other prostacyclin analogues are known to increase the expression of Id proteins via activation of cyclic AMP (cAMP) (Yang et al., 2010). The cumulative effect of estrogen and treprostinil activating adenyl cyclase and cAMP may explain why, in the present study there was a greater induction of Id1 and Id3 mRNA in female rats. Interestingly, the increase in Id mRNA expression was not supported by increases in protein expression. However, in the second hypoxic study we observed increases in Id1 and Id3 mRNA in male rats under hypoxic conditions and an increase in Id1 protein expression in female and male hypoxic rats. This may be a compensatory response to hypoxic conditions. Lowery and colleagues have demonstrated that in hypoxic mice both Id1 and Id3 expression was increased and may play a role in regulating responses to chronic hypoxia (Lowery et al., 2010). SMURF1 is a E3 ubiquitin protein ligase, which suppress BMP signalling by degrading SMAD proteins (Fukunaga et al., 2008). Interestingly, the mRNA expression of the E3 ubiquitin protein ligase SMURF1 was found to be increased in female hypoxic rats treated with treprostinil. Previously, SMURF1 was shown to be increased in the pulmonary arteries of hypoxic and monocrotaline rat models of PH (Murakami et al., 2010). Also, the deletion of SMURF1 has been demonstrated to protect mice from PH development (Rothman et al., 2016). The main roles of the KCNK3 channel is to control cellular resting membrane potential and to contribute to the relaxation of vessels (Patel et al., 1999) The role of missense variants of the KCNK3 gene in PAH development have recently been identified, these variants lead to reduced potassium channel current thereby
contributing to PAH development (Ma et al., 2013). Presently, both studies demonstrate increases in KCNK3 mRNA under hypoxic conditions, although the first study only showed a significant increase in female hypoxic rats. Conversely, it was recently shown that both PAH patient and monocrotaline rat lungs have reduced expression of KCNK3 (Antigny et al., 2016). We did not observe treprostinil altering the expression of KCNK3, however treprostinil has been previously shown to activate KCNK3 via cAMP dependent phosphorylation to mediate vasodilatory effects in hPASMCs (Olschewski et al., 2006a).

Over the two in-vivo studies, both prostaglandin receptors and the BMPR-II signalling pathway were examined by processing whole lung tissue isolated from female and male rats. Although this technique is often used due to experimental limitations or time constraints, it may portray changes in gene expression that are not reflecting in more physiologically relevant areas like pulmonary arteries. This has been reflected in lung airway studies, these demonstrated the differences in specific gene expression (CYP2F4) from whole lung, to proximal then to distal airways (Baker et al., 2004). This was also highlighted was the differing expression of ET receptors from proximal to distal pulmonary arteries (Soma et al., 1999). For future studies, pulmonary arteries could be isolated from untreated/treated animals, however the level of protein or RNA isolated would be significantly lower than from whole lung. Alternatively, immunohistochemistry (IHC) could confirm if changes to the BMPR-II pathway or prostaglandin receptors are localised to pulmonary arteries and more specifically to smooth muscle cells or endothelial cells.

It is possible that the level of remodelling observed in the hypoxic rats played a role in leading to the sex difference observed in response to treprostinil. In the current in-vivo studies hypoxic male rats have higher pressures than female rats, this was in line with previously published research (Rabinovitch et al., 1981). It may be possible that the weaker response to treprostinil in males may be due to the greater PH severity. Clinical studies involving the use of treprostinil do not split the end-points into female and male data therefore it is difficult to conclude if what was seen in the hypoxic rats can be extrapolated into humans.

Recently it has been hypothesised that better right ventricular compensation is a reason why female PAH patients have greater survival than males (Jacobs et al., 2014). Females were also found to have better improvements in right ventricular ejection fraction (RVEF) after PAH therapy (Jacobs et al., 2014). In the present study, RVEF was found significantly decreased in both studies under hypoxic conditions, although, females exhibited small but insignificant increases in RVEF with treprostinil (100ng/kg/min) treatment. In accordance with RVSP and remodelling analysis, there was a female-specific reversal of hypoxic-induced RVH by treprostinil at 100ng/kg/min. This effect appeared to be dose-dependent as 400ng/kg/min treprostinil dosing reduced RVH in male rats. Previously treprostinil was
found to reduce RVH in a monocrotaline rat model of PH (Yang et al., 2010). However, it was not determined if the reduction in hypertrophy was an indirect result of the therapeutic effects of treprostinil in the lungs or direct action in the right ventricle. However, treprostinil was found to have no significant therapeutic effect on a pulmonary trunk banding rat model of PH, which induces pronounced right ventricular hypertrophy and near heart failure (Axelgaard et al., 2016). Both treprostinil and MRE-269 were found to improve RV function in isolated healthy rat heart, however both drugs had no effect on hearts that had been subjected to pulmonary trunk banding (Holmboe et al., 2016).

In conclusion, the use of sub-cutaneous pellets to release treprostinil was a valid method of drug delivery in the chronic hypoxic rat model of PH. Firstly, the data indicates a novel observation that treprostinil can have sex-specific therapeutic effects in a chronic hypoxic model of PH. The results also suggest that the sex-specific effects were dose dependant, with a greater dose of treprostinil leading to greater therapeutic effects in both sexes of hypoxic rats.
Chapter 4

4 The influence of prostaglandin receptors, BMPR-II signalling and sex on the in-vitro effects of treprostinil
4.1. Introduction

Pulmonary arterial hypertension (PAH) is associated with reduced levels of vasodilators, such as prostacyclin and increased levels of vasoconstrictors such as endothelin and thromboxane (Tuder et al., 1999, Christman et al., 1992, Giaid et al., 1993). The ability to manipulate these pathways has been a basis of therapy for PAH. Originally prostacyclin was investigated for its effects in peripheral vascular disease (Hossmann et al., 1984). This led to the investigation into the effectiveness of prostacyclin (epoprostenol) as a therapy for PAH (Barst et al., 1996). Despite clinical outcomes improving, the short half-life of epoprostenol and associated side effects of a constant catheter infusion of epoprostenol led to the development of prostacyclin analogues. Prostacyclin analogues such as treprostinil and iloprost were developed as alternatives to epoprostenol therapy. Prostacyclin analogues offered a variety of dosing methods including inhalation and oral dosing, they also provided greater durations of vasodilation and better stability at room temperature (Fitscha et al., 1987).

The major signalling mechanism of treprostinil and other prostacyclin analogues is via activation of G-protein coupled prostaglandin receptors. The prostacyclin (IP), the prostaglandin E2 receptor 2 (EP2) and prostaglandin D2 receptor 1 (DP1) are receptors that treprostinil binds to with the greatest affinity (Whittle et al., 2012). These receptors are associated with the Gs subunit and increase cAMP levels in smooth muscle cells (Breyer et al., 2001). Additionally, the anti-proliferative effects of treprostinil have been examined in the idiopathic patient subset of PAH (IPAH). It has also been shown that treprostinil mediates its effects via the IP receptor and peroxisome proliferator-activated receptor gamma (PPAR-γ) (Falcetti et al., 2010). It has been well established that reduced expression of the BMPR-II signalling pathway has a deleterious role in the development of PAH (Atkinson et al., 2002). Treprostinil has been found to play a role in activating BMPR-II signalling in both BMPR-II mutants and non-mutant human pulmonary artery smooth muscle cells (hPASMCs), either dependent or independent of SMAD activation (Yang et al., 2010).

This was the first study to examine if sex could influence the effects of treprostinil in an in-vitro setting. As stated previously (chapter 3) the influence of sex on drug therapy has already been demonstrated (Mathai et al., 2015, Gabler et al., 2012). However, by examining the underlying mechanisms of treprostinil in hPASMCs we hoped to gain more clinically relevant observations than those found in animal models of PH.

Treprostinil and prostaglandin receptor antagonist concentrations were chosen due to previously published research (Falcetti et al., 2007, Falcetti et al., 2010, Patel et al., 2014, Jigisha et al., 2015). The data demonstrates that in control hPASMCs, female cells have a greater response to treprostinil stimulation than male cells. Interestingly, the pro-
proliferative vasoconstrictor endothelin-1 (ET-1) was found to significantly increase the anti-proliferative effect of treprostinil in female control hPASMCs. The addition a dual endothelin receptor antagonist (SB-217242) blunted the anti-proliferative effect of treprostinil in combination with ET-1. The IP receptor antagonist (RO1138452) partially inhibited the anti-proliferative effects of treprostinil in the presence of ET-1 in female control hPASMCs. In PAH patient hPASMCs, the addition of the EP<sub>2</sub> receptor antagonist (PF-04418948) partly blocked the anti-proliferative effects of treprostinil. Treprostinil was found to increase the Id mRNA and proteins of the BMPR-II signalling pathway in hPASMCs. Female control hPASMCs were consistently found to have higher induction of the Id mRNA and protein at two different time-points compared to male control hPASMCs. Female PAH patients hPASMCs, were found to have a slightly higher induction of the Id3 protein compared to male PAH patient hPASMCs after 24-hour stimulation with treprostinil. The induction of Id proteins in female control and PAH patient hPASMCs was found to be partially dependant on the activation of the IP and EP<sub>2</sub> prostaglandin receptors suggesting a role for cAMP in the activation of the Id proteins under these experiment conditions.

Hypotheses and aims of this chapter:

Sex has been shown to influence the clinical outcomes of patients receiving ERAs and PDE-5 inhibitors (Gabler et al., 2012, Mathai et al., 2015). We hypothesise that the PGI<sub>2</sub> analogue treprostinil can have sex specific effects in isolated hPASMCs. To investigate this hypothesis, the following aim was carried out.

1. Characterisation of the influence of sex on treprostinil in human pulmonary artery smooth muscle cells

BMPR-II signalling has been previously demonstrated to mediate the effect of prostacyclin analogues and other PAH therapies (Yang et al., 2010, Yang et al., 2013a). Also, sex differences have been identified in BMPR-II signalling in hPASMCs (Mair et al., 2015). The hypothesis is that increased BMPR-II signalling mediated by treprostinil accounted for the differences in anti-proliferative properties of treprostinil. To investigate this hypothesis, the following aim was carried out.

2. Investigation into BMPR-II signalling role in the effects of treprostinil in human pulmonary artery smooth muscle cells
4.2. Results

4.2.1. The anti-proliferative effects of treprostinil in the presence of endothelin-1 in control and PAH patient hPASMCs

Treprostinil is an established clinical treatment for PAH. As a prostacyclin analogue, its effects are mediated though vasodilation of pulmonary arteries and proliferation of hPASMCs. The effect of treprostinil on the proliferation of hPASMCs derived from women and men were examined as was the influence of endothelin-1 on the anti-proliferative effects of treprostinil. These results were determined by cell counts via a haemocytometer after 72-hour stimulation with treprostinil. Treprostinil (100nM, 1µM and 10µM) significantly decreased serum induced proliferation in female control hPASMCs (Figure 4-1A). The addition of 30nM endothelin-1, at the beginning of the experiment, increased the anti-proliferative effect of treprostinil. This was exhibited by significant decreases in proliferation induced by treprostinil at 10nM, 100nM, 1µM and 10µM (Figure 4-1B). In male control hPASMCs, treprostinil (10µM) significantly reversed serum-induced proliferation (Figure 4-1C). The addition of endothelin-1 did not significantly increase the anti-proliferative effect at any additional treprostinil concentrations (Figure 4-1D). The anti-proliferative effects of treprostinil were also explored in female and male PAH patient hPASMCs. Treatments with treprostinil (1µM and 10µM) significantly reduced proliferation in female patient hPASMCs (Figure 4-2A). The addition of endothelin-1 led to a significant reduction in proliferation after 10nM and 100nM stimulation with treprostinil (Figure 4-2B). Serum-induced proliferation was significantly reduced in male patient hPASMCs by treprostinil (10µM) (Figure 4-2C). The addition of endothelin-1 led to a significant reduction in proliferation with treprostinil (1µM) (Figure 4-2D). The combination of treprostinil and endothelin-1 was investigated across a greater range of concentrations of treprostinil. Each hPASMCs group was stimulated with treprostinil ranging from 1pM-10µM. The greatest effect of endothelin-1 was observed in female control hPASMCs (Figure 4-3A). Endothelin-1 was found to significantly increase the anti-proliferative effect of treprostinil, specifically at 100pM (Figure 4-3A). As before, there was no significant effect of endothelin-1 in male control hPASMCs, however the overall anti-proliferative effect of treprostinil was lower in male control hPASMCs compared to female controls (Figure 4-3B). Endothelin-1 did not significantly increase the anti-proliferative effect of treprostinil in female and male PAH patient hPASMCs (Figure 4-4A-B6). Area under the curve (AUC) analysis was used as an overall measure of the anti-proliferative response of treprostinil. The presence of endothelin-1 significantly reduced the AUC compared to treprostinil without endothelin-1 in female control hPASMCs (Figure 4-5A). Endothelin-1 did not significantly change the AUC in either male control or female and male PAH patient hPASMCs (Figure 4-5B-D). This data suggests that treprostinil has anti-proliferative effects in all hPASMCs groups, however differences in anti-proliferation can be observed between female and male control hPASMCs.
Figure 4-1 The anti-proliferative effect of treprostinil is increased by the addition of 30nM endothelin-1 in female but not male control hPASMCs

HPASMCs from (A, B) female and (C, D) male control patients were quiesced for 24 hours in 0.5% FBS phenol free DMEM media. (A, C) PASMCs were then stimulated by treprostinil (10nM, 100nM, 1µM and 10µM) in the presence of 7% FBS. (B, D) Identical experiments carried out in the presence of endothelin-1 (30nM). Cell counts were assessed 72 hours later by haemocytometer. N=3 human samples per group, in duplicate/triplicate, **P<0.01, ***P<0.001 cf. 0.5% FBS; ϕ P<0.05, ϕϕ P<0.01, ϕϕϕ P<0.001 cf. 7% FBS; ξξ P<0.01, ξξξ P<0.001 cf. 7% FBS + Endothelin-1 (30nM). One-way ANOVA with Bonferroni’s post-hoc test. Data expressed as % of 7% FBS.
Figure 4-2 The anti-proliferative effect of treprostinil is increased by the addition of 30nM endothelin-1 in female and male patient hPASMCs

HPASMCs from (A, B) female and (C, D) male PAH patients were quiesced for 24 hours in 0.5% FBS phenol free DMEM media. (A, C) PASMCs were then stimulated by treprostinil (10nM, 100nM, 1µM and 10µM) in the presence of 7% FBS. (B, D) Identical experiments carried out in the presence of endothelin-1 (30nM). Cell counts were assessed 72 hours later by haemocytometer. N=2-3 human samples per group, in duplicate/triplicate (N=2 male patient hPASMCs), **P<0.01, ***P<0.001 cf. 0.5% FBS; ¤ P<0.05, ¤¤ P<0.01, ¤¤¤ P<0.001 cf. 7% FBS; ¥ P<0.05, ¥¥ P<0.01, ¥¥¥ P<0.001 cf. 7% FBS + Endothelin-1 (30nM). One-way ANOVA with Bonferroni’s post-hoc test. Data expressed as % of 7% FBS.
Figure 4-3 The anti-proliferative effect of treprostinil is significantly increased by the addition of 30nM endothelin-1 in female control hPASMCs not in male control hPASMCs

HPASMCs from (A) female controls and (B) male controls were quiesced for 24 hours in 0.5% FBS phenol free DMEM media. PASMCs were then stimulated by treprostinil (1pM, 10pM, 100pM, 1nM, 10nM, 100nM, 1µM and 10µM) in the presence or absence of endothelin-1 (30nM). Experiments were carried out in the presence of 7% FBS phenol free DMEM media. Cell counts were assessed 72 hours later by haemocytometer. N=4-5 human samples per group, in duplicate/triplicate, **P<0.01 cf. Trep, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as % of 7% control. Trep= treprostinil, ET-1= endothelin-1.
Figure 4-4 The anti-proliferative effect of treprostinil is not significantly increased by the addition of 30nM endothelin-1 in female and male patient hPASMCs

HPASMCs from (A) female and (B) male PAH patients were quiesced for 24 hours in 0.5% FBS phenol free DMEM media. PASMCs were then stimulated by treprostinil (1pM, 10pM, 100pM, 1nM, 10nM, 100nM, 1µM and 10µM) in the presence or absence of endothelin-1 (30nM). Experiments were carried out in the presence of 7% FBS phenol free DMEM media. Cell counts were assessed 72 hours later by haemocytometer. N=3-4 human samples per group, in duplicate/triplicate, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as % of 7% control. Trep= treprostinil, ET-1 = endothelin-1.
Figure 4-5 The area under the curve of treprostinil is significantly decreased by the addition of 30nM endothelin-1 in female control hPASMCs

HPASMCs from (A) female control, (B) male control, (C) female PAH patient and (D) male PAH patients were quiesced for 24 hours in 0.5% FBS phenol free DMEM media. PASMCs were then stimulated by treprostinil (1pM, 10pM, 100pM, 1nM, 10nM, 100nM, 1µM and 10µM) in the presence or absence of endothelin-1 (30nM). Experiments were carried out in the presence of 7% FBS phenol free DMEM media. N=3-5 human samples per group, *P<0.05 cf. Trep, un-paired t-test. Data expressed as mean ± SEM. Trep = treprostinil, ET-1 = endothelin-1.
4.2.2. The enhanced treprostinil response in combination with endothelin-1 may be partially dependent on the ET<sub>A,B</sub> receptors

To investigate the greater treprostinil response in combination with ET-1 occurs, the role of the endothelin receptors was examined. Expression of endothelin receptors (ET) in hPASMCs were examined by both Taqman® analysis (mRNA) and Western blot (protein) respectively. There were no significant differences in endothelin receptor mRNA expression between sex and control/PAH hPASMCs (Figure 4-6A-B). Interestingly, prepro-ET-1 mRNA expression was significantly lower in female control hPASMCs compared to male control and male patient hPASMCs (Figure 4-6C). However, ET<sub>A</sub> mRNA expression was found to be significantly higher than ET<sub>B</sub> expression in female/male control hPASMCs (Figure 4-7A) and in female PAH patient hPASMCs (Figure 4-7B) but not in male PAH patient hPASMCs (Figure 4-7B). Endothelin receptor protein expression did not change between females and males or control/PAH patient hPASMC group (Figure 4-8A-B). The lack of significant change may be partially due to the high variability in the expression of the endothelin receptors, specifically the ET<sub>B</sub> receptor in individual hPASMCs examined (Figure 4-9C-D). The addition of a specific ET<sub>A</sub> antagonist sitaxentan sodium did not significant change the anti-proliferative effect of treprostinil combined with ET-1 (Figure 4-10A). The dual endothelin receptor antagonist SB-217242 significantly reduced the anti-proliferative effect of treprostinil, at 100pM, combined with ET-1 (Figure 4-10B). Sitaxentan sodium and did not significantly change the AUC (Figure 4-11A), however the addition of SB-2172424 did significantly change the AUC (Figure 4-11B). The data also suggests that the additive effects of endothelin-1 on treprostinil may be mediated by ET<sub>A</sub> and ET<sub>B</sub> receptors.
Figure 4-6 Endothelin receptors mRNA and in female and male hPASMC

MRNA expression of (A) ET\(_A\), (B) ET\(_B\) and (C) Prepro-ET-1 were assessed in female/male control and PAH patient hPASMCs by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=3-4 per group in triplicate for Taqman®. *P<0.05, **P<0.01 cf. Female Control, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ\(_{\text{max}}\)/RQ\(_{\text{min}}\) and mean ± SEM. RQ= relative quantification.
Figure 4-7 Comparison of endothelin receptor mRNA expression in female and male hPASMC

MRNA expression of ET\textsubscript{A} and ET\textsubscript{B} receptors in (A) female/male control hPASMCs and (B) female/male PAH patient hPASMCs by Taqman® quantitative Real Time-PCR. Results were normalised to \(\beta\)-2-microglobulin. N=3-4 per group in triplicate for Taqman®. *\(P<0.05\), ***\(P<0.001\) cf. Female ET\textsubscript{A}, \(\phi\phi\) \(P<0.01\) cf. Male ET\textsubscript{A}, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ \(\pm\) RQ\textsuperscript{max}/RQ\textsuperscript{min} and mean \(\pm\) SEM. RQ= relative quantification.
Figure 4-8 Endothelin receptors protein expression in female and male hPASMC

Protein expression of (A) ET\textsubscript{A} (A) and (B) ET\textsubscript{B} were assessed in female/male control and PAH patient hPASMCs by western blot. Representative immunoblot (E). Results were normalised to α-tubulin was used as the internal loading control. N=3 per group, Two-way ANOVA with Bonferroni’s post-hoc test. ET\textsubscript{A} = Endothelin receptor A, ET\textsubscript{B} = Endothelin receptor B.
Figure 4-9 Endothelin receptor non-pulled protein expression in female and male hPASMC
Protein expression of ET$_A$ and ET$_B$ were assessed in female/male control and PAH patient hPASMCs by western blot. Results were normalised to α-tubulin was used as the internal loading control. N=3-4 per group. Data expressed as individual values. ET$_A$ = Endothelin receptor A, ET$_B$ = Endothelin receptor B.
Figure 4-10 Treprostinil in combination with endothelin-1: effect of an ET\textsubscript{A} and ET\textsubscript{A,B} receptor antagonist

HPASMCs from female controls were quiesced for 24 hours in 0.5% FBS phenol free DMEM media. PASMCs were pre-incubated with (A) endothelin-A receptor (ET\textsubscript{A}) antagonist sitaxentan sodium (1\mu M) or a (B) dual endothelin receptor (ET\textsubscript{B}) antagonist SB-217242 (1\mu M) for 30 minutes prior to the addition treprostinil (1pM, 10pM, 100pM, 1nM, 10nM, 100nM, 1\mu M and 10\mu M) in the presence or absence of endothelin-1 (30nM). Experiments were carried out in the presence of 7% FBS phenol free DMEM media. Cell counts were assessed 72 hours later by haemocytometer. N=3-4 human samples per group, in duplicate/triplicate, \#P<0.01 cf. Trep + ET-1, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as % of 7% control. Sita Ant= sitaxentan sodium, SB Ant= SB-217242, Trep= treprostinil, ET-1= endothelin-1, ET\textsubscript{A} = Endothelin receptor A, ET\textsubscript{B} = Endothelin receptor B.
Figure 4-11 The area under the curve is changed by the addition of an ET$_{A+B}$ antagonist in female control hPASMCs

HPASMCs from female controls were quiesced for 24 hours in 0.5% FBS phenol free DMEM media. PASMCs were pre-incubated with either an ET$_A$ receptor antagonist sitaxentan sodium (1µM) (A) or an ET$_{A,B}$ receptor antagonist SB-217242 (1µM) (B) for 30 minutes prior to the addition treprostinil (1pM, 10pM, 100pM, 1nM, 10nM, 100nM, 1µM and 10µM) in the presence or absence of endothelin-1 (30nM). Experiments were carried out in the presence of 7% FBS phenol free DMEM media. N=3-4 per group, *P<0.05 cf. Trep + ET-1, un-paired t-test. Data expressed as mean ± SEM. Sita Ant= sitaxentan sodium, SB Ant= SB-217242, Trep= treprostinil, ET-1= endothelin-1, ET$_A$= Endothelin receptor A, ET$_B$= Endothelin receptor B.
4.2.3. Prostaglandin receptors mRNA and protein in control and PAH patient hPASMCs

Treprostinil is prostacyclin analogue that is known to act through multiple prostaglandin receptors, primarily treprostinil binds with the highest affinity to the DP₁, IP and EP₂ receptors respectively (Whittle et al., 2012). It is currently unknown if expression of these receptors differs between sexes therefore the expression of the prostaglandin receptors was measured by Taqman® analysis and Western blot. In hPASMCs from women and men, mRNA expression of the IP, EP₁, EP₂ and DP₁ receptors did not significantly change between sexes or control and PAH groups (Figure 4-12A-D). Interestingly, the mRNA expression of the EP₂ receptor was found to be significantly higher in both control and PAH patient hPASMCs groups than IP, EP₁ and DP₁ expression (Figure 4-13A-B). Also, the mRNA expression of the DP₁ receptor was found to be significantly lower than the other prostaglandin receptors in all hPASMCs groups (Figure 4-13A-B). Comparing IP, EP₂ and DP₁ mRNA expression to whole lung homogenates from the Chapter 3 second hypoxic study led to different patterns between hPASMCs and rats (Figure 4-14-Figure 4-15). The main difference from comparing control hPASMC to normoxic rats was that EP₂ mRNA expression is much higher than IP mRNA in hPASMCs, whereas the opposite occurs in rats (Figure 4-14A&B). The same pattern is clear in the comparison of patient hPASMCs with hypoxic rats (Figure 4-15A&B). Interestingly, the DP₁ was identified as the lowest expressing receptor in both hPASMCs and whole rat lung (Figure 4-14-Figure 4-15). The level of IP receptor protein was not reduced in the PAH patient hPASMCs examined as has been previously reported by Falcetti and colleagues (Falcetti et al., 2010) (Figure 4-16A). Protein expression of the EP₂ and DP₁ receptors were also found not to change across sex and hPASMCs groups (Figure 4-16B-C). To account for any variability within each hPASMC group, individual densitometric values for each prostaglandin receptor was plotted (Figure 4-17-Figure 4-18).
Figure 4-12 Expression of prostaglandin receptors mRNA under basal conditions in control and PAH hPASMCs
Expression of (A) IP, (B) EP₁, (C) EP₂ and (D) DP₁ mRNA were assessed from female and male control and PAH patient hPASMCs by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=3-4 per group in triplicate, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ_{max}/RQ_{min}. RQ= relative quantification.
Figure 4-13 Differential expression of prostaglandin receptors mRNA under basal conditions in control and PAH hPASMCs

Expression of IP, EP₁, EP₂ and DP₁ mRNA were assessed from (A) female and male control and (B) female and male PAH patient hPASMCs by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=3-4 per group in triplicate. ***P<0.001 cf. IP (Female), ****P<0.001 cf. EP₁ (Female), ⦼⦼⦼ P<0.001 cf. EP₂ (Female), ⦼⦼⦼ P<0.001 cf. IP (Male), ⦼⦼⦼⦼ P<0.001 cf. EP₁ (Male), # # P<0.01 cf. EP₂ (Male), Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ_{max} \div RQ_{min}. RQ= relative quantification.
Figure 4-14 Comparison of prostaglandin receptors mRNA under basal conditions in control hPASMCs with vehicle dosed normoxic rats

Expression of IP, EP$_2$ and DP$_1$ mRNA were assessed from (A) female control hPASMCs and female normoxic (Veh) rats and (B) male control hPASMCs and female normoxic (Veh) rats by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=3-6 per group in triplicate. Data expressed as RQ ± RQ$^{\text{max}}$/RQ$^{\text{min}}$. RQ= relative quantification. Veh= Vehicle dosed.
Figure 4-15 Comparison of prostaglandin receptors mRNA under basal conditions in PAH patient hPASMCs with vehicle dosed hypoxic rats

Expression of IP, EP_2 and DP_1 mRNA were assessed from (A) female PAH patient hPASMCs and female hypoxic (Veh) rats and (B) male PAH patient hPASMCs and female normoxic (Veh) rats by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=3-6 per group in triplicate. Data expressed as RQ ± RQ^{max}/RQ^{min}. RQ= relative quantification. Veh= Vehicle dosed.
Figure 4-16 Expression of prostaglandin receptors protein under basal conditions in control and PAH hPASMCs

Expression of (A) IP, (B) EP₂ and (C) DP₁ were assessed from female/male control and PAH patient hPASMCs homogenates by western blot. (D) Representative immunoblot (D), α-tubulin was used as the internal loading control. N=3-4 in duplicate. Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM.
Figure 4-17 Expression of prostaglandin receptors protein (non-pulled) under basal conditions in control hPASMCs

Expression of (A) IP, (B) EP₂ and (C) DP₁ were assessed from female/male control hPASMCs homogenates by western blot. α-tubulin was used as the internal loading control. 
N=3-4 (per group) in duplicate. Data expressed as individual values.
Figure 4-18 Expression of prostaglandin receptors protein (non-pulled) under basal conditions in PAH hPASMCs

Expression of (A) IP, (B) EP$_2$, and (C) DP$_1$ were assessed from female/male PAH patient hPASMCs homogenates by western blot. α-tubulin was used as the internal loading control. N=3 (per group) in duplicate. Data expressed as individual values.
4.2.4. Prostaglandin receptor antagonists can influence the anti-proliferative effect of treprostinil

Treprostinil is prescribed therapy for the treatment of PAH. As a prostacyclin analogue, treprostinil carries out its effects primarily through the prostaglandin receptors. The impact of blocking the IP, EP$_2$ and DP$_1$ receptors with selective antagonists; RO1138452, PF-04418948 and BW 245C respectively, was examined by haemocytometer cell counts. Briefly, the effectiveness and specificity of the antagonists were examined in female control hPASMCs. The IP receptor antagonist RO1138452 (1µM) significantly reversed the anti-proliferative effect of the IP receptor agonist MRE-269 (Figure 4-19A). The anti-proliferative effects of the EP$_2$ receptor agonist butaprost were successfully blocked by the addition of the EP$_2$ receptor antagonist (PF-04418948) (Figure 4-19B). The DP$_1$ receptor antagonist BW A868C inhibited the anti-proliferative actions of the DP$_1$ receptor agonist BW 245C (Figure 4-19C). The effect of the antagonists on the anti-proliferative effects of treprostinil were examined in the presence or absence of endothelin-1. These were compared to previously run experiments in the absence of the antagonists. Without endothelin-1 the IP and EP$_2$ antagonists had no significant effects on the anti-proliferative effects of treprostinil in female control hPASMCs (Figure 4-20A-B). However, the addition of the DP$_1$ antagonist did significantly shift the anti-proliferative effect of treprostinil at 1nM (Figure 4-20C). Previously, the combination of treprostinil and endothelin-1 led to an increase in the anti-proliferative effect of treprostinil in female control hPASMCs (Figure 4-3A). In the presence of endothelin-1, the effects of treprostinil at 10nM were significantly reduced by the addition of the IP receptor antagonist (Figure 4-21A). Both the EP$_2$ and DP$_1$ antagonists had no effect on the anti-proliferative actions of treprostinil at any concentration (Figure 4-21B-C). In female PAH patient hPASMCs, none of the antagonists significantly altered the effects of treprostinil (Figure 4-22A-C). In the presence of endothelin-1, the effects of treprostinil at 10nM and 100nM were significantly reduced by the addition of the EP$_2$ receptor antagonist (Figure 4-23B). The addition of endothelin-1 did not lead to any significant changes for the IP or DP$_1$ receptor antagonists (Figure 4-23A, C). As demonstrated in the female PAH patient hPASMCs, the addition of the IP, EP$_2$ and DP$_1$ antagonists had no significant effect on the anti-proliferative effects of treprostinil in male PAH patient hPASMCs (Figure 4-24A-C). The anti-proliferative effects of treprostinil at 100nM were inhibited by the EP$_2$ receptor antagonist, in the presence of endothelin-1 (Figure 4-25B). In male PAH patient hPASMCs, the IP and DP$_1$ receptor antagonists had no significant effect on the anti-proliferative actions of treprostinil in the presence of endothelin-1 (Figure 4-25A, C).
Figure 4-19 The IP, EP₂ and DP₁ prostaglandin receptor antagonists block their respective agonists anti-proliferative actions in female control hPASMCs

HPASMCs from female controls were quiesced for 24 hours in 0.5% FBS phenol free DMEM media. PASMCs were pre-incubated with 1µM of either an (A) IP (RO1138452), (B) EP₂ (PF-04418948) or (C) DP₁ (BW A868C) receptor antagonists (1µM) for 30 minutes prior to the addition of 100nM and 1µM of respective receptor agonists, (A) MRE-269, (B) Butaprost and (C) BW 245C. Experiments were carried out in the presence of 7% FBS phenol free DMEM media. Cell counts were assessed 72 hours later by haemocytometer. N=3-4 human samples per group, in triplicate, ***P<0.001 cf. 0.5% FBS, ♢♢ P<0.01, ♢♢♢ P<0.001 c.f 7% FBS, ♣♣ P<0.01, ♣♣♣ P<0.001 cf. 7% + respective antagonist. One-way ANOVA with Bonferroni’s post-hoc test. Data expressed as % of 7% control. IP Antagonist = RO1138452, EP₂ antagonist = PF-04418948, DP₁ antagonist = BW A868C.
Figure 4-20 The IP and EP\textsubscript{2} prostaglandin receptor antagonists have no significant effect on the anti-proliferative effect of treprostinil in the absence of endothelin-1

HPASMCs from female controls were quiesced for 24 hours in 0.5% FBS phenol free DMEM media. PASMCs were pre-incubated with 1µM of either an (A) IP (RO1138452), (B) EP\textsubscript{2} (PF-04418948) or (C) DP\textsubscript{1} (BW A868C) receptor antagonists (1µM) for 30 minutes prior to the addition treprostinil (1pM, 10pM, 100pM, 1nM, 10nM, 100nM, 1µM and 10µM). Experiments were carried out in the presence of 7% FBS phenol free DMEM media. Cell counts were assessed 72 hours later by haemocytometer. N=3-4 human samples per group, in duplicate/triplicate. *P<0.05 cf. Trep, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as % of 7% control. IP Antagonist = RO1138452, EP\textsubscript{2} antagonist = PF-04418948, DP\textsubscript{1} antagonist = BW A868C, Trep= treprostinil.
Chapter Four

In-vitro

Figure 4-21 The IP receptor antagonist significantly reduced the anti-proliferative effect of treprostinil in female control hPASMCs in the presence of endothelin-1

HPASMCs from female controls were quiesced for 24 hours in 0.5% FBS phenol free DMEM media. PASMCs were pre-incubated with 1µM of either an (A) IP (RO1138452), (B) EP₂ (PF-04418948) or (C) DP₁ (BW A868C) receptor antagonists (1µM) for 30 minutes prior to the addition treprostinil (1pM, 10pM, 100pM, 1nM, 10nM, 100nM, 1µM and 10µM) in the presence endothelin-1 (30nM). Experiments were carried out in the presence of 7% FBS phenol free DMEM media. Cell counts were assessed 72 hours later by haemocytometer. N=3-4 human samples per group, in duplicate/triplicate. **P<0.01 cf. Trep + ET-1. Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as % of 7% control. IP Antagonist = RO1138452, EP₂ antagonist = PF-04418948, DP₁ antagonist = BW A868C, ET-1 = endothelin-1, Trep= treprostinil.
HPASMCs from female PAH patients were quiesced for 24 hours in 0.5% FBS phenol free DMEM media. PASMCs were pre-incubated with 1µM of either an (A) IP (RO1138452), (B) EP₂ (PF-04418948) or (C) DP₁ (BW A868C) receptor antagonists (1µM) for 30 minutes prior to the addition treprostinil (1pM, 10pM, 100pM, 1nM, 10nM, 100nM, 1µM and 10µM). Experiments were carried out in the presence of 7% FBS phenol free DMEM media. Cell counts were assessed 72 hours later by haemocytometer. N=3-4 human samples per group, in duplicate/triplicate. Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as % of 7% control. IP Antagonist = RO1138452, EP₂ antagonist = PF-04418948, DP₁ antagonist = BW A868C, Trep= treprostinil.
Figure 4-23 The EP₂ receptor antagonist significantly reduced the anti-proliferative effect of treprostinil in female PAH patient hPASMCs in the presence of endothelin-1

HPASMCs from female PAH patient were quiesced for 24 hours in 0.5% FBS phenol free DMEM media. PASMCs were pre-incubated with 1µM of either an (A) IP (RO1138452), (B) EP₂ (PF-04418948) or (C) DP₁ (BW A868C) receptor antagonists (1µM) for 30 minutes prior to the addition treprostinil (1pM, 10pM, 100pM, 1nM, 10nM, 100nM, 1µM and 10µM) in the presence endothelin-1 (30nM). Experiments were carried out in the presence of 7% FBS phenol free DMEM media. Cell counts were assessed 72 hours later by haemocytometer. N=3-4 human samples per group, in duplicate/triplicate, *P<0.05, **P<0.01 cf. Trep + ET-1. Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as % of 7% control. IP Antagonist = RO1138452, EP₂ antagonist = PF-04418948, DP₁ antagonist = BW A868C, ET-1 = endothelin-1, Trep= treprostinil
Figure 4-24 The IP, EP₂ and DP₁ prostaglandin receptor antagonists have no significant effect on the anti-proliferative effects of treprostinil in male PAH patient hPASMCs in the absence of endothelin-1

HPASMCs from male PAH patients were quiesced for 24 hours in 0.5% FBS phenol free DMEM media. PASMCs were pre-incubated with 1µM of either an (A) IP (RO1138452), (B) EP₂ (PF-04418948) or (C) DP₁ (BW A868C) receptor antagonists (1µM) for 30 minutes prior to the addition treprostinil (1pM, 10pM, 100pM, 1nM, 10nM, 100nM, 1µM and 10µM). Experiments were carried out in the presence of 7% FBS phenol free DMEM media. Cell counts were assessed 72 hours later by haemocytometer. N=2-3 human samples per group, in duplicate/triplicate, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as % of 7% control. IP Antagonist = RO1138452, EP₂ antagonist = PF-04418948, DP₁ antagonist = BW A868C, Trep= treprostinil.
Figure 4-25 The EP$_2$ receptor antagonist significantly reduced the anti-proliferative effect of treprostinil in male PAH patient hPASMCs in the presence of endothelin-1

HPASMCs from male PAH patients were quiesced for 24 hours in 0.5% FBS phenol free DMEM media. PASMCs were pre-incubated with 1µM of either an IP (RO1138452) (A), EP$_2$ (PF-04418948) (B) or DP$_1$ (BW A868C) (C) receptor antagonists (1µM) for 30 minutes prior to the addition treprostinil (1pM, 10pM, 100pM, 1nM, 10nM, 100nM, 1µM and 10µM) in the presence endothelin-1 (30nM). Experiments were carried out in the presence of 7% FBS phenol free DMEM media. Cell counts were assessed 72 hours later by haemocytometer. N=2-3 human samples per group, in duplicate/triplicate, *P<0.05 cf. Trep + ET-1, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as % of 7% control. IP Antagonist = RO1138452, EP$_2$ antagonist = PF-04418948, DP$_1$ antagonist = BW A868C, ET-1 = endothelin-1, Trep= treprostinil.
4.2.5. The effects of treprostinil on BMPR-II protein signalling in control and PAH patient hPASMCs

As the BMPR-II signalling pathway is important in cases of HPAH and IPAH. The effect of treprostinil, in the presence or absence of endothelin-1, on BMPR-II signalling was examined. This was carried out under the same conditions as the proliferation cell count experiments. BMPR-II, pSmad1/5/9, Id1 and Id3 were examined by western blot protein analysis. In female control hPASMCs, it was found that BMPR-II, pSmad1/5/9 and Id1 protein expression were not altered by the addition of treprostinil with or without endothelin-1 (Figure 4-26A-C). However, 1µM treprostinil stimulation significantly increased Id3 protein expression. The addition of endothelin-1 did not significantly increase this effect (Figure 4-26D). No significant changes in BMPR-II, pSmad1/5/9, Id1 or Id3 were observed following treprostinil stimulation in male control hPASMCs (Figure 4-27A-D). Both female (Figure 4-28A-C) and male (Figure 4-29A-C) PAH hPASMCs had no significant increase in BMPR-II, pSmad 1/5/9 and Id1 protein with treprostinil stimulation. However, Id3 protein expression was increased by treprostinil stimulation (1µM) in female PAH patient hPASMCs (Figure 4-28D). There was no significant increase in Id3 expression in male PAH patient hPASMCs (Figure 4-29D). The data suggests that although treprostinil can activate components of the BMPR-II signalling pathway endothelin-1 does not increase this effect.
Figure 4-26 Expression of Id3 protein is increased by the addition of treprostinil (1µM) at 72 hours in female control hPASMCs; the addition of endothelin-1 (30nM) has no additional effect on BMPR-II signalling

Expression of (A) BMPR-II, (B) pSmad 1/5/9, (C) Id1 and (D) Id3 were assessed from female control hPASMCs stimulated with treprostinil (100nM and 1µM) in presence or absence of endothelin-1 (30nM) over 72 hours by western blot. (E) Representative immunoblot, α-tubulin was used as the internal loading control. N=4 (n=3 in duplicate). *P<0.05, **P<0.01 cf. Basal, ɸɸP<0.01, ɸɸɸP<0.001 cf. 7% FBS, ξP<0.05, ξξP<0.01 cf. 7% FBS + Endothelin-1. One-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media. ET-1 = endothelin-1, Trep= treprostinil.
Figure 4-27 Treprostinil does not affect BMPR-II signalling at 72 hours in male control hPASMCs; the addition of endothelin-1 (30nM) has no additional effect on BMPR-II signalling. Expression of (A) BMPR-II, (B) pSmad 1/5/9, (C) Id1 and (D) Id3 were assessed from male control hPASMCs stimulated with treprostinil (100nM and 1µM) in presence or absence of endothelin-1 (30nM) over 72 hours by western blot. (E) Representative immunoblot, α-tubulin was used as the internal loading control. N=3-4 (n=3 in duplicate). One-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media. ET-1 = endothelin-1, Trep= treprostinil.
Figure 4-28 Expression of Id3 protein is increased by the addition of treprostinil (1µM) at 72 hours in female patient hPASMCs; the addition of endothelin-1 (30nM) has no additional effect on BMPR-II signalling

Expression of (A) BMPR-II, (B) pSmad 1/5/9, (C) Id1 and (D) Id3 were assessed from female patient hPASMCs stimulated with treprostinil (100nM and 1µM) in presence or absence of endothelin-1 (30nM) over 72 hours by western blot. (E) Representative immunoblot, α-tubulin was used as the internal loading control. N=3 in duplicate. *P<0.05 cf. Basal, ϕ P<0.05 cf. 7% FBS, ξ P<0.05 cf. 7% FBS + Endothelin-1. One-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media. ET-1 = endothelin-1, Trep= treprostinil.
Figure 4-29 Treprostinil does not affect BMPR-II signalling at 72 hours in male patient hPASMCs

Expression of (A) BMPR-II, (B) pSmad 1/5/9, (C) Id1 and (D) Id3 were assessed from male patient hPASMCs stimulated with treprostinil (100nM and 1µM) in presence or absence of endothelin-1 (30nM) over 72 hours by western blot. (E) Representative immunoblot, α-tubulin was used as the internal loading control. N=3 in duplicate (n=2 for BMPR-II blot). One-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media. ET-1 = endothelin-1, Trep= treprostinil.
4.2.6. The effects of treprostinil on BMPR-II signalling in control and PAH patient hPASMCs: mRNA

As endothelin-1 had no effect on treprostinil activation of BMPR-II signalling, the effects of treprostinil alone were examined by Taqman® analysis. BMPR-II, Id1 and Id3 mRNA was assessed in female and male control/PAH hPASMCs groups. BMPR-II mRNA expression was not significantly affected by any experimental condition in control or PAH patient hPASMC group (Figure 4-30A, Figure 4-31A). 100nM and 1µM treprostinil stimulations caused a significant increase in Id1 mRNA in female control hPASMCs (Figure 4-30B). No reciprocal increase in Id1 mRNA expression was observed in male control hPASMCs (Figure 4-28B) or in PAH patient hPASMCs (Figure 4-31B). Treprostinil stimulation (100nM and 1µM) significantly increased Id3 mRNA expression in female control hPASMCs, this effect was not observed in male control hPASMCs (Figure 4-30C). However, treprostinil (1µM) significantly up-regulated Id3 mRNA expression in both female and male PAH hPASMCs (Figure 4-31C). The significant increase in Id3 was much greater in female PAH patients (Figure 4-31C). In female control hPASMCs 100nM treprostinil stimulation led to a significant increase in Id1 mRNA expression over the equivalent BMPR-II mRNA level (Figure 4-32A). Also, basal and 7% FBS mRNA expression of Id3 was significantly lower than BMPR-II expression under the same conditions (Figure 4-32A). No differences were detected between BMPR-II, Id1 or Id3 in male control hPASMCs (Figure 4-32B). In female PAH patient hPASMCs 1µM treprostinil stimulation led to higher Id3 mRNA levels than observed in BMPR-II mRNA under the same conditions (Figure 4-33A). As with male control hPASMCs, male PAH patient hPASMCs expressed no significant difference in BMPR-II, Id1 or Id3 mRNA expression (Figure 4-33B).
Figure 4-30 Id1 and Id3 mRNA expression is increased by treprostinil in female control hPASMCs but not in male control hPASMCs

Expression of (A) BMPR-II, (B) Id1 and (C) Id3 mRNA were assessed from female and male control hPASMCs stimulated with treprostinil (100nM and 1µM) over 72 hours by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=4 per group in triplicate, **P<0.01, ****P<0.0001 cf. Basal, ɸ P<0.05, ɸɸ P<0.01, ɸɸɸ P<0.001 cf. 7% FBS. Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ\text{max}/RQ\text{min}. RQ= relative quantification. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media, Trep= treprostinil.
Expression of (A) BMPR-II, (B) Id1 and (C) Id3 mRNA were assessed from female and male PAH patient hPASMCs stimulated with treprostinil (100nM and 1µM) over 72 hours by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=3 per group in triplicate, ***P<0.001 cf. Basal, φφ P<0.01 cf. 7% FBS. Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ$^{max}$/RQ$^{min}$. RQ= relative quantification. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media, Trep=treprostinil.
Figure 4-32 Comparison of overall BMPR-II, Id1 and Id3 mRNA expression within female and male control hPASMCs

Expression of BMPR-II, Id1 and Id3 mRNA were assessed from (A) female and (B) male control hPASMCs stimulated with treprostinil (100nM and 1µM) over 72 hours by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=4 per group in triplicate. *P<0.05 cf. BMPR-II (respective group), Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ = RQ_{max}/RQ_{min}. RQ= relative quantification. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media, Trep= treprostinil.
Figure 4-33 Comparison of overall BMPR-II, Id1 and Id3 mRNA expression within female and male PAH patient hPASMCs

Expression of BMPR-II, Id1 and Id3 mRNA were assessed from (A) female and (B) male PAH patient hPASMCs stimulated with treprostinil (100nM and 1µM) over 72 hours by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=3 per group in triplicate. *P<0.05 cf. BMPR-II (respective group), Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ_{max}/RQ_{min}. RQ= relative quantification. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media, Trep= treprostinil.
4.2.7. The increase in Id3 protein, by treprostinil (1µM) for 72 hours, is greater in female control hPASMCs

A comparison of BMPR-II signalling after 72-hour treprostinil stimulation between female and male control hPASMCs was carried out by western blot protein analysis. Sex or treprostinil had no effect on in BMPR-II, pSmad 1/5/9 or Id1 protein levels (Figure 4-34A-C). However, 1µM treprostinil stimulation caused a significant induction of Id3 protein expression specifically in female control hPASMCs (Figure 4-34D). This effect was not observed in male control hPASMCs (Figure 4-32D).
Figure 4-34 The increase in Id3 protein is greater in female control hPASMCs over male control hPASMCs when stimulated with treprostinil (1µM) for 72 hours

Expression of (A) BMPR-II, (B) pSmad 1/5/9, (C) Id1 and (D) Id3 were assessed from female and male control hPASMCs stimulated with treprostinil (100nM and 1µM) over 72 hours by western blot. Representative immunoblot (E), α-tubulin was used as the internal loading control. N=3-4 (n=3 for BMPR-II blot) in duplicate. ****P<0.0001 cf. Basal, ⧿⧿⧿⧿ P<0.0001 cf. 7% FBS, ⧿ P<0.01 cf. Trep (0.1µM), Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media, Trep= treprostinil.
4.2.8. BMPR-II signalling protein is increased in both control and patient hPASMCs groups after 24 hour treprostinil stimulation

Due to the low levels of induction of the BMPR-II signalling pathway after 72-hour of stimulation with treprostinil, effects of treprostinil on BMPR-II signalling after 24 hours. The BMPR-II pathway and the impact of endothelin-1 on the effect of treprostinil were investigated by western blot protein analysis. In female control hPASMCs, BMPR-II and pSmad 1/5/9 were not significantly changed by the addition of treprostinil in the presence or absence of endothelin (Figure 4-35A, B). Both concentrations of treprostinil significantly increased Id1 protein (Figure 4-35C), this differed to the results observed at 72-hours (Figure 4-26C). Previously, treprostinil only significantly induced Id3 protein at 1µM (Figure 4-26D). Presently, a much greater significant increase was observed after treprostinil stimulation at both 100nM and 1µM (Figure 4-35D). The addition of endothelin-1 did not have significant influence on the effects of treprostinil. Previously, in male control hPASMCs, no changes to the BMPR-II pathway were observed (Figure 4-27). Presently, 1µM of treprostinil was found to increase the induction of Id3 protein when combined with endothelin-1 above the FBS level (Figure 4-36D). No significant changes were observed in BMPR-II, pSmad 1/5/9 or Id1 protein after treprostinil stimulation (Figure 4-36A-C). The shorter experimental time also improved the female PAH patient response to treprostinil. Despite no significant increases in BMPR-II, pSmad 1/5/9 and Id1 protein (Figure 4-37A-C), 100nM and 1µM of treprostinil significantly increased Id3 protein induction (Figure 4-37D). Treprostinil did not significantly increase protein expression of BMPR-II, pSmad 1/5/9, Id1 or Id3 in male PAH patient hPASMCs (Figure 4-38A-D).
Figure 4-35 Expression of Id1 and Id3 protein is increased by the addition of treprostinil (100nM and 1µM) for 24 hours in female control hPASMCs

Expression of (A) BMPR-II, (B) pSmad 1/5/9, (C) Id1 and (D) Id3 were assessed in female control hPASMCs stimulated with treprostinil (100nM and 1µM) over 24 hours by western blot. (E) Representative immunoblot, α-tubulin was used as the internal loading control. N=4 in duplicate. **P<0.01 cf. Basal, ϕ P<0.05, ϕϕ P<0.01, ϕϕϕ P<0.001 cf. 7% FBS, ξξ P<0.01, ξξξ P<0.001 cf. 7% FBS + ET-1. One-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media, ET-1 = endothelin-1, Trep= treprostinil.
Expression of (A) BMPR-II, (B) pSmad 1/5/9, (C) Id1 and (D) Id3 were assessed in male control hPASMCs stimulated with treprostinil (100nM and 1μM) over 24 hours by western blot. (E) Representative immunoblot, α-tubulin was used as the internal loading control. N=4 in duplicate. *P<0.05 cf. 7% FBS. One-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media, ET-1 = endothelin-1, Trep= treprostinil.
Figure 4-37 Expression of Id3 protein is increased by the addition of treprostinil (100nM and 1µM) for 24 hours in female patient hPASMCs

Expression of (A) BMPR-II, (B) pSmad 1/5/9, (C) Id1 and (D) Id3 were assessed in female patient hPASMCs stimulated with treprostinil (100nM and 1µM) over 24 hours by western blot. (E) Representative immunoblot, α-tubulin was used as the internal loading control. N=4 in duplicate (n=3 for pSmad 1/5/9). *P<0.05, **P<0.01 cf. Basal, °P<0.05, °°P<0.01 cf. 7% FBS, ηP<0.05, ηηP<0.01 cf. 7% FBS + ET-1. One-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media, ET-1 = endothelin-1, Trep= treprostinil.
Figure 4-38 BMPR-II signalling is not significantly changed by 24 hour treprostinil stimulation in male PAH patient hPASMCs

Expression of (A) BMPR-II, (B) pSmad 1/5/9, (C) Id1 and (D) Id3 were assessed from male patient hPASMCs stimulated with treprostinil (100nM and 1µM) over 24 hours by western blot. (E) Representative immunoblot, α-tubulin was used as the internal loading control. N=3 in duplicate. One-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media, ET-1 = endothelin-1, Trep= treprostinil.
4.2.9. The effect of treprostinil on BMPR-II signalling pathway mRNA in control and PAH patient hPASMCs at 24 hours

The changes in the BMPR-II (mRNA) signalling was investigated by Taqman® analysis. Consistent with previous results, treprostinil stimulation led to no change in BMPR-II mRNA expression in control and PAH patient hPASMCs (Figure 4-39A, Figure 4-40A). Interestingly, treprostinil did not change Id1 mRNA expression in female control hPASMCs (Figure 4-39B). This differs from the increase that was observed at 72-hours (Figure 4-30B). Id1 mRNA was not up-regulated in male control hPASMCs (Figure 4-39B), this was consistent with previous results (Figure 4-30B). Treprostinil increased Id3 mRNA expression at 100nM and 1µM in both female and male control hPASMCs (Figure 4-39C). Previously after 72-hours, treprostinil increased Id3 expression in female control hPASMCs (Figure 4-30C). Id1 mRNA was upregulated in female and male PAH patient hPASMCs. However, the increase was observed at both 100nM and 1µM of treprostinil in females (Figure 4-40B) but only at 1µM in male patients (Figure 4-40B). Similarly, Id3 was increased in female and male PAH patients, although in females the increase was observed after treprostinil stimulation at 100nM and 1µM (Figure 4-40C). In female control hPASMCs, 1µM treprostinil stimulation significantly increased both Id1 and Id3 mRNA expression over equivalently treated BMPR-II mRNA (Figure 4-41A). Only Id3 mRNA was significantly higher than BMPR-II mRNA after 1µM treprostinil in male control hPASMCs (Figure 4-41B). Both 100nM and 1µM treprostinil stimulation led to significantly higher mRNA expression Id3 compared to Id1 and BMPR-II under the same stimulations (Figure 4-42A). In male PAH patients 100nM and 1µM treprostinil stimulation significantly increased Id3 mRNA expression compared to BMPR-II mRNA (Figure 4-42B). Also, Id1 mRNA was significantly higher than BMPR-II mRNA after 1µM treprostinil stimulation (Figure 4-42B).
Figure 4-39 Id3 mRNA expression is increased in both female and male control hPASMCs after 24 hours of treprostinil stimulation

Expression of BMPR-II (A), Id1 (B) and Id3 (C) mRNA were assessed from female and male control hPASMCs stimulated with treprostinil (100nM and 1µM) over 24 hours by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=4 per group in triplicate, \( \Phi \) P<0.01 cf. (Female) 7% FBS, \( \xi \) P<0.05, \( \xi \xi \) P<0.01 cf. (Male) 7% FBS, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ\(_{\text{max}}\)/RQ\(_{\text{min}}\). RQ= relative quantification. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media, Trep= treprostinil.
Figure 4-40 Id1 and Id3 mRNA expression is increased in both female and male patient hPASMCs after 24 hours of treprostinil stimulation

Expression of BMPR-II (A), Id1 (B) and Id3 (C) mRNA were assessed from female and male PAH patient hPASMCs stimulated with treprostinil (100nM and 1µM) over 24 hours by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=3-4 per group in triplicate, *P<0.05 cf. (Female) Basal, ϕ P<0.05, ϕϕ P<0.01 cf. (Female) 7% FBS, ξ P<0.05 cf. (Male) 7% FBS, Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQ_{max}/RQ_{min}. RQ= relative quantification. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media, Trep = treprostinil.
Figure 4-41 Comparison of overall BMPR-II, Id1 and Id3 mRNA expression within female and male control hPASMCs

Expression of BMPR-II, Id1 and Id3 mRNA were assessed from (A) female and (B) male control hPASMCs stimulated with treprostinil (100nM and 1µM) over 24 hours by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=3-4 per group in triplicate, *P<0.05 cf. BMPR-II (respective group), Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as RQ ± RQmax/RQmin. RQ= relative quantification. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media, Trep= treprostinil.
Figure 4-42 Comparison of overall BMPR-II, Id1 and Id3 mRNA expression within female and male PAH patient hPASMCs

Expression of BMPR-II, Id1 and Id3 mRNA were assessed from (A) female and (B) male PAH patient hPASMCs stimulated with treprostinil (100nM and 1µM) over 24 hours by Taqman® quantitative Real Time-PCR. Results were normalised to β2-microglobulin. N=3-4 per group in triplicate, *P<0.05, ****P<0.0001 cf. BMPR-II (respective group), ﹁﹁ P<0.01, ﹁﹁﹁﹁ P<0.0001 cf. Id1 (respective group), Two-way ANOVA with Bonferroni’s post-hoc test Data expressed as RQ ± RQmax/RQmin. RQ= relative quantification. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media, Trep= treprostinil.
4.2.10. The effect of treprostinil on BMPR-II signalling after 24-hour treprostinil stimulation demonstrates greater induction of Id proteins in female control hPASMCs

BMPR-II signalling was investigated in the absence of endothelin-1 by western blot analysis after 24-hour stimulation with treprostinil. BMPR-II and pSmad 1/5/9 were unchanged with treprostinil stimulation, in both female and male control hPASMCs (Figure 4-43A-B). As observed previously, treprostinil elevated Id3 protein significantly in female control hPASMCs (Figure 4-43D). Consistent with previous results, there was no significant increase in Id3 protein expression in male control hPASMCs (Figure 4-43D). In PAH patients, there was no significant increase in BMPR-II, pSmad 1/5/9 and Id1 protein expression after 24-hour treprostinil stimulation (Figure 4-44A-C). In female PAH patients, both 100nM and 1µM of treprostinil significantly increase Id3 protein, however this only 1µM of treprostinil increased Id3 in male PAH hPASMCs (Figure 4-44D). The data demonstrates that females have moderately better induction of the Id proteins after 24-hour of treprostinil stimulation. However, as observed previously the greatest difference was between female and male control hPASMCs.
Figure 4-43 The increase Id3 protein is greater in female control hPASMCs than in male control hPASMCs when stimulated with treprostinil at 100nM and 1µM for 24 hours

Expression of (A) BMPR-II, (B) pSmad 1/5/9, (C) Id1 and (D) Id3 were assessed from female and male control hPASMCs stimulated with treprostinil (100nM and 1µM) over 24 hours by western blot. (E) Representative immunoblot, α-tubulin was used as the internal loading control. N=4 in duplicate. **P<0.01 cf. Basal, ***P<0.001 cf. 7% FBS (Female). Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media, Trep=treprostinil.
Figure 4-44 The increase in Id3 protein is greater in female PAH patient hPASMCs over male PAH patient hPASMCs when stimulated with treprostinil (1µM) for 24 hours

Expression of (A) BMPR-II, (B) pSmad 1/5/9, (C) Id1 and (D) Id3 were assessed from female and male PAH patient hPASMCs stimulated with treprostinil (100nM and 1µM) over 24 hours by western blot. (E) Representative immunoblot, α-tubulin was used as the internal loading control. N=3 in duplicate. *P<0.05, **P<0.01 cf. Basal, ϕϕ P<0.01 cf. 7% FBS (Female), ξ P<0.05 cf. 7% FBS (Male), Two-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media, Trep= treprostinil.
4.2.11. Dual prostaglandin receptor antagonism block treprostinil’s induction of Id proteins

The influence of the prostaglandin receptors on Id protein induction was investigated by western blot. Female control and PAH patient hPASMCs were stimulated with treprostinil in the absence or combined with prostaglandin receptor antagonists, for 24 hours. In female control hPASMCs, treprostinil increased both Id1 and Id3 (Figure 4-45A-B). Id1 and Id3 increases at 100nM were significantly blocked by the addition of 1µM of IP and EP2 receptor antagonists; RO1138452 and PF-04418948 (Figure 4-45A-B). Interestingly, the combination of the IP and EP2 antagonists with 1µM of a DP1 receptor antagonist (BW A868C) did not lead to a significant reduction in Id1 and Id3 (Figure 4-45C-D). In female PAH patient hPASMCs, treprostinil at 100nM and 1µM increased Id3 protein expression (Figure 4-46B). Similarly, to female control hPASMCs the increase in Id3 at 100nM of treprostinil was abolished by the addition of the IP and EP2 receptor antagonists (Figure 4-46B). As before, the combination of the IP and EP2 antagonists with the DP1 receptor antagonist did not preserve the significant reversal of the Id3 protein (Figure 4-46D).
Figure 4-45 Id1 and 3 protein expression in female control hPASMCs after treatment with treprostinil +/- IP and EP₂ antagonists

Expression of (A, C) Id1 and (B, D) Id3 were assessed from female control hPASMCs pre-incubated for 30 minutes with a combination of 1µM of IP (RO1138452) and EP₂ (PF-04418948) antagonists or IP, EP₂ and DP₁ (BW A868C) antagonists, then stimulated with treprostinil (100nM and 1µM) over 24 hours by western blot. (E) Representative immunoblot, α-tubulin was used as the internal loading control. N=3 in duplicate, *P<0.05, **P<0.01 cf. Basal, Φ P<0.05, ΦΦ P<0.01 cf. 7% FBS, ξ P<0.05, ξξξ P<0.001 cf. Trep (0.1µM). One-way ANOVA with Bonferroni’s post-hoc test. Data expressed as mean ± SEM. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media, Trep= treprostinil.
Figure 4-46 Increase in Id3 protein, by treprostinil, is partially reversed by the dual antagonism of the IP and EP<sub>2</sub> prostaglandin receptors in female patient hPASMCs

Expression of (A, C) Id1 and (B, D) Id3 were assessed from female PAH patient hPASMCs pre-incubated for 30 minutes with a combination of 1µM of IP (RO11138452) and EP<sub>2</sub> (PF-04418948) antagonists or IP, EP<sub>2</sub> and DP<sub>1</sub> (BW A868C) antagonists, then stimulated with treprostinil (100nM and 1µM) over 24 hours by western blot. (E) Representative immunoblot, α-tubulin was used as the internal loading control. N=3 in duplicate. **P<0.01, ***P<0.001 cf. Basal, φ P<0.01, φφ P<0.01 cf. 7% FBS, ξξξ P<0.001 cf. Trep (0.1µM). One-way ANOVA with Bonferroni's post-hoc test. Data expressed as mean ± SEM. Basal = non-quiesced hPASMCs in 10% FBS phenol red DMEM media, Trep = treprostinil.
4.3. Discussion

There are many therapies that are prescribed to treat PAH, these include endothelin receptor antagonists, phosphodiesterase 5 inhibitors and prostacyclin analogues (Jasinska-Stroschein and Orszulak-Michalak, 2014). Treprostinil and other prostacyclin analogues are known to act through the prostaglandin receptors to mediate vasodilatory and anti-proliferative effects (Clapp and Gurung, 2015). Male PAH patients have a poorer survival outcome compared to female PAH patients (Benza et al., 2010). Previously, a phosphodiesterase 5 inhibitor and a endothelin receptor antagonist have exhibited greater beneficial effects in male and female respectively (Mathai et al., 2015, Gabler et al., 2012).

This was the first study to investigate the potential role of sex in the anti-proliferative effects of treprostinil. Through in-vitro investigations, sex was found to have an influencing role in the anti-proliferative effects of treprostinil however, the main differences were observed between control hPASMCs not PAH patient hPASMCs. The female control hPASMCs had a more diverse and significant anti-proliferative response to treprostinil than male control hPASMCs. The addition of endothelin-1 (30nM) to the FBS moderately improved the anti-proliferative activity of treprostinil in all groups, although, significant improvement was observed only in female control hPASMCs. The BMPR-II signalling pathway was implicated in the anti-proliferative effects of treprostinil. After 72-hour stimulation with treprostinil female control hPASMCs had significant increases in Id mRNA and protein whereas male control hPASMCs did not. 72-hour stimulation with treprostinil led to increases in Id3 mRNA expression in both female and patient PAH hPASMCs, however protein expression did not increase. A shorter 24-hour stimulation with treprostinil led to greater induction in both Id1 and Id3 protein in female control hPASMCs. A smaller female bias was observed in PAH patient hPASMCs. The role prostaglandin receptors play in treprostinil’s effects, was observed not only in anti-proliferation, but also in the drugs’ ability to increase Id protein expression.

Until recently, the underlying pharmacological reasons for treprostinil’s actions were not well understood. Treprostinil was discovered to increase levels of cAMP in hPASMCs (Clapp et al., 2002) and latterly found to reduce proliferation in both HEK cells and hPASMCs (Falcetti et al., 2007). Presently, this study demonstrates that female control hPASMCs have a greater significant response to treprostinil than equivalently treated male control hPASMCs. Much of the research into sex influencing drug effects is limited to the study of adverse drug reactions, primarily occurring in women (Fattinger et al., 2000). However, it has been shown that serotonin receptor 1B receptor expression was much higher in females, whereas serotonin transporter binding potential was significantly lower compared to males (Jovanovic et al., 2008). Sex differences in the serotonin system in PAH have also been demonstrated recently in animal models and in hPASMCs (Wallace et al., 2015). In this study, endothelin-1 combined with FBS was shown to partially improve
the anti-proliferative effects of treprostinil in female control hPASMCs. This appears counter-intuitive as endothelin-1 is a pro-proliferative vasoconstrictor that is elevated in PAH patients (Stewart et al., 1991, Giaid et al., 1993, Hall et al., 2011). Interestingly, there was no significant difference in endothelin receptor protein or mRNA in the hPASMCs tested that could adequately explain the increased treprostinil effect in female control hPASMCs. However, significantly lower levels of preproET-1 mRNA were identified in female control hPASMCs suggesting lower local ET-1 production. The actions of anti-proliferative therapies for PAH were examined under similar conditions (Patel et al., 2014). However, these were not compared to equivalent experiments without endothelin-1. The additive effects of treprostinil and endothelin were also demonstrated in isolated human pulmonary arteries. Pre-constriction with endothelin-1 led to a greater treprostinil mediated vasodilation than in those vessels pre-constricted with norepinephrine (Benyahia et al., 2015). In the present study, the treprostinil response in combination with ET-1 was blunted by the addition of the dual endothelin receptor antagonist SB-217242, suggesting that involvement of both endothelin receptors. Treprostinil has also previously been found to inhibit serum induced increases in ET-1 in PAH patient hPASMCs, this effect was hampered by the addition of the dual endothelin receptor antagonist bosentan and ET\textsubscript{A} specific antagonist BQ-788 (Jigisha et al., 2012). Consensus about ET-1 in PAH suggests that it is a causative factor in disease development. However, there may be potential benefits to its presence in pulmonary circulation. In proximal hPASMCs, endothelin-1 was found to increase the release of cAMP via a cyclooxygenase-2/IP (COX-2/IP) receptor autocrine loop (Deacon and Knox, 2010). This autocrine loop involved the local prostacyclin synthesis which then could act on the IP receptor, triggering cAMP increase and activation of cAMP response binding elements (CREBs). Deacon and Knox also identified the ET-1 increased cyclooxygenase 1 and 2 (COX1/2) protein levels, however any ET-1 mediated cAMP increase was removed by COX-1/2 inhibition and IP receptor inhibition (Deacon and Knox, 2010). ET-1 was also shown to increase PGI\textsubscript{2} and PGE\textsubscript{2} secretion from hPASMCs (Deacon and Knox, 2010). Since treprostinil can bind and activate the IP receptor as well as other prostaglandin receptors it may be a possible explanation for the increased treprostinil effect in combination with ET-1. However, as the previously published work was carried out in proximal hPASMCs, it would need to be repeated in distal hPASMCs.

A primary consequence of activating Gs coupled receptors like the IP, EP\textsubscript{2} and DP\textsubscript{1} is an increase in cAMP levels (Hata and Breyer, 2004). Although not measured in this study cAMP induction may differ between the hPASMC groups, specifically it may explain the weaker response to treprostinil in male hPASMCs. For future studies, it would be prudent to investigate cAMP induction from each prostaglandin receptor in the absence and presence of the specific antagonists. The specificity of treprostinil to each receptor would
then be able to be ascertained. Although the ability of treprostinil to increase cAMP levels has been determined previously, it has not been investigated regarding the EP\textsubscript{2} and DP\textsubscript{1} receptors in hPASMCs (Falcetti et al., 2010, Nikam et al., 2011). Yang and colleagues successfully identified a link between prostacyclin analogues increasing cAMP and the activation of BMPR-II signalling (Yang et al., 2010). Therefore, measuring cAMP levels may also offer insight into the different levels of BMPR-II signalling induction observed between sexes and control/PAH patient hPASMCs.

The differing effects of treprostinil across the hPASMCs groups led to investigation of the role of prostaglandin receptors. Presently, the IP receptor mRNA and protein expression was not downregulated in PAH patient hPASMCs. The results achieved from this study differ from established research, specifically the IP receptor was shown to be downregulated in adult IPAH and child IPAH patient hPASMCs both at mRNA and protein levels (Falcetti et al., 2010). However, the PAH patient hPASMCs used in the current experiment were a mix of IPAH, HPAH and APAH suggesting that the IP receptor may not universally down-regulated in all forms of PAH. Although not investigated in the current study, IP receptor desensitisation has been shown to occur after stimulation with the prostacyclin analogue iloprost (Smyth et al., 1998). Rat pulmonary arteries demonstrate a desensitisation of the IP receptor after cicaprost stimulation, which was sustained for up to 12 hours after the removal of the agonist (Sobolewski et al., 2004). Presently, the PAH patient hPASMCs used in the experiments may have been treated extensively with prostacyclin analogues which may have desensitised the IP receptor but not lowered the expression. There was no observable sex difference in the mRNA or protein of the prostaglandin receptors. Due to the lack of suitable anti-proliferative response to treprostinil in male control hPASMC, the other hPASMCs groups were studied in the presence of prostaglandin receptor antagonists. The combination of endothelin-1 and treprostinil was again found to influence all the hPASMCs when responding to specific antagonists. The reduction in treprostinil’s effects by the IP receptor antagonist has been demonstrated previously (Falcetti et al., 2010). A possible reason for observing a significant reduction in the presence of endothelin-1 may be due to the increased anti-proliferative effect in the presence of treprostinil, therefore the antagonist has a greater effect to block. This is also demonstrated within the PAH patient hPASMCs, where the EP\textsubscript{2} antagonist blocks the effects of treprostinil but only in the presence of endothelin-1. The influence of the EP\textsubscript{2} receptor on the anti-proliferative actions of treprostinil have been recently explored (Jigisha et al., 2015). This suggests that the EP\textsubscript{2} receptor plays a role with the IP receptor in accounting for anti-proliferative effects of treprostinil. It is important to note that the IP receptor has been shown to be desensitised and internalised after agonist stimulation (Smyth et al., 1998). Recently, Askai and colleagues discovered that the IP specific agonist selexipag did not promote the recruitment of β-arrestin, which is
essential for the internalisation process (Asaki et al., 2015). This may be due to the non-prostanoid structure of selexipag. Interestingly, other IP agonists like treprostinil and iloprost did promote the recruitment of β-arrestin (Asaki et al., 2015). Interestingly, EP₂ receptors do not demonstrate receptor desensitisation in human airway smooth muscle cells under conditions where other prostaglandin receptors like EP₄ do desensitise (Penn et al., 2001). A further comparison of EP₂ and EP₄ desensitisation found that EP₄ receptor underwent short term agonist induced desensitisation that did not occur in EP₂ receptors (Nishigaki et al., 1996). Also discovered was that long-term exposure to PGE₂ led to equal desensitisation of both receptors (Nishigaki et al., 1996). As stated previously, the non-prostanoid nature of selexipag prevented the recruitment of β-arrestin, this reaction to different types of agonists may be occurring in the EP receptors. This emphasises the importance of the EP₂ receptor in the effects mediated by treprostinil. This study was the first to examine the potential anti-proliferative role of DP₁ receptors in hPASMCs. Like the IP and EP₂ receptors, the DP₁ receptor has been previously identified as a Gₛ coupled receptor that leads to cAMP production in eosinophils (Chiba et al., 2011) and airway smooth muscle cells (Maher et al., 2015). The interaction of treprostinil and the DP₁ receptor has been explored within the pulmonary vein, where treprostinil was found to be a potent relaxant due to its high affinity to the DP₁ receptors (Benyahia et al., 2013). Presently, we detect the DP₁ receptor mRNA and protein expression in all hPASMCs groups, however the lack of an inhibitory effect by the antagonist suggests it may not play a key role in anti-proliferation of hPASMCs under these conditions. As previously stated, treprostinil has high binding affinities for the IP, EP₂ and DP₁ receptors however, this study has not investigated the influence of other prostaglandin receptors like Gᵢ/Gₛ subunit associated receptors EP₁, TP or EP₄ receptors. When activated the EP₄ receptor increases cAMP levels whereas the EP₁ and TP receptors are associated with vasoconstriction and are known to increase intracellular calcium or reduce cAMP levels (Woodward et al., 2011). In monocrotaline rats, iloprost has been found to activate the EP₄ receptor to compensate for the reduced expression of the IP receptor (Lai et al., 2008). Treprostinil and other prostacyclin analogues can bind and activate the EP₁ receptor, which has the potential to limit their anti-proliferative or vasodilatory properties (Whittle et al., 2012). Presently, protein expression of the EP₁ receptor was not confirmed in hPASMCs however, mRNA expression was identified although it was unchanged across both sexes and in control/PAH patient hPASMC groups. The thromboxane receptor has been previous shown to dimerise with the IP receptor and increase cAMP levels (Wilson et al., 2004). This could be significant in PAH due to the high levels of thromboxane that exist in the disease (Christman et al., 1992). These results provide insight into the differing actions of treprostinil between sex, control and PAH patients.
Disrupted BMPR-II signalling is known to play a role in smooth muscle and endothelial cell proliferation in PAH (Machado et al., 2001, Yang et al., 2005, Machado et al., 2009a). Proliferation is mediated through reduction of Smad signalling and Id proteins, which inhibit cell cycle progression (Yang et al., 2005). Much of the original research into the Id proteins focused on cancer development, however more recently the Id proteins have been implicated in angiogenesis and atherosclerosis (Nishiyama et al., 2005, Doran et al., 2010). Iloprost and treprostinil have been shown previously to directly induce both pSmad 1/5/9 and Id1 protein levels in hPASMCs (Yang et al., 2010). The PDE5 inhibitor sildenafil has also been shown to activate Smad signalling via activation of a cGMP dependant kinase 1 (cGKI) (Schwappacher et al., 2009). Also, sildenafil could recover reduced BMPR-II signalling in BMPR-II mutant hPASMCs (Yang et al., 2013a). Therefore, investigation of this signalling pathway under the present experimental conditions was undertaken. The data demonstrated that across all hPASMCs groups there was no change in the BMPR-II receptor mRNA or protein upon treprostinil stimulation. However, the present study investigated the changes in BMPR-II signalling under different conditions to those previously published. Presently, BMP-4 was not added to pre-activate the BMPR-II pathway. This may account for the lack of significant change observed in pSmad 1/5/9 across the hPASMCs groups. After 72-hour stimulation with treprostinil, female control and female PAH patient hPASMCs expressed a significant increase in Id3 protein expression. The influence of sex on the BMPR-II pathway has been investigated previously however, it was demonstrated that female hPASMCs have lower expression of BMPR-II, Smad 1, Id1 and Id3 (Mair et al., 2015). These suppressed levels identified in female control hPASMCs may be a possible reason for the ability of treprostinil to significantly increase the expression of the Id proteins. The enhanced effect of treprostinil combined with ET-1 was not explained by any changes in BMPR-II signalling, suggesting that these effects are mediated down alternative signalling pathways or through an un-investigated branch of BMPR-II/TGF-β signalling. To account for variability in separate western blot analysis, the female and male control hPASMCs were re-examined on the same western blot gel. This re-affirmed the sex difference observed, with much greater Id3 protein induction in female control hPASMCs, the Id3 mRNA levels also support this increased female control hPASMC response. The Id3 induction observed in female control hPASMCs may explain the slightly greater anti-proliferative response compared to male control hPASMCs. However, the lack of response in male PAH patient hPASMC would suggest that the BMPR-II signalling pathway, specifically, the Id proteins are not the only mechanism for the anti-proliferative effects of treprostinil. The data presented a contradiction with mRNA levels of Id1 increasing in female control hPASMCs with no reciprocal increase in protein levels. This also occurred with increasing Id3 mRNA in male PAH patient hPASMCs. Interestingly, there was no difference in BMPR-II, Id1 and Id3 mRNA expression levels in unstimulated hPASMCs. Correlation of mRNA and protein data has been a difficult process.
due to the potential of post-translational changes (Maier et al., 2009) and the potential short half-life of specific proteins (Doherty et al., 2009). Previously, Id proteins have been associated with a short half-life although this can vary between different cell types (Deed et al., 1996). Therefore, in line with previous Id studies carried out in hPASMCs, a shorter experimental time of 24 hours was chosen to examine the effect of treprostinil stimulation on BMPR-II signalling (Yang et al., 2010, Yang et al., 2013b, Yang et al., 2013a). The shorter experimental time point appeared to be vindicated with a combination either Id1, Id3 or both protein levels increasing with treprostinil stimulation. Earlier it was hypothesised that the mixture of PAH patient disease types may influence the expression of prostaglandin receptors, this may have also occurred in BMPR-II signalling. It was not possible to test one PAH disease across the different sexes, therefore there was a mix of IPAH, HPAH and APAH tested.

The current study has demonstrated increased expression of the Id genes across all both control and PAH patient hPASMC groups. However, we did not have a definitive link between Id gene increase and the anti-proliferative effects of treprostinil. A possible method for scrutinising this would be to knock-out aspects of the BMPR-II signalling pathway using small interfering RNA (siRNA) (Dunmore et al., 2013). Using a siRNA for the BMPR-II receptor was found to blunt BMP4 induced Smad1/4 phosphorylation in hPASMCs (Zhang et al., 2014). Additional published research has shown the knocking out the BMPR-II receptor reversed the anti-proliferative effects of TGF-β1 in control hPASMCs (Davies et al., 2012). Currently, we have found that treprostinil has no direct effect on BMPR-II receptor expression suggesting that neither antagonising or knocking out the receptor would significantly change the increase in Id genes. Previously it was shown by Yang and colleagues that using a siRNA specific to Id1 increased the level of proliferation in hPASMCs under FBS conditions, but also removed the anti-proliferative effect of iloprost on hPASMCs (Yang et al., 2010). Additionally Yang and colleagues demonstrated that transfecting a lentivirus to overexpress Id3 led to a reduction in proliferation in control hPASMCs and in hPASMCs with a BMPR-II mutation (Yang et al., 2013b). Both these studies demonstrate that increasing Id genes can lead to anti-proliferative effects in hPASMCs. To confirm if one or both Id genes are responsible for the effects of treprostinil, siRNAs specific to Id1 and Id3 genes would be used in combination with treprostinil stimulation. This would link activation of BMPR-II signalling directly with anti-proliferative effects of treprostinil.

This study investigated the effect of treprostinil on BMPR-II signalling, however many of the underlying interactions that occur in BMPR-II signalling are not completely understood in PAH. The degradation of both Smad and Id proteins, while not examined in this study, may play an important role in observations reported. Ubiquitin ligases like Smad specific E3 ubiquitin protein ligase 1 and 2 (SMURF1/2) have been implicated in reducing BMPR-
II levels in the pulmonary arteries of the hypoxic mouse model of PH (Murakami et al., 2010). SMURF1 has been found to be elevated in newly diagnosed PAH patients and highly expressed in the endothelial and smooth muscle cells in pulmonary vascular lesions of HPAH patients with BMPR-II mutations and APAH patients (Rothman et al., 2016). In the present study, this may explain the weaker induction of Id proteins in male PAH hPASMCs. Genetic deletion of SMURF1 was also found to offer protection against the development of sugen hypoxic PH (Rothman et al., 2016). In breast cancer studies, SMURF2 was found to induce the degradation of SMURF1 to prevent cell migration (Fukunaga et al., 2008).

An analogue of cAMP, dibutyryl cAMP (dbcAMP) was previously found to stimulate an increase in Id1 through direct action on a cyclic AMP response element (CRE) found in the Id1 promoter (Ohta et al., 2008). The study also found that the increase in Id1 could be blocked by the addition of H89, a protein kinase A inhibitor. This was replicated in hPASMCs (Yang et al., 2010). Presently, the data demonstrates that a blockade of the IP and EP2 receptors can significantly reduce the treprostinil mediated induction observed in Id1 and Id3 protein in female control hPASMCs. Under these experimental conditions, the induction of Id proteins may be dependent on the cAMP generated from more than one prostaglandin receptor. The ability of treprostinil to induce a cAMP increase by activating the IP receptor is well defined (Clapp et al., 2002); however, treprostinil was also found to induce cAMP activation through activation of the EP2 receptor in macrophages (Aronoff et al., 2007). As this study, did not investigate cAMP levels in hPASMCs it is not possible to definitively conclude that the induction of Id proteins was dependent on cAMP. A possible influence of the results obtained in this study would be the actions of PKA on adenyl cyclase (AC) activity. In cardiac tissue, PKA has been identified as directly phosphorylating the major isoform of AC by inhibiting its catalytic activity (Iwami et al., 1995). This reduces the catalytic rate of AC not the affinity for ATP (Iwami et al., 1995). Greater response in female hPASMCs could be explained by different localisation of PKA relative to AC. Kim and colleagues discovered that when A-kinase anchoring proteins (AKAPs) anchor PKA close to AC, this generates significantly higher PKA activity compared to PKA anchored away from AC (Kim et al., 2011). It is also possible that treprostinil is not exclusively activating Id proteins through cAMP activation of protein kinase A but also through activation of the exchange protein directly activated by cAMP (Epac) (Cheng et al., 2008). Epac has been implicated, together with PKA, in inhibiting vascular smooth muscle proliferation (Hewer et al., 2011) and also it was found to be down-regulated, along with Rap-1, in both PAH patient hPASMCs and a monocrotaline rat model of PH (Murray et al., 2009). Epac has also been shown to influence Id1 expression in angiogenesis studies in endothelial cells (Doebele et al., 2009). In neural stem cells Id1 has been identified as repressing the activity of Rap1GAP, which downregulates the activity of Rap-1 (Niola et
It may also be possible that Epac/Rap-1 may play a role in the sex difference in Id protein observed in control hPASMCs, however further study is required to confirm this. Another influencer of cAMP signalling is the presence of phosphodiesterases (PDEs), which catalyses the breakdown of cAMP. Murray and colleagues identified that the PDE isoforms PDE1 and PDE3 had increased expression in PAH patients (Murray et al., 2007). These increases in PDEs influenced the cAMP generating ability of beraprost (Murray et al., 2007). More recently, both PDE3A and PDE5A were found to have the highest expression of any PDE isoforms in PASMCs. In PAH patients increased expression of PDE1 and PDE3 was linked to an increased PDE1 and PDE3 contribution to total cAMP-PDE activity (Murray et al., 2011). Sex differences have also been identified in the mRNA expression of PDE isoforms in endothelial cells isolated from female and male rats (Wang et al., 2010b). Expression of PDE3B was found to be higher in females compared to males and the reverse was true of PDE1B (Wang et al., 2010b). It may be possible that different PDE expressions or activity could be effect the anti-proliferative response to treprostinil, therefore changes in PDEs should be investigated in the future. Interestingly, the combination of a PDE2 inhibitor with prostacyclin analogues was found to augment cAMP increases in IPAH hPASMCs thereby increasing the anti-proliferation effects (Bubb et al., 2014). This suggests that other PDEs can play a role in the effectiveness of cAMP increasing PAH therapies.

Over the course of the current study a haemocytometer was used to manual cell count to ascertain the anti-proliferative effects of treprostinil. A primary concern of manual cell counting is the potential subjective nature of the results. However, replacing the haemocytometer with an automated counting machine may remove any doubts about the result. Recently, a comparison was made of haemocytometer counting versus a semi-automated counter and a fully automated counter (Cadena-Herrera et al., 2015). Interestingly, all three methods of counting produced very similar results, the main advantage that the other counting methods had over the haemocytometer was the ability to process a higher throughput of samples (Cadena-Herrera et al., 2015). An alternative to cell counting would be the measurement of the incorporation of thymidine or a close analogue into cellular DNA, as a measurement of DNA replication. This can be achieved in by the addition of radioactive tritiated thymidine or the addition of 5-bromo-2'-deoxyuridine (BrdU) incorporation, which is an analogue of thymidine, into cells. The main advantage of BrdU assays is the lack of radioactivity, which may damage the cells. Another alternative measure of proliferation is the measurement of cell metabolism. This is an indirect method of measuring cell proliferation, a common assay is the 2-(4,5-Dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Both BrdU and MTT assays can be scaled to high or low throughput options also both assay produce colour changes that can be analysed by spectrophotometer. Although, BrdU can also be combined with
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In-vitro flow cytometry analysis. However, in a carcinoma cell line BrdU was deemed to be more sensitive and accurately reflect the proliferative state of the cells (Bergler et al., 1993).

In conclusion, this study has demonstrated that sex can have an influence on the anti-proliferative activity of treprostinil in hPASMCs. The influence of sex was predominately observed in control hPASMCs and can be partially explained by differing inductions of the Id proteins of the BMPR-II signalling pathway. We also observed that induction of the Id proteins by treprostinil is dependent on the activation of more than one prostaglandin receptor. Ultimately, it is difficult to draw clinical significance from these results as individual PAH patients could respond differently to treatment. However, in PAH treatment the influence of sex must be considered in both research and clinical settings.
Chapter 5

5 General Discussion
5.1. General Discussion

PH is an incurable vascular disease that is subdivided into five major categories; PAH, pulmonary hypertension due to left heart disease, pulmonary hypertension due to chronic lung disease and/or hypoxia, chronic thromboembolic pulmonary hypertension and pulmonary hypertension with unclear multifactorial mechanisms (Simonneau et al., 2013). Dysfunctional signalling pathways play a major role in the pathogenesis of PAH. Three examples include; endothelin (ET), nitric oxide (NO) and prostacyclin (PGI$_2$). Increased production of endothelin-1 (ET-1) and its major pre-cursor, preproendothelin-1 (preproET-1) in PAH patients were linked to increases in pulmonary vascular resistance (PVR) and the severity of structural abnormalities in small resistance pulmonary arteries (Stewart et al., 1991, Giaid et al., 1993, Bressollette et al., 2001). Giaid and Saleh demonstrated that greater vasoconstriction in PAH patient pulmonary arteries was associated with reduced endothelial nitric oxide synthase expression (Giaid and Saleh, 1995). PAH patients demonstrated reduced expression of prostacyclin and prostacyclin synthase (Christman et al., 1992, Tuder et al., 1999). The ET, NO and PGI$_2$ pathways are therefore considered to be the major targets of therapeutic intervention for PAH.

The greater number of females diagnosed with PAH is well described. An increased female predisposition to PAH was highlighted by Dresdale as early as the 1950s (Dresdale et al., 1951). Recent UK/Ireland and French epidemiology studies identified that 70% and 65% respectively of diagnosed patients were female (Ling et al., 2012, Montani et al., 2010). The increased incidence in PAH in females led to the investigation of estrogen as a causative factor in the development of PAH. This hypothesis is supported by studies showing that inhibition of the estrogen synthesising enzyme aromatase can reverse a PH phenotypes in hypoxic mice and sugen hypoxic rats (Mair et al., 2014). An initial clinical trial has demonstrated that treatment with anastrazole, which targets aromatase, increased 6MWD in treated patients vs placebo controls (Kawut et al., 2016). Removal of ovaries prevented development of PH in female-specific experimental models such as SERT+ and dexfenfluramine mice (White et al., 2011a, Dempsie et al., 2013). Alternatively, estrogen treatment has been found to reduce RVSP, RVH and remodelling in ovariectomised sugen hypoxic rats (Frump et al., 2015a). At higher doses, estrogen has also been found to inhibit proliferation in hPASMCs (Tofovic et al., 2008). These differing effects had led to the description of an estrogen paradox.

In the clinic, despite increased female incidence of PAH, male sex is a predictor of poorer survival (Humbert et al., 2010a). Greater survival in females has been attributed to better right ventricle adaptation after treatment specifically the right ventricle ejection fraction (Jacobs et al., 2014). Sex has been shown to influence clinical outcomes in two different therapies of PAH. 6-minute walk distances (6MWD) improvements were found to be greater in females after treatment with endothelin receptor antagonists than those
observed in men (Gabler et al., 2012). Conversely, the phosphodiesterase 5 inhibitor tadalafil was found to have better outcomes effects in males (Mathai et al., 2015). This was thought to be caused by lower male retention of NO thereby they would respond better to a NO raising therapy (Forte et al., 1998).

Here, we sought to examine the influence of sex on treprostinil in both a well described animal model of PH and in isolated human PASMCs from both control and PAH patients. The potential mechanisms by which the effects of treprostinil are mediated were also examined, as was the role of the prostaglandin receptors and BMPR-II signalling in mediating the effects of treprostinil.

In Chapter 3 the effects of two separate doses of treprostinil were examined in a well-defined animal model of PH. We observed difference in response to both treprostinil doses, where females had greater reduction in RVSP. Relating the results to clinical research is difficult due to the differences that exist in the chronic hypoxic model of PH versus PAH. The use of the monocrotaline (MCT) or sugen-5416 hypoxic (SU-Hx) models of PH would allow a closely matched comparison due to their similarities to human PAH (Colvin and Yeager, 2014). Also, as treprostinil is usually prescribed to late stage PAH disease, it may offer a better comparison with isolated PAH patient tissue. Due to the less severe PH that chronic hypoxia induces we were unable to measure survival however interestingly, female patients receiving the synthetic prostacyclin epoprostenol were found to have less hospitalisations and a greater survival rate than male patients suggesting a female bias (Frantz et al., 2015). It is unknown if this was due to a greater severity of disease in male patients or better treatment response in female patients, however this is one aspect of PAH that the chronic hypoxic model replicates. A common action of research in pre-clinical animal models of disease is to give a larger dose of a therapy than you would to human patients, this is due to differences in metabolic rates and physiological processes (Nair and Jacob, 2016). With our study, a comparison to clinical data can be made between the dose and levels of treprostinil in the blood. In Chapter 3, the blood plasma concentration of treprostinil ranges from 10pg/ml to 27pg/ml for the 100ng/kg/min study and 12pg/ml to 55pg/ml for the 400ng/kg/min study. Clinically, prescribed doses of treprostinil range from an initial dose of 1.25ng/kg/min, this can be increased incrementally to 40ng/kg/min, in some cases this can increase to 125ng/kg/min (Lang et al., 2006, McSwain et al., 2008). Blood plasma concentrations of treprostinil can range from 14.5pg/ml to 18,248pg/ml post treatment (McSwain et al., 2008). In the clinic, treprostinil doses can be adjusted due to changes in patient’s weight or if the current dose is not tolerated. However, the slow-release pellets do not allow for this adjustment. To treat rats with a dose that would closely resemble that of a human, Axelgaard and colleagues used 900ng/kg/min of treprostinil administered via osmotic mini-pumps (Axelgaard et al., 2016). This produced the closest blood plasma concentration to that observed in clinic (Axelgaard et al., 2016). It is also
important to observe that the rats treated with 900ng/kg/min also suffered from a significant
decrease in mean arterial pressure (MAP) (Axelgaard et al., 2016).

PGI$_2$ plays a key role in maintaining cardiovascular homeostasis, initially it was found to
mediate the anti-thrombotic properties of the endothelium (Moncada et al., 1977). The
vasoactive effects of PGI$_2$ also extend to the pulmonary vasculature although its levels are
known to be diminished in PAH patients (Christman et al., 1992). The effects of PGI$_2$ are
primarily mediated by the IP receptor. In Chapter 4 of this study, it was demonstrated that
the IP receptor plays a role in mediating the anti-proliferative effects of treprostinil, as well
partially accounting for the increases in Id protein expression in female control and PAH
patient hPASMCs. We also did not find any expression differences between sexes or
control/PAH patient groups to account for the different responses to treprostinil that were
observed. Although previous studies highlighted lower expression of the receptor in IPAH
patients (Lai et al., 2008, Falcetti et al., 2010). Functionality of receptors has long been of
interest in PAH research, this has included the study of many known receptor mutation
that are positively correlated with PAH development. These include the BMPR-II, KCNK3,
Angiotensin II type I receptor (AGTR1), ENG and ALK1 (Machado et al., 2009b). Indeed,
in this study we examined treprostinil in female and male PAH patients with BMPR-II
mutations. However, it may be possible that a reason for a sex difference in the response
to treprostinil could be due mutations in the IP or other prostaglandin receptor. Mutations
in the prostacyclin synthase gene have already been identified as protective in non-PAH
carriers of BMPR-II mutations (Stearman et al., 2014). Stitham and colleagues highlighted
that the polymorphism R212H located at the third intracellular loop of the receptor led to
significantly lessened cAMP response after iloprost stimulation (Stitham et al., 2002). The
EC$_{50}$ of the cAMP response was 6-fold higher for the IP receptor with R212H polymorphism
that a wild type IP receptor (Stitham et al., 2002). This polymorphism was latterly identified
as effecting arginine codons (Stitham et al., 2007). Interestingly, in a clinical setting an
arginine variant was associated with reduced response to a beta blocker and decrease G-
protein coupling (Liggett et al., 2006, Mason et al., 1999). The L104R and M113T
polymorphisms have been identified within the IP receptor, that disrupt the structural
integrity leading to protein misfolding (Stitham et al., 2011). Importantly, in Chapter 4 we
observed a role for the EP$_2$ receptor in mediating the effects of treprostinil both in
hPASMCs counts experiments and in the induction of the Id proteins of the BMPR-II
pathway, also that hPASMC EP$_2$ mRNA expression was higher than the other
prostaglandin receptors. Interestingly, it was thought that due to the 100% conservation
across the prostaglandin receptors, the L104R and M113T polymorphisms may affect
other receptors (Stitham et al., 2011). However, within the EP$_2$ receptor only one single
nucleotide polymorphism (SNP) has been implicated as a marker of essential hypertension
(Sato et al., 2007). Also, research into polymorphism in prostaglandin receptors usually
finds varying susceptibility in different races (Jinnai et al., 2004, Shimizu et al., 2013, Liang et al., 2016), while in this current study we cannot say if that played any part in the results we obtained. Within the current study it was not possible to identify any polymorphism in the receptors that were examined, so it is not possible to confirm if they are responsible for the differences in response to treprostinil. However, it would be crucial to identify any prostaglandin receptor mutations in the future as they could impact the effectiveness of all prostacyclin analogues not just treprostinil.

5.1.1. Targeting cAMP in PAH

This current study has proposed a link the anti-proliferative actions of treprostinil to cAMP, although cAMP levels were not measured in this study. The initial therapies for PAH included the use of either synthetic prostacyclin or prostacyclin analogues, which are cAMP elevating agents (Duarte et al., 2013). Classically, receptor expression was thought to be a primary influence on the effectiveness of an agonist (Milligan et al., 1998). In this study, no differences were observed in prostaglandin receptor expression that could account for the sex difference observed. For the generation of cAMP, the activation of a receptor like IP, EP\textsubscript{2} or DP\textsubscript{1} is only the initial step. Expression of the receptor/s does not give the complete stoichiometry of cAMP. To take this into account the levels of receptor occupancy, G-protein subunit, adenylyl cyclase (AC), adenosine triphosphate (ATP) and protein kinase A (PKA) should be considered. Alousi and colleagues reviewed the stoichiometry of the adenylyl cyclase pathway focusing on the \(\beta\)-adrenergic receptor in murine lymphoma cells. They highlighted that the ratio of receptor to G-protein subunit to adenylyl cyclase was \(1:100:3\) (Alousi et al., 1991). They concluded that the main limiting factors on efficacy was the receptor or AC, these results were later repeated in rat cardiomyocytes (Alousi et al., 1991, Post et al., 1995). More recently, heightened expression of the \(\beta\)-adrenergic receptor or the \(G_{\alpha}\) protein subunit only accounted for a small increase in cAMP maximum response in mouse cardiac tissue (Gaudin et al., 1995). Whereas other groups discovered that overexpression of AC6 increased the cAMP response and improved cardiac function through cAMP release via the \(\beta\)-adrenergic receptor (Gao et al., 1998, Roth et al., 1999). Although, induction of ACs and cAMP is intrinsically linked with the action of the \(G_{\alpha}\) subunit, the AC2 isoform has been shown to be activated by the \(G_{\beta\gamma}\) subunit (Federman et al., 1992, Pian and Dobbs, 1995). This could be a compensatory mechanism when other AC isoforms are inhibited by the \(G_{i}\) subunit. Within the context of PAH, specific AC isoforms have been identified as being important in cAMP regulation. Interestingly, Jourdan and colleagues found that hypoxia did not change the expression of ACs and that AC2, 3, 5 and 6 were found in smooth muscle cells from the pulmonary artery and bronchus (Jourdan et al., 2001). In Chapter 3 of our current study, we observed an increase in EP\textsubscript{2} receptor mRNA expression in female rats under hypoxic conditions with treprostinil treatment. This combined with Jourdan and colleagues’
observation that AC isoforms do not decrease in hypoxia may partially explain the increased effect we observed in female rats treated with treprostinil.

As previously stated, increasing cAMP signalling is a feature of synthetic and prostacyclin analogues therapies for PAH. Targeting cAMP directly may prove difficult as it is an important second messenger in many systems not just the pulmonary vasculature, potentially leading to an increased risk of side effects. In-directly targeting cAMP may offer more precise targets like specific isoforms of AC. Jourdan and colleagues discovered that the AC5 isoform plays a key role in mediating cAMP increases and anti-proliferative actions on rat PASMCs (Jourdan et al., 2001). NKH-477, a specific AC5 stimulator was also found to elevate cAMP levels greater than forskolin (Toya et al., 1998, Jourdan et al., 2001). To further avoid unwanted side-effects of universal drug dosing, adenovirus can be used to obtain tissue specificity (Robson and Hirst, 2003). Adenovirus containing AC5 was demonstrated to increase left ventricular function and increase cAMP levels in the hearts of pigs (Lai et al., 2000). Elevating AC6 expression has been previously proposed as way reducing endothelial cell permeability. Bundey and Insel linked overexpression of AC6 via an adenovirus to a reduction in endothelial barrier permeability through prostacyclin action (Bundey and Insel, 2006). An adenovirus expressing AC6 was also introduced to heart failure patients, where it increased LV function over standard heart failure therapies (Hammond et al., 2016). As endothelial dysfunction is hallmark of PAH development targeting AC6 may be a novel method of treating the disease and it may act a compliment to PGI$_2$ analogue therapy (Budhiraja et al., 2004). Additionally, AC6 overexpression co-localised with the IP receptor was linked to a reduction in cardiac fibroblast activity via a cAMP increase (Liu et al., 2008). Morecroft and colleagues demonstrated that adenoviruses can also knock-down mediators of PH, specifically tryptophan hydroxylase-1 (TPH-1) in pulmonary endothelial cells (Morecroft et al., 2012). As well as increase aspects of cAMP signalling adenoviruses could be used to knock-down PDEs, which breakdown cAMP. Knock-down of PDE2A by an adenovirus reduced alveolar inflammation and doubled the production of cAMP (Rentsendorj et al., 2011). As PDE1, specifically PDE1A and PDE1C, are known to be elevated and animal models of PH and PAH patients (Schermuly et al., 2007, Murray et al., 2007), it may be a novel target of adenovirus mediated knockdown. The combination of therapies specifically increasing aspects of cAMP signalling like AC with established prostacyclin analogues may open new routes in the treatment of PAH.

5.1.2. Links between BMPR-II signalling and cAMP: Where does treprostinil fit in?
The BMPR-II signalling pathway is an important therapeutic target in PAH. Targeting of BMP signalling is also a developing field within cancer research, with small molecule inhibitors used to reduce expression of Id genes (Anido et al., 2010). In Chapter 4 of this study, we demonstrated that treprostinil up-regulates both mRNA and protein expression
of downstream components of BMPR-II signalling namely Id1 and Id3. We did not completely identify the reason/s for the increase in Id expression however we hypothesise that the cAMP triggers the upregulation. The actions of cAMP on BMPR-II signalling has been explored in osteoblasts, through investigation into both cAMP-response elements (CREs), cAMP-response binding protein (CREB) and s BMP response elements (BREs) (Ohta et al., 2008). The Id1 gene was previously identified as having a CRE site in its promoter region (Korchynskyi and ten Dijke, 2002). The use of a siRNA targeting CREB led to a reduction in cAMP mediated enhancement of Smad-mediated transcription (Ohta et al., 2008). In Chapter 4 we did not identify a significant increase in Smad signalling accompanying Id increase therefore if cAMP was involved it may be bypassing Smad. In PAH context, Yang and colleagues investigated the effects of iloprost and treprostinil on Id1 expression. Both prostacyclin analogues increased expression of Id1 but not Smad 1/5/8 (Yang et al., 2010). Interestingly, Yang did not observe an increase in Id1 protein expression after 100nm treprostinil stimulation after 24 hours, however we did observe a change in female control at the same timepoint. This may due to protocol differences, although it emphasises the stronger response we observed in female hPASMCs. Id3 induction was a feature of treprostinil stimulation in our current study that has not observed before, however the link between cAMP and Id3 has not been studied directly within the PAH field. Research with PC12 cells isolated rat adrenal medulla identified that after stimulation with a cAMP-analogue, Id3 expression significantly increased suggesting that Id3 is a cAMP-responsive gene like Id1 (Ghzili et al., 2006). Also, overexpression of the oncoprotein Lyl1 was identified to increase expression of Id1 and Id3 through the action of CREB (San-Marina et al., 2008). With the previously discussed research we can estimate the link between the upregulation of cAMP with the increase in Id expression (Figure 5-1). We have examined the effects of treprostinil in one cell type, however in PAH both hPASMCs and pulmonary endothelial cells (PAECs) contribute to disease development (Zhang et al., 2003, Teichert-Kuliszewska et al., 2006). Clinically, treprostinil therapy increased the level of endothelial colony forming cells in children with PAH (Smadja et al., 2011, Smadja et al., 2015). Whereas, targeting BMPR-II signalling with BMP-9 in PAECs was associated with a reversal of PAH in three models of animal PAH (Long et al., 2015). An analysis that incorporates an investigation into the actions of treprostinil on BMPR-II signalling in PAECs may offer a chance to have a more complete understanding of the effects of treprostinil in the pulmonary vasculature.

5.1.3. Concluding remarks
In summary, through in-vivo and in-vitro investigations sex has been shown to have potential role in the therapeutic effects of treprostinil. The major novel observations from this study were that females have a greater response to treprostinil stimulation and that this is partially mediated through greater increases in BMPR-II signalling, specifically the
Id genes. Additionally, increase in BMPR-II signalling by treprostinil was mediated by activation of more than one prostaglandin receptor. Further research is required to confirm if the general hypothesis of the study is true. For future perspective, to account for greater survival amongst female PAH patients, the role of sex influencing therapeutic response must be considered.
Figure 5-1 Treprostinil mediates BMPR-II signalling through an increase in cAMP levels (Legend on next page)
Chapter Five General Discussion

Figure 5-1: Treprostinil mediates BMPR-II signalling through an increase in cAMP levels

Treprostinil activates Gαs coupled receptors (IP and EP2) triggering activation of adenyyl cyclase leading to an increase in cAMP. cAMP activates protein kinase A, which then translocates to the nucleus and activates cAMP response binding protein, which in turn binds to the cAMP response element in the promotor region of the Id genes. BMPR-II receptor activation can also lead to increase Id expression. Activation leads to phosphorylation of Smad 1/5/9. Smad 1/5/9 forms a complex with Smad4 that then translocates to the nucleus where it can regulate Id transcription through binding to transcription factors. cAMP= cyclic AMP, PKA=protein kinase A, EPAC= exchange factor directly activated by cAMP 1, CREB= cAMP response binding protein, CRE= cAMP response element, SMAD= Sma and MAD (mothers against decapentaplegic), TF= Transcription factor, Id= inhibitor of DNA binding.
Chapter Five General Discussion

5.2 Future Perspective

The primary goal of biological scientific research is the ability to translate attributes determined in the laboratory to a clinical setting where they can benefit patients. During this study, the effects of treprostinil were examined in female and male chronic hypoxic rats and in isolated hPASMCs from both control and PAH patients.

Treprostinil is a recommended therapy for PAH patients with WHO classification III and IV, (Galie et al., 2009b, Badesch et al., 2007). As the chronic hypoxic model of PH resembles early forms of group 1 human PH (PAH) but is also more reflective of group 3 human PH, it would be useful to confirm the effects of treprostinil in both female and males using the Su-Hx model of PH as it closely recapitulates late phase human PAH. Also, it would useful to confirm if the influence of sex exists in an animal model that has a greater relevance to PAH. In PAH, higher survival rates in women are attributed to the ability of the right ventricle to adapt to increasing pressure (Jacobs et al., 2014). The underlying molecular mechanisms in the RV should be investigated in the future. This could be achieved by IHC, qRT-PCR, western blot. Interestingly, Holbome and colleagues observed that treprostinil can improve RV function in healthy rats and that this improvement is lost in pulmonary artery banded rats (Holmboe et al., 2016). Recently, BMPR-II mutant patients were discovered to have lower RV function in comparison to non-mutant patients (Van Der Bruggen et al., 2016). However, there were no significant differences in BMPR-II signalling found in the cardiomyocytes of the heart (Van Der Bruggen et al., 2016).

Presently, we have identified the potential role of sex in influencing the anti-proliferative effects of treprostinil. Here, we have shown increased BMPR-II associated signalling attributed to treprostinil stimulation. However, recent research has identified that high levels of glucose can increase specific ubiquitin ligases (SMURF-1), which can disrupt canonical BMPR-II signalling (Barnes et al., 2016). As discussed earlier in this chapter, a greater examination of GPCRs signalling is required to fully understand the effects of treprostinil. This would help elucidate the reasons for the differences in anti-proliferative and BMPR-II signalling induction we observed in this study. Personalised medicine, where treatments are individually tailored to a specific patient’s circumstances, is a growing trend in the medical profession. In PAH, there are many underlying molecular processes that are not fully understood, this also extends to therapeutic interventions. This study suggests that in the drive towards personalised medicine the influence of sex on therapeutic intervention needs to be quantified.
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