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

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B.Sc (Hons). MRes.

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

College of Medical, Veterinary and Life Sciences

University of Glasgow

July/2015
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 where acknowledgement has been made. 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 and Professor A.H. Baker.
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What’s for you won’t go by you.
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<tbody>
<tr>
<td>16α-OHE1</td>
<td>16α-Hydroxyestrone</td>
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<td>2-OHE2</td>
<td>2-Hydroxyestradiol</td>
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<td>4-OHE2</td>
<td>4-Hydroxyestradiol</td>
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<td>5-CT</td>
<td>5-carboxamidotryptamine</td>
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<td>5-HIIA</td>
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<td>5-HT</td>
<td>5-Hydroxytryptamine</td>
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<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
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<tr>
<td>AF1</td>
<td>Activation function 1</td>
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<td>Argonaute 2</td>
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<tr>
<td>ALK-1</td>
<td>Activin receptor-like kinase 1</td>
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<tr>
<td>AMI</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>AMO</td>
<td>Anti-miRNA-oligonucleotides</td>
</tr>
<tr>
<td>ANG-II</td>
<td>Angiotensin-II</td>
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<tr>
<td>APAH</td>
<td>Associated pulmonary arterial hypertension</td>
</tr>
<tr>
<td>APO-E</td>
<td>Apolipoprotein-E</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BHLH</td>
<td>Basic helix loop helix</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BMPR-II</td>
<td>Bone morphogenic protein receptor type II</td>
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<tr>
<td>Bp</td>
<td>Base pair</td>
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<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
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<td>Ca^{2+}</td>
<td>Calcium</td>
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</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>Chloride</td>
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<td>CO₂</td>
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<td>DHT</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<tr>
<td>DNA</td>
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<tr>
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<td>Endothelial cell</td>
</tr>
<tr>
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<td>Electrocardiogram</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<tr>
<td>ERA</td>
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GPCRs  G-protein-coupled-receptors
GPER  G protein-coupled estrogen receptor 1
GPR30  G-protein coupled receptor 30
GTP  Guanosine 5’ triphosphate
HCV  Hepatitis C viral
HHT  Hemorrhagic telangiectasia type 2
HPAH  Heritable pulmonary arterial hypertension
hPASMCs  Human pulmonary artery smooth muscle cells
HPG  Hypothalamic-pituitary-gonadal
HPV  Hypoxic pulmonary vasoconstriction
HR  Heart rate
HRP  Horse radish peroxidase
HRT  Hormone replacement therapy
HSD  3β- hydroxysteroid dehydrogenase
HSP  Heat shock protein
Id  Inhibitor of DNA
IgG  Immunoglobulin G
IL-6  Interleukin-6
IP3  Inositol-1, 4, 5 triphosphate
IPAH  Idiopathic pulmonary arterial hypertension
I-SMAD  Inhibitory SMAD
Jak/STAT  Janus kinase/signal transducers and activators of transcription
JNK1/2/3  c-Jun amino (N)-terminal kinases 1/2/3
K+  Potassium
kb  Kilobases
KCNK3  Potassium channel subfamily K, member 3
KO  Knockout
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kv</td>
<td>Voltage dependent potassium channels</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>LNA</td>
<td>Locked-nucleic-acid</td>
</tr>
<tr>
<td>IncRNA</td>
<td>Long non-coding RNA</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>M.SssI</td>
<td>CpG methyltransferase</td>
</tr>
<tr>
<td>MAD</td>
<td>Mothers against decapentaplegic</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCT</td>
<td>Monocrotaline</td>
</tr>
<tr>
<td>MEK1/2</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MOA</td>
<td>Monoamine oxidase-A</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxide</td>
</tr>
<tr>
<td>α-sma</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chains</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin-light-chain-kinase</td>
</tr>
<tr>
<td>mPAP</td>
<td>Mean pulmonary arterial pressure</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MTS1</td>
<td>Calcium binding protein S100A4/Mts1</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium</td>
</tr>
<tr>
<td>NIH</td>
<td>National institutes of health</td>
</tr>
<tr>
<td>ncRNA</td>
<td>Non-coding ribonucleic acid</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>NYHA</td>
<td>New York Heart Association</td>
</tr>
<tr>
<td>NBF</td>
<td>Neutral buffered formalin</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>p21</td>
<td>Cyclin-dependent kinase inhibitor 1A</td>
</tr>
<tr>
<td>PACT</td>
<td>Protein activator of PKR</td>
</tr>
<tr>
<td>PAEC</td>
<td>Pulmonary artery endothelial cell</td>
</tr>
<tr>
<td>PAH</td>
<td>Pulmonary arterial hypertension</td>
</tr>
<tr>
<td>PAP</td>
<td>Pulmonary arterial pressure</td>
</tr>
<tr>
<td>PASMC</td>
<td>Pulmonary artery smooth muscle cell</td>
</tr>
<tr>
<td>p-bodies</td>
<td>Processing bodies</td>
</tr>
<tr>
<td>PCPA</td>
<td>P-chlorophenylalanine</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PDCD4</td>
<td>Programmed cell death 4</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PGI₂</td>
<td>Prostaglandin I₂</td>
</tr>
<tr>
<td>PGIS</td>
<td>Prostacyclin synthase</td>
</tr>
<tr>
<td>PH</td>
<td>Pulmonary Hypertension</td>
</tr>
<tr>
<td>PKG</td>
<td>cGMP-Dependent Protein Kinases</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLB</td>
<td>Passive lysis buffer</td>
</tr>
<tr>
<td>PPHTN</td>
<td>Portopulmonary Hypertension</td>
</tr>
<tr>
<td>PPHN</td>
<td>Persistent pulmonary hypertension of the newborn</td>
</tr>
<tr>
<td>pre-miRNA</td>
<td>Precursor miRNA</td>
</tr>
<tr>
<td>pri-miRNA</td>
<td>Primary miRNA</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real time-polymerase chain reaction</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PVR</td>
<td>Pulmonary vascular resistance</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>REVEAL</td>
<td>Registry to evaluate early and long-term PAH disease management</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>R-SMAD</td>
<td>Receptor regulated SMAD</td>
</tr>
<tr>
<td>RHC</td>
<td>Right heart catheterisation</td>
</tr>
<tr>
<td>RV</td>
<td>Right ventricle</td>
</tr>
<tr>
<td>RVH</td>
<td>Right ventricular hypertrophy</td>
</tr>
<tr>
<td>RVP</td>
<td>Right ventricular pressure</td>
</tr>
<tr>
<td>RVSP</td>
<td>Right ventricular systolic pressure</td>
</tr>
<tr>
<td>RV/LV+S</td>
<td>Right ventricle/ Left ventricle + septum</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio immuno precipitation assay</td>
</tr>
<tr>
<td>RQ</td>
<td>Relative quantification</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>Ser/Thr</td>
<td>Serine/threonine</td>
</tr>
<tr>
<td>S-phase</td>
<td>Synthesis phase</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>SERT</td>
<td>Serotonin transporter</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SHP</td>
<td>Short heterodimer partner</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Na(^+)/Cl(^-)</td>
<td>Sodium/chloride</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------------</td>
<td>----------------------------------------------------------------</td>
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<tr>
<td>SMAD</td>
<td>SMA and Mothers against decapentaplegic</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>SMURF</td>
<td>SMAD ubiquitination and regulatory factor</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin re-uptake inhibitor</td>
</tr>
<tr>
<td>SERT+</td>
<td>SERT over-expression</td>
</tr>
<tr>
<td>SM22-5-HTT+</td>
<td>SERT over-expression in smooth muscle cells</td>
</tr>
<tr>
<td>STAT-3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>SU-HX</td>
<td>Sugen-Hypoxic</td>
</tr>
<tr>
<td>mSAP</td>
<td>Mean systemic arterial pressure</td>
</tr>
<tr>
<td>SAP</td>
<td>Systemic arterial pressure</td>
</tr>
<tr>
<td>TA2</td>
<td>Thromboxane A2</td>
</tr>
<tr>
<td>TASK-1</td>
<td>TWK-related acid sensitive potassium channel</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TPH</td>
<td>Tryptophan hydroxylase</td>
</tr>
<tr>
<td>TRBP</td>
<td>Transactivating response dsRNA-binding protein</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline tween</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetra-methylbenzidine</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</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>vWF</td>
<td>Von williebrand factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
</tr>
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</table>
Abstract

Women develop pulmonary arterial hypertension (PAH) more frequently than men suggesting that female sex and/or female sex hormones i.e. estrogens play a role in disease pathogenesis. Building evidence also implicates a role for microRNAs (miRNAs) in PAH. Little is known surrounding the interplay between sex/estrogens and miRNAs in PAH. Examining the sexual dymorphism in miRNAs with regards to PAH disease may provide insight into the sex bias observed in PAH.

Loss-of-function BMPR-II mutations underlie heritable PAH. Here, we showed that in pulmonary artery smooth muscle cells (PASMCs) explanted from a pulmonary hypertensive mouse model with a knock-in BMPR-II mutation (BMPR-II$^{R899X+/}$) there were differences in miRNA expression between sexes. Among the 20 miRNAs examined, 9 miRNAs exhibited significant change in expression within female BMPR-II$^{R899X+/}$ compared to female wild-type (WT) PASMCs but remained unchanged in male PASMCs. Of interest miRNA-96 demonstrated significant down-regulation in female BMPR-II$^{R899X+/}$ but remained unchanged in male BMPR-II$^{R899X+/}$. In silico prediction software demonstrates the 5-HT$_{1B}$ receptor as a putative target of miRNA-96. The 5-HT$_{1B}$ receptor has previously been implicated in PAH development as it is thought to play a role in pulmonary artery vasoconstriction and pulmonary artery remodelling, two major hallmarks of PAH. To verify if 5-HT$_{1B}$ was a true target of miRNA-96 we carried out a 3’UTR luciferase reporter assay. Indeed we found over-expression of miRNA-96 could down-regulate the luciferase output of the luciferase reporter construct containing the 3’UTR of the 5-HT$_{1B}$ receptor. The down-regulation of miRNA-96 within the female BMPR-II$^{R899X+/}$ mouse PASMCs was associated with a concomitant increase in 5-HT$_{1B}$ mRNA and protein. This expression pattern was re-iterated in PASMCs explanted from female PAH patients. Here, we found female PAH patients had a decrease in miRNA-96 expression and an increase in 5-HT$_{1B}$ mRNA and protein but again expression remained unchanged in male patients compared to non-PAH controls. Interestingly we also found only female PAH patient PASMCs were proliferative to the mitogen serotonin (5-HT). This could be explained by the expression pattern of 5-HT$_{1B}$ observed. Next we examined the effect of miRNA-96 over-expression in human PASMCs. Here, we demonstrated that over-expressing miRNA-96 had the ability to reduce 5-HT$_{1B}$ protein expression; however, mRNA expression remained unchanged. In addition, we found that over-expressing...
miRNA-96 prevented serotonin-induced proliferation in PASMCs from female PAH patients.

We have previously shown a relationship between estrogen and 5-HT$_{1B}$ as 17β-estradiol, the main pre-menopausal circulating estrogen, increases the protein expression of 5-HT$_{1B}$ in human PASMCs. Here, we examined the effect of 17β-estradiol on miRNA-96 expression. We found that 17β-estradiol decreased miRNA-96 expression suggesting 17β-estradiol’s effect on 5-HT$_{1B}$ expression could be mediated through a decrease in miRNA-96. To determine if endogenous 17β-estradiol also influences miRNA-96 expression we assessed the expression of miRNA-96 and 5-HT$_{1B}$ expression within whole lung homogenates from female and male mice that had been dosed with an aromatase (estrogen synthesising enzyme) inhibitor anastrozole. These mice have depleted circulating and local lung synthesis of estrogen, and elevated BMPR-II signalling. MiRNA-96 was elevated in the lungs from the estrogen-depleted female mice and this was accompanied by a decrease in 5-HT$_{1B}$ mRNA expression. No changes in miRNA-96 and 5-HT$_{1B}$ mRNA expression were observed within male lung tissue. These results further implicated a role for estrogen in regulating miRNA-96 and subsequently 5-HT$_{1B}$.

As over-expression of miRNA-96 in cell culture prevented a proliferative phenotype in human PASMCs we sought to assess the effect of over-expressing miRNA-96 in vivo. We utilised both the BMPR-II$^{R899X+/−}$ and hypoxic mouse model of pulmonary hypertension (PH) to examine whether a miRNA-96 mimic could both reverse and prevent a PH phenotype. The miRNA-96 mimic was administered intravenously via the tail vein once a week for 2 weeks using the MaxSuppressor$^\text{TM}$ In Vivo RNA-LANCEr II delivery method. We first confirmed that in both of these models miRNA-96 was significantly depleted in the lungs of diseased mice. Secondly we showed that intravenous injection delivered miRNA-96 mimic to the pulmonary arteries. Our in vivo results demonstrated that dosing with miRNA-96 mimic reduced the right ventricular systolic pressure (RVSP), right ventricular hypertrophy (RVH) and % of remodelled vessels in hypoxic and BMPR-II$^{R899X+/−}$ female mice. Mice dosed with a negative control mimic showed no effect on PAH indices. Interestingly we also showed that hypoxic and BMPR-II$^{R899X+/−}$ mice dosed with miRNA-96 mimic had a reduction in 5-HT$_{1B}$ protein expression compared to those dosed with negative control mimic.

This is the first study to observe sexual dimorphism in miRNA expression with regards to PAH. We have provided novel data demonstrating how miRNA-96, under the potential...
influence of estrogen, plays a role in the development of PH in a sex-dependent manner, by regulating 5-HT$_{1B}$ expression and serotonin-induced proliferation. Restoring depleted miRNA-96 levels may present a novel therapeutic approach in PAH.
Chapter One

1. Introduction
1.1 The Pulmonary Circulation

1.1.1 Development of the Pulmonary Circulation

Throughout embryological development the pulmonary circulation is being continuously prepared for a maturation and transition phase which occurs at birth. Development and growth of the pulmonary vasculature involves both the initial formation of blood vessels and their subsequent outward branching in vasculogenic and angiogenic processes respectively (deMello et al., 1997). Endothelial cells (ECs) initiate the formation of tube like vessels. The presence of growth factors e.g. vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β) have a fundamental role in mediating and shaping pulmonary vascular development. Abnormalities in either of their signalling pathways have detrimental consequences in both the development and maintenance of the normal pulmonary circulation (Morrell et al., 2001; Grover et al., 2003). ECs play a role in attracting mesenchymal cells which can then differentiate into smooth muscle cells (SMCs) and contribute to the architecture of the lung vasculature (Hall et al., 2000). Further vessel branching encompasses migration and proliferation of both ECs and SMCs until a dense vascular network is present and ready to function in one of the body’s most vital tasks: gaseous exchange. ECs from different tissue vascular beds are thought to originate from distinct embryological sites and have a heterogeneous population with specific phenotypes (Stevens et al., 2001). Distinct populations can also exist for SMCs (Frid et al., 1994). The embryonic origins of proximal and distal pulmonary arteries are different with proximal pulmonary arteries deriving from the truncus arteriosus and distal pulmonary arteries deriving from the mesenchymal primary capillary plexus (Hall et al., 2000). Tissue specific phenotypes of both ECs and SMCs can contribute to the observed differences to stimuli, such as hypoxia in the pulmonary vs. systemic circulations (Stevens et al., 2001).

1.1.2 The Structure of the Pulmonary Circulation

In comparison to the systemic vasculature where thick-walled high pressure arteries distribute oxygenated blood to peripheral tissues, the pulmonary vasculature consists of thin-walled low pressure arteries which carry deoxygenated blood to terminal alveoli. In the pulmonary arteries systolic pressure is ~25 mmHg compared to 120 mmHg in systemic arteries. Branching from the right ventricle (RV) of the heart the main pulmonary artery bifurcates into the right and left pulmonary arteries before entering the parenchyma of the right and left lung respectively. The pulmonary arteries continue branching, tracing the
course of the bronchial tree until the terminal alveoli at the periphery of the lung pleural are reached. Pulmonary arteriograms demonstrate there are 15 orders of pulmonary artery (Huang et al., 1996). The orders are categorised using the Strahler ordering system starting from the peripheral pre-capillary arteries, termed order 1, up to the main pulmonary artery, termed order 15. Order 1 pulmonary arteries are ~20 μm in diameter compared to order 15 pulmonary arteries which are ~15.12 mm in diameter (Huang et al., 1996). From the main pulmonary artery (order 15), there are a predicted ~72 million order 1 pulmonary arteries arising, forming the basis of an extensive capillary network structure in conjunction with alveolar ducts to ensure optimal gaseous exchange (Huang et al., 1996).

Similarly to systemic arteries, pulmonary arteries have three concentric layers within the vascular wall however differences within the composition of these layers do exist between systemic arteries. The inner most layer of the vessel is termed the tunica intima and consists of a single layer of ECs which line the lumen of the vessel. The ECs are in constant contact with blood and therefore vulnerable to insult from shear stress and circulating mediators. ECs can act as environmental sensors, working in conjunction with underlying SMCs to adapt the artery to the environment accordingly i.e. mechanical stretch and hypoxia increases serotonin synthesis and release from ECs which can act in a paracrine fashion to SMCs to elicit an effector response e.g. vasoconstriction (Morecroft et al., 1999; Pan et al., 2006). The tunica intima is separated from the middle layer by an internal elastic lamina. The middle layer of the vessel is termed the tunic media and consists of SMCs arranged around the circumference of the vessel. In between the tunica media and the tunica adventitia, the outer most layer, is the external elastic lamina. The tunica adventitia consists of fibroblasts and collagen fibres important for vessel stability and is tethered to the corresponding segment of the bronchial tree. As the pulmonary circulation is a low resistance, low pressure circuit the need for highly muscularised arteries is not required. Throughout the 15 orders of pulmonary artery the structure of the artery wall changes with regards to the distribution of the elastic lamina and the degree of muscularity. The pulmonary arteries in order 15-13 have an abundant elastic portion within their tunica media to aid vessel compliance (Elliott et al., 1965). In orders 13-4 there is a higher presence of SMCs within the tunica media, however, the continuous layer of SMCs gradually becomes more sparsely distributed as the branch extends and continues down to order 4 (Elliott et al., 1965; Jones et al., 2011). These pulmonary arteries are termed muscularised and partially muscularised accordingly. Further to this the pulmonary arteries transition into non-muscularised pulmonary arteries in orders 3-1 upon reaching the
capillary network (Jones et al., 2011). The most peripheral distal non-muscularised pulmonary arteries consist of a single thin elastic lamina lined by ECs. Pericytes are also known to be present within the most distal non-muscularised pulmonary arteries. Pericytes are undifferentiated perivascular cells which can contribute to the remodelling of a pulmonary artery by transforming into SMCs and by excreting collagen fibres (Wu et al., 1995; Ricard et al., 2014).

### 1.1.3 The Physiology of the Pulmonary Circulation

During foetal life gaseous exchange between the alveoli and pulmonary arteries is not required and the pulmonary vasculature is considered a hypoxic environment. In utero blood is oxygenated through the placenta. As a result there is low oxygen tension upon the pulmonary vasculature contributing to vasoconstriction of pulmonary arteries and a high pulmonary vascular resistance (PVR). In utero, when blood returns to the right atrium of the heart the majority of blood flow bypasses the pulmonary circulation crossing the foramen ovale (FO) into the left side of the heart. Additionally, blood within the pulmonary artery can directly move into the aorta via the ductus arteriosus (DA). Lung tissue is adequately perfused through bronchial arteries which branch from the aorta. Upon birth and intake of the first breath, the oxygen tension increases, the medial layer of the distal pulmonary arteries thins, the PVR decreases and the FO and DA close forcing blood from the RV to flow into the pulmonary artery. No longer a hypoxic environment the pulmonary arteries fully dilate and partake in gaseous exchange with terminal alveoli. If the FO and DA do not successfully close and the PVR remains high after birth, persistent pulmonary hypertension of the newborn (PPHN) can result. Vasoconstriction of the pulmonary arteries in response to hypoxia (hypoxic pulmonary vasoconstriction, HPV) in utero contributes to the hypoxic response in adult pulmonary arteries. In contrast, systemic arteries dilate in response to hypoxia.

After birth the pulmonary vasculature becomes responsible for carrying deoxygenated blood returning to the right side of the heart to the pulmonary capillary system before transporting oxygenated blood to the left side of the heart where it is subsequently distributed throughout the systemic peripheral vasculature. The fundamental role of re-oxygenation is sophisticatedly engineered by the integration of the dense pulmonary capillary network with terminal alveoli. At this interface gaseous exchange occurs via diffusion. As the partial pressure of oxygen (O\textsubscript{2}) in the terminal alveoli is higher than in the pulmonary capillaries, O\textsubscript{2} received by inspiration diffuses down the pressure gradient and into the
blood. Likewise as the partial pressure of carbon dioxide (CO$_2$) is higher in the pulmonary capillaries compared to terminal alveoli, it diffuses to the alveoli and is removed upon expiration.

1.1.4 The Regulation of Pulmonary Vascular Tone

In pulmonary arteries the basal vascular tone is low to facilitate a low resistance, low pressure and high flow circuit. This is as a result of a thinner layer of SMCs in comparison to the systemic circulation where a high vascular tone exists. At rest the major contributor to pulmonary vascular tone (or the resting membrane potential) is the activity of potassium channels within pulmonary artery smooth muscle cells (PASMCs). In human PASMCs, the voltage dependent potassium channels (Kv) are largely responsible for deciding the resting membrane potential (Yuan, 1995). When the cell membrane depolarises the voltage sensor within the Kv triggers the Kv channel to open to allow potassium ions (K$^+$) to flow out of the cell and restore the membrane potential. With regards to PASMCs this restores a low vascular tone. Inhibition of Kv channels initiates a sustained increase in membrane potential allowing calcium (Ca$^{2+}$) channels to remain open. This leads to increased intracellular concentrations of Ca$^{2+}$ resulting in sustained vasoconstriction of PASMCs. Hypoxia has shown to inhibit Kv contributing to the vasoconstrictive phenotype observed in PASMCs by hypoxia. Interestingly PASMCs from pulmonary arterial hypertension (PAH) patients exhibited dysfunctional Kv channels which resulted in more depolarised PASMCs and contributed to enhanced vasoconstriction in PASMCs from PAH patients vs. non-patients (Yuan et al., 1998). Further to this a loss of function mutation within the potassium channel KCNK3 has recently been identified in hereditary forms of PAH (HPAH) (Ma et al., 2013). Although a different class of K$^+$ channel than Kv, it further emphasises the importance K$^+$ movement has on pulmonary vascular tone.

As briefly mentioned above Ca$^{2+}$ mobilisation also has an influence on pulmonary vascular tone. PASMC contraction and relaxation cycle is determined by free intracellular Ca$^{2+}$ levels. Influx of Ca$^{2+}$ can occur via different routes including voltage-gated Ca$^{2+}$ channels. When the cell membrane is depolarised voltage-gated Ca$^{2+}$ channels open allowing the entry of Ca$^{2+}$. Intracellular Ca$^{2+}$ can initiate further Ca$^{2+}$ release from the sarcoplasmic reticulum resulting in a further increase in free Ca$^{2+}$ levels. Ca$^{2+}$ then exerts function through interaction with the Ca$^{2+}$-binding protein calmodulin. Ca$^{2+}$-calmodulin then binds and activates the kinase, myosin-light-chain-kinase (MLCK) which subsequently phosphorylates myosin light chains (MLC). A conformational change occurs and allows
cross-bridge interaction between MLC and contractile actin filaments leading to contraction of the PASMC. This action is terminated by myosin phosphatase which initiates relaxation of the PASMC.

Pulmonary artery endothelial cells (PAECs) are additionally an important regulator of pulmonary vascular tone. As briefly mentioned ECs function to sense and mediate signalling to underlying SMCs. Communication between both cell types occurs via the release of vasoactive mediators which can be categorised into those which result in either vasoconstriction or vasodilation of PASMCs. Prostanoids including prostaglandin I\textsubscript{2} (PGI\textsubscript{2}, prostacyclin) and nitric oxide (NO) are both synthesised within PAECs and cause vascular relaxation. Another factor secreted by PAECs is endothelin-1 (ET-1) which is a chief vasoconstrictor of PASMCs. Orchestrating a fine balance between the vasoactive mediators is critical to ensure normal vascular homeostasis. This control from PAECs can be disrupted as a result of insult to the endothelial layer e.g. shear stress or hypoxia. This disruption is characteristic of many systemic and pulmonary vascular pathologies.

### 1.2 Pulmonary Hypertension

A dense and complete pulmonary vascular network is vital in ensuring sufficient gaseous exchange to meet the energy requirements of the body. A diminished pulmonary vascular network can occur via either loss or obstruction of pulmonary arteries. Pulmonary hypertension (PH) is the term given to a group of progressive, and presently incurable, vasculopathies affecting the pulmonary arteries that can ultimately result in their obliteration. In simple terms, PH concerns an increase in the pulmonary arterial pressure (PAP). Normally a low pressure circuit, an elevation in PAP can contribute towards and trigger a cascade of pathogenic characteristics with fatal consequences.

#### 1.2.1 Clinical Definition and Diagnosis

Within the clinic PH is defined by a mean PAP (mPAP) of >25 mmHg at rest and >30 mmHg during exertion (Badesch et al., 2009). The registry to evaluate early and long-term PAH disease management (REVEAL) documented an mPAP of ~50 mmHg within PAH patients (Badesch et al., 2010). This is in comparison to a normal mPAP of ~14-20 mmHg. PH can exist in many different forms (Table 1.1). However, most patients will typically present at clinic with symptoms including dyspnoea (breathlessness), dizziness and fatigue which are all suggestive of an insufficient oxygen supply to demand. These symptoms are ambiguous and not specific to PH and as symptoms usually present at
advanced stages of disease there is delayed disease onset time to diagnosis time. Due to the multiple classifications of PH, a more specific diagnosis is determined after a series of investigations which can include electrocardiogram (ECG), echocardiography, ventilation/perfusion pulmonary scan, pulmonary angiography, cardiac magnetic resonance imaging (MRI), blood tests, abdominal ultrasound scan and finally, right heart catheterisation (RHC) and vasoreactivity test. RHC is used to assess patients with an unknown cause of PH and is currently the gold standard diagnostic test to confirm the classification of pulmonary arterial hypertension (PAH). If a patient is diagnosed with PAH they will routinely undergo a vasoreactivity test to examine the potential effectiveness of calcium channel blockers (CCBs) as a therapeutic strategy. This evaluation involves challenging the patient with a vasodilator compound e.g. inhalational nitric oxide (NO), intravenous adenosine or intravenous prostacyclin. A positive acute vasodilator response is defined as a reduction in mPAP ≥10mmHg and is only observed within 5-10% of patients (Sitbon et al., 2005).

1.2.2 WHO and NYHA Classification

The fifth world symposium on PH was documented in 2013 detailing updated recommendations in clinical classifications (Table 1.1). Each clinical classification shares common pathological features however the purpose of categorising the disease into groups aids identification of disease aetiology, patient prognosis and therapeutic management. Irrespective of disease aetiology PH patients are additionally classified functional to give a standardised measure of disease severity. Functional classification integrates patient symptoms with patient limitations and is derived from the New York Heart Association (NYHA) scale on heart failure (Table 1.2). Functional classes I-IV rank PH patients physical abilities from no daily limitations (class I) to complete incapacity even at rest (class IV). Patients within class III represent a widely heterogeneous PH population with an extensive range of abilities and limitations and therefore caution must be given when extrapolating data from this group. The NYHA scale is beneficial in recommending treatment selection and is a broadly accepted predictor of patient survival.
Table 1.1 World Health Organisation Classification of Pulmonary Hypertension

<table>
<thead>
<tr>
<th>Dana Point Clinical Classification of Pulmonary Hypertension Updated 2013</th>
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<tbody>
<tr>
<td>1. Pulmonary arterial hypertension (PAH)</td>
</tr>
<tr>
<td>1.1 Idiopathic PAH (IPA)</td>
</tr>
<tr>
<td>1.2 Heritable (HPAH)</td>
</tr>
<tr>
<td>1.2.1 BMPR-II</td>
</tr>
<tr>
<td>1.2.2 ALK-1, ENG, SMAD9, CAV1, KCNK3</td>
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<tr>
<td>1.2.3 Unknown</td>
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<tr>
<td>1.3 Drug- and toxin-induced</td>
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<tr>
<td>1.4 Associated PAH (APA)</td>
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<tr>
<td>1.4.1 Connective tissue diseases</td>
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<tr>
<td>1.4.2 HIV infection</td>
</tr>
<tr>
<td>1.4.3 Portal Hypertension (PPHTN)</td>
</tr>
<tr>
<td>1.4.4 Congenital heart diseases</td>
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<tr>
<td>1.4.5 Schistosomiasis</td>
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<tr>
<td>1.4.6 Chronic haemolytic anemia</td>
</tr>
<tr>
<td>1. Pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary hemangiomatosis (PCH)</td>
</tr>
<tr>
<td>1” Persistent pulmonary hypertension of the newborn (PPHN)</td>
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<tr>
<td>2. Pulmonary hypertension owing to left heart disease</td>
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<tr>
<td>2.1 Systolic dysfunction</td>
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<tr>
<td>2.2 Diastolic dysfunction</td>
</tr>
<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 owing to lung diseases and/or hypoxia</td>
</tr>
<tr>
<td>3.1 Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>3.2 Interstitial lung disease</td>
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<tr>
<td>3.3 Other pulmonary diseases with mixed restrictive and obstructive pattern</td>
</tr>
<tr>
<td>3.4 Sleep-disordered breathing</td>
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<tr>
<td>3.5 Alveolar hypoventilation disorders</td>
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<tr>
<td>3.6 Chronic exposure to high altitude</td>
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<tr>
<td>3.7 Developmental lung diseases</td>
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<tr>
<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 hemolytic anemia, myeloproliferative disorders, splenectomy</td>
</tr>
<tr>
<td>5.2 Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis</td>
</tr>
<tr>
<td>5.3 Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders</td>
</tr>
<tr>
<td>5.4 Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure, segmental PH</td>
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</tbody>
</table>


1.2.3 Epidemiology of Pulmonary Arterial Hypertension

The epidemiology of PAH has been revised over the years due to new and advanced data from more recent longitudinal studies and centralised registries (Humbert et al., 2006; Badesch et al., 2010; Ling et al., 2012). In the United Kingdom (U.K.) and Ireland registry and the French registry the incidence is 1.1 and 2.4 cases per million respectively (Humbert et al., 2006; Ling et al., 2012). A national institutes of health (NIH) study conducted in the 1980’s showed the PAH patient had a mean age of 36 years with 62.5% of patients being female (female to male ratio was 1.7:1) (Rich et al., 1987b). Within the most recent U.K. and Ireland registry, Ling and colleagues collated data from all 8 PAH centres and found the mean age was 50 years with 70% of patients being female (Ling et al., 2012). In agreement with this recent data, both the French and REVEAL registries show an older mean age of 50 and 53 years respectively with 65% and 80% of patients being female (Humbert et al., 2006; Badesch et al., 2010). The reasons underlying the high female to male ratio regarding PAH prevalence remain relatively obscure and the female susceptibility in PAH is the focus of much current research. Interestingly the 5 year mortality rate has reduced over the years from 66% in the NIH registry to 40% in the U.K. and Ireland registry (Rich et al., 1987b; Ling et al., 2012).

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### Table 1.2 New York Heart Association Classification of Functional Status of Patients with Pulmonary Hypertension

<table>
<thead>
<tr>
<th>Class</th>
<th>Description</th>
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<tbody>
<tr>
<td>Class I</td>
<td>Patients with pulmonary hypertension but without resulting limitation of physical activity. Ordinary physical activity does not cause undue dyspnea or fatigue, chest pain or near syncope.</td>
</tr>
<tr>
<td>Class II</td>
<td>Patients with pulmonary hypertension with slight limitation of physical activity. They are comfortable at rest. Ordinary physical activity causes undue dyspnea or fatigue, chest pain or near syncope.</td>
</tr>
<tr>
<td>Class III</td>
<td>Patients with pulmonary hypertension with marked limitation of physical activity. They are comfortable at rest. Less than ordinary physical activity causes undue dyspnea or fatigue, chest pain or near syncope.</td>
</tr>
<tr>
<td>Class IV</td>
<td>Patients with pulmonary hypertension with inability to carry out any physical activity without symptoms. These patients manifest signs of right heart failure. Dyspnea and/or fatigue may even be present at rest. Discomfort is increased by any physical activity.</td>
</tr>
</tbody>
</table>
1.2.4 Pathobiology of Pulmonary Arterial Hypertension

PAH is categorised within the group 1 classification and includes idiopathic PAH (IPAH), heritable PAH (HPAH), drug- and toxin-induced PAH, associated PAH (APAH) and persistent pulmonary hypertension of the newborn (PPHN). The peripheral and most distal pulmonary arteries of the lungs are responsible for the initial genesis of disease. As discussed earlier, under normal physiological conditions these pulmonary arteries have an absence of SMCs with only a monolayer of ECs and elastic lamina. Evidence from both clinical and experimental data highlights within the origins of PAH there is a pathogenic transformation within the EC layer. This detrimental alteration can be triggered by either exogenous or endogenous insult. For example, exogenous stimuli could arise from either circulating mediators or a hypoxic environment. PAECs exposed to hypoxia demonstrate reduced structural and functional plasma membranes (Block et al., 1989). Furthermore, circulating reactive oxygen species (ROS) have also been documented to reduce EC membrane integrity (Thies et al., 1991). Or endogenously through heritable genetic mutations which could trigger the switching on/off of genes with fatal consequences. The majority of HPAH patients present with a loss of function mutation within the bone morphogenic protein receptor type II (BMPR-II) gene (Deng et al., 2000; International et al., 2000). Loss of BMPR-II in PAECs increases the level of PAECs apoptosis which can then expose underlying PASMCs (Teichert-Kuliszewska et al., 2006). Conversely, but equally detrimental, loss of BMPR-II signalling in PASMCs leads to their aberrant proliferation (Zhang et al., 2003). A dysfunctional endothelial layer can lead to the release of either vasoconstrictors or mitogens which can act in a paracrine fashion to neighbouring PAECs or underlying PASMCs in the medial layer of the pulmonary artery. For example, ET-1 is synthesised in PAECs and expression is increased in PAH patients (Giaid et al., 1993). Abluminal release of ET-1 from PAECs to underlying PASMCs can trigger sustained vasoconstriction and mediate proliferative processes (MacLean et al., 1994; Davie et al., 2002). In addition the dysfunctional ECs may cease to produce vasodilatory and anti-proliferative mediators. It is demonstrated that levels of NO production are attenuated in PAECs from IPAH patients compared to PAEC from healthy controls (Giaid et al., 1995; Xu et al., 2004). NO is a potent vasodilator and has anti-proliferative properties therefore its loss can augment vasoconstriction and remodelling processes of pulmonary arteries. The loss of the EC barrier and the change in the balance of pathological vs. physiological mediators creates a destructive micro-environment within the distal pulmonary arteries. In normal physiology the peripheral distal pulmonary arteries lack a defined SMC layer. However, during the onset and development of PAH,
dysfunctional ECs can influence pericytes present in the basement membrane to differentiate into SMCs (Ricard et al., 2014). Further to this PASMCs from further up the pulmonary arterial branch can migrate to distal portions of the vasculature (Sheikh et al., 2014). Eventually these once non-muscularised pulmonary arteries will evolve to exhibit a muscular phenotype (Figure 1.1). Proliferation of PASMCs within the medial layer of distal pulmonary arteries defines the presence of a double elastic lamina which is used experimentally to indicate evidence of pulmonary arterial remodelling. PASMCs can also contribute to a double elastic lamina through increased production of elastin and collagen fibres (Todorovich-Hunter et al., 1988; Rabinovitch, 1998). In addition, enhanced muscularisation of already muscular pulmonary arteries in order 4-9 will occur with hypertrophy and proliferation of SMCs within their medial layer and neointima formation within the lumen. Altogether these actions within the pulmonary arteries will lead to a diminution of the luminal area. This distinct loss of blood flow area within the peripheral vasculature is an effect termed “vascular pruning” as evident from arteriograms from PAH patients (Figure 1.2). Pathological remodelling of pulmonary arteries is currently an irreversible process and regression of this PAH hallmark is an attractive therapeutic target.
Pulmonary arterial hypertension (PAH) is a severe vasculopathy affecting the pulmonary arteries. PAH results from muscularisation of distal non-muscularised pulmonary arteries which can lead to their obliteration. Pulmonary arteries already muscularised will undergo hypertrophy of the medial smooth muscle cell layer which can lead to a severe reduction in lumen size. The overall increase in pulmonary vascular resistance increases the pulmonary arterial pressure.

Figure 1-1 Diagrammatic scheme of pulmonary arterial hypertension pathogenesis
Figure 1-2 Pulmonary Vascular Arteriograms

Pulmonary vascular arteriogram from a healthy (non-PAH) adult lung (left) and from an idiopathic PAH patient lung (right). Note the extensive loss of peripheral vasculature in the idiopathic PAH patient lung (Reid, 1986).

The consequent increase in pulmonary vascular resistance substantially raises the PAP. The REVEAL registry reports a mean PAP of ~50mmHg which is significantly higher than the mean PAP of a healthy individual of ~14-20mmHg (Badesch et al., 2010). Experimentally within in vivo animal models measurement of right ventricular systolic pressure (RVSP) is used as an indicative measurement of PAP. Anatomically the RV of the heart precedes the pulmonary artery therefore can be directly influenced by hemodynamic properties within the pulmonary circulation. Normally the RV deals with a low pressure pulmonary circuit and structurally has a thin ventricular muscle wall, less so than the left ventricle (LV) which has to pump against the high pressure systemic circuit (Voelkel et al., 2006). In light of high pulmonary vascular resistance and PAP in PAH pathology the RV is not adequately structurally equipped. Consequently the RV undergoes an adaptive hypertrophic transformation to compensate against the increased after-load. Hypertrophy of the RV is indicated by an increase in wall thickness and the total gross weight (Voelkel et al., 2006). Initially the hypertrophic process is beneficial as it combats the high PVR and maintains cardiac output of the RV. However, the adaptive process will eventually become maladaptive and the RV will begin to lose its functional contractile ability and fail.
failure is the primary cause of death within PAH patients thus their survival is largely governed by the adaptability of the RV to the increased after-load.

1.2.5 Current Pulmonary Arterial Hypertension Therapeutics

Therapeutic success in PAH is impeded by the complex multifactorial nature of the disease. In addition, incomplete understanding of the molecular pathways involved, adds to the lack of sufficient treatment strategies. In most scenarios the disease manifestation predates the onset of symptoms and diagnosis meaning the disease pathology can be well progressed by the time therapies are initiated. Present drugs are modelled on mechanisms and pathways identified around 15-20 years ago. That said, life expectancy is greater than it was as indicated by a reduction in the 5 year mortality rate from 66% in the NIH registry from the 1980’s to 40% in the U.K. and Ireland registry from 2012 (Rich et al., 1987b; Ling et al., 2012). Even though current therapeutics have greatly improved and prolonged patient life quality and survival they are unable to reverse the progression of PAH disease characteristics e.g. pulmonary arterial remodelling, and mortality rates remain unacceptably high. Thus there is great rationale to investigate new therapeutic routes.

1.2.5.1 Calcium Channel Blockers

Intense vasoconstriction of small pulmonary arteries is a major recognised hallmark of PH pathogenesis (Rich et al., 1992). Vasoconstriction of PASMCs is ultimately governed by the availability of intracellular Ca^{2+}. As previously discussed, Ca^{2+} enters SMCs via voltage gated channels on the cell surface and initiates a vasoconstrictive cascade increasing PVR. Thus the ability to prevent Ca^{2+} influx by blocking the L-type channels presents an approach to suppress SMC vasoconstriction and promote vasodilation. Almost three decades ago Rich and colleagues described the first use of Ca^{2+} channel blockers in pursuit of decreasing PVR in PAH (Rich et al., 1987a). Since then the use of nifedipine, amlodipine, or diltiazem has been recognised in the clinic. It is worthy to note Ca^{2+} channel blockers are only recommended for use in patients who respond positively to an initial vasodilatory test which is roughly 5-10% of patients (Sitbon et al., 2005). Thus the target and therefore responsive PH patient population is a small minority.

1.2.5.2 Endothelin Receptor Antagonists

In 1988 Yanagisawa and colleagues identified endothelin-1 (ET-1) as a highly potent vasoconstrictive molecule with fundamental importance in regulation of vascular tone (Yanagisawa et al., 1988). Within the vascular network the predominant source of ET-1 is
from ECs with the majority of synthesis occurring within the pulmonary circulation (Firth et al., 1992). PAECs are critically involved in the pathogenesis of PAH and their dysfunction can be attributed by ET-1 (Galie et al., 2004). Through endothelin receptor A (ET_A) and endothelin receptor B (ET_B), ET-1 can exert vasoconstrictive and proliferative properties on PASMCs and PAECs creating a detrimental environment and initiating structural arterial remodelling (MacLean et al., 1994; Davie et al., 2002). In PH patients circulating ET-1 concentrations are increased and correlate with disease severity (Stewart et al., 1991; Giaid et al., 1993). Up-regulation of ET_A and ET_B receptors has also been documented in PH and could account for heightened effects driven by ET-1 (Davie et al., 2002). The most effective approach at blocking the action of ET-1 is through dual antagonism of ET_A and ET_B as selective inhibition of one receptor can be compensated through the action of the other. Experimental data highlights dual receptor blockade can reduce human PASMC proliferation, pulmonary haemodynamics and RVH (Eddahibi et al., 1995; Davie et al., 2002). However, ET_B plays a major role in the clearance of ET-1 and therefore blockade of this receptor by ET_B antagonists could lead to heightened circulating ET-1 levels and limits ET_B antagonists therapeutic appeal (Fukuroda et al., 1994). There are currently three FDA (Food and Drug Administration) approved ET-1 receptor antagonists (ERAs) for the use of PAH; bosentan, macitentan and ambrisentan. Bosentan and macitentan are dual receptor antagonists whereas ambrisentan is an ER_A selective antagonist. Bosentan was the first oral treatment prescribed for use in PAH and longitudinal patient studies have shown 2 year survival at 70% (McLaughlin et al., 2005). Interestingly in 2012 Gabler and colleagues reported sex differences in treatment response to ERAs with women demonstrating better clinical outcome than men (Gabler et al., 2012). This suggests that stratification of PAH therapies may improve treatment effectiveness.

1.2.5.3 Phosphodiesterase 5 Inhibitors

Nitric Oxide (NO) has long been recognised as an important regulator of vascular tone (Furchgott et al., 1980; Ignarro et al., 1988). Deficiencies in NO have been identified in PAH patients (Giaid et al., 1995). Within the pulmonary vasculature NO is a critical local mediator synthesised in the endothelial layer via conversion of L-arginine by endothelial nitric oxide synthase (eNOS). Acting in a paracrine fashion to underlying PASMCs endogenous NO activates soluble guanylate cyclase. This activation triggers the conversion of guanosine 5’ triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) increasing intracellular cGMP levels. cGMP is a second messenger and through cGMP-Dependent Protein Kinases (PKG) signalling, is key in initiating vasodilation of small
pulmonary arteries. The action of cGMP is terminated by cyclic nucleotide phosphodiesterase (PDE) enzymes, primarily PDE-5. Within the pulmonary vasculature PDE-5 is abundantly and predominantly expressed in PASMCs. Increased levels of PDE-5 are reported in animal models of PH (Maclean et al., 1997; Corbin et al., 2005). Selective inhibition of PDE-5 prevents the hydrolysis of cGMP and thus potentiates the beneficial vasodilatory effects brought on by PKG. Selective PDE-5 inhibitors approved for PAH therapy include sildenafil, verdenafil and tadalafil. Experimentally sildenafil has been shown to restore deficiencies in BMPR-II signalling in in vitro and in vivo models which exhibit disrupted BMPR-II pathway (Yang et al., 2013a). Clinically treatment with sildenafil reduced PAH patients mPAP, PVR and increased their cardiac index compared to placebo controls however, clinical worsening was indifferent between groups (Galie et al., 2005). Recently it was observed that male PAH patients were more likely to respond better to tadalafil treatment than female PAH patients reiterating the impact gender and/or sex has on treatment response (Mathai et al., 2014).

1.2.5.4 Prostacyclin Analogues

Prostacyclin is a member of the prostaglandin family sharing a common molecular structure to other members such as thromboxane A2 (TA2). Prostacyclin and TA2 have paradoxical effects on vascular tone, vasodilation and vasoconstriction respectively, emphasising the importance prostaglandins play in maintaining vascular homeostasis. Prostacyclin is also a major inhibitor of platelet aggregation; an additional contributing factor in PAH development (Moncada et al., 1976; Moncada et al., 1979). Abnormal urinary ratios between prostacyclin and TA2 metabolites in favour of TA2 have been observed in PH patients (Christman et al., 1992). Within the pulmonary circulation prostacyclin synthesis occurs primarily in PAECs and acts in a paracrine fashion to underlying PASMCs where it activates G-protein-coupled-receptors (GPCRs) to increase intracellular cAMP levels. More recent evidence has also highlighted a role for prostacyclin in proliferation (Clapp et al., 2002). Subsets of PH patients present with deficiencies in endogenous prostacyclin synthase (PGIS) and subsequent decreased levels of circulating prostacyclin (Christman et al., 1992; Tuder et al., 1999). This decrease in expression is mirrored in animal models of pulmonary hypertension (Badesch et al., 1989) and replenishing prostacyclin decreases disease indices in the mouse and rat hypoxic model of pulmonary hypertension (Rabinovitch et al., 1988; Geraci et al., 1999). Experimentally the prostacyclin analogue treprostinil has been shown to activate KCNK3 in PASMCs restoring the K+ current and resting membrane potential (Olschewski et al.,
Similarly it has been demonstrated that the prostacyclin analogue iloprost has the ability to inhibit PASMC proliferation through enhancing the BMPR-II signalling pathway (Yang et al., 2010). Clinically the approach of augmenting endogenous prostacyclin in PH patients arose ~30 years ago and remains at the forefront in treating advanced cases of PAH (NYHA class III and IV) (Rubin et al., 1982). However, the short half-life of prostacyclin derived therapies still poses a hurdle in today’s treatments. To overcome the short half-life, prostacyclin analogues can be administered via a continuous intravenous infusion but this can subsequently result in recipient patients being highly susceptible to sepsis (Kitterman et al., 2012). Inhaled and orally active prostacyclin analogues are thus more appealing.

1.2.6 Emerging Pulmonary Arterial Hypertension Therapeutics

Current analysis has highlighted the effectiveness of prescribing double or even triple therapy of existing PAH drugs for optimal results (Sitbon et al., 2014). This reinforces the concept that PAH needs a multi-hit treatment plan. The intricate and diverse mechanisms involved in pulmonary arterial remodelling and distal pulmonary artery obliteration in PAH pathogenesis remain relatively elusive. Discovering novel treatments targeting these aspects of disease will certainly prove beneficial. Ongoing research has identified fundamental pathways including those involving reactive oxygen species (ROS) (Liu et al., 2006; Mittal et al., 2007), K+ ion channels (Xie et al., 2004), tyrosine kinases (Schermuly et al., 2005), epigenetic modulation (non-coding RNAs and histone deacetylases) (Caruso et al., 2012; Zhao et al., 2012) and regeneration utilising endothelial progenitor cells (Wang et al., 2007).

1.3 Genetic Control of Pulmonary Arterial Hypertension

A heritable component of PAH has been recognised since Dresdale observed repeat occurrence of the disease within a single family in the 1950’s (Dresdale et al., 1951) (Dresdale et al., 1954). From this it took almost 50 years for clinicians and researchers to fully identify the first genetic risk factor in heritable PAH (HPAH) as mutations within the BMPR-II gene (Deng et al., 2000) (International et al., 2000). HPAH is known to be inherited in an autosomal dominant manner meaning only one parent is required to pass on the affected gene for the child to be at risk. Genetic mutations are known to occur with reduced disease penetrance as only 20% of mutation carriers actually develop disease indicating other additional contributing factors are involved. These additional factors are thought to be of both genetic and environmental origin. Together this implies that a
Chapter One

Introduction

correspondence from a PAH-associated gene mutation is required but not sufficient for HPAH to develop. Other genetic risk factors identified include mutations within activin receptor-like kinase 1 (ALK-1) and potassium channel subfamily K, member 3 (KCNK3). The theory of HPAH exhibiting genetic anticipation i.e. disease onset occurring earlier in age with each affected generation, has recently been dismissed as there is currently insufficient longitudinal data to support such a claim. However, on average HPAH patients present earlier in age with more severe haemodynamic impairment and die around 10 years earlier compared to non-HPAH patients (Sztrymf et al., 2008; Girerd et al., 2010). Around 6% of the PAH population will be classed as a HPAH patient (disease presentation within one or more first order blood relative) (Rich et al., 1987b). A gender disparity is observed in HPAH with a 2.7:1 female to male ratio suggesting interplay of sex with disease prevalence (Loyd et al., 1995).

1.3.1 BMPR-II Mutations

Mutations within the gene encoding BMPR-II are associated with ~75% of HPAH patients (Machado et al., 2006a). Interestingly ~20% of idiopathic PAH (IPAH) patients also present with BMPR-II mutations (Atkinson et al., 2002; Hamid et al., 2009). The nature of BMPR-II mutations documented vary between affected families and to date there have been ~300 observed (Machado et al., 2006a; Soubrier et al., 2013). Evidence suggests disease penetrance of BMPR-II mutation carriers can depend on the location of mutation in the BMPR-II gene however data cannot be fully interpreted due to study cohorts involving few non-affected carriers (Austin et al., 2009b). Additionally, among affected BMPR-II mutation carriers disease severity appears to depend on mutation type e.g. missense mutations carry more detrimental ramifications than truncating mutations (Austin et al., 2009b). The mutations described result in reduced expression or reduced function of the BMPR-II receptor. Therefore a reduction in BMPR-II mediated signalling is associated with HPAH development.

1.3.1.1 BMPR-II Signalling Pathway

BMPR-II is a receptor subtype member of the TGF-β superfamily type II receptors and has four functional domains including a ligand binding domain, a transmembrane domain, a kinase domain and a cytoplasmic tail domain. In the pulmonary circulation BMPR-II signalling is crucial for vascular cell growth and differentiation playing an important role in cellular processes such as angiogenesis and vascular repair (Pardali et al., 2012; Sountoulidis et al., 2012). BMPR-II receptors are serine-threonine receptor kinases
activated by a group of cytokines called bone morphogenetic proteins (BMPs) (Cai et al., 2012). BMPs regulate both cell proliferation and apoptosis (Figure 3). These pleiotropic effects mediated by BMPs are dependent on the cell type involved i.e. BMP signalling in PASMCs is anti-proliferative but proliferative in PAECs (Zhang et al., 2003; Teichert-Kuliszewska et al., 2006). BMP signalling is crucial for the initial development of the pulmonary circulation promoting PAEC survival and their vital role in tube-like vessel formation (Teichert-Kuliszewska et al., 2006). Canonical BMPR-II signalling is initiated by ligand binding to the cell surface BMPR-II receptors (Figure 1.3). The ligand activated BMPR-II receptor then recruits a TGF-β type I receptor (i.e. BMPR-I) to form a heterodimer complex before signalling proceeds (Derynck et al., 2003). The kinase activity of the BMPR-II receptor then phosphorylates and activates the transmembrane region of the BMPR-I receptor. The activated BMPR-I receptor is then able to interact with intracellular signalling proteins called Sma and MAD (mothers against decapentaplegic) known collectively as SMAD proteins (Massague et al., 2005). There are three types of SMAD proteins: receptor regulated SMADs (R-SMADs) which include SMAD1/5/8, common mediator SMAD (co-SMAD) which includes SMAD4 and inhibitory SMADs (I-SMADs) which include SMAD6/7. The BMPR-I receptor phosphorylates the R-SMADs which form a hetero-oligomer complex with the co-SMAD4. This new SMAD complex translocates to the nucleus where it plays a role in the transcription of target genes. SMAD-target genes include inhibitor of DNA binding (Id) of which there are 4 subtypes. Id proteins are functional inhibitors of the basic helix loop helix (BHLH) transcription factors and act by prohibiting their DNA binding ability. BHLH transcription factors usually direct expression of proliferative and pro-survival genes but this action is lost when they are bound to Id (Lasorella et al., 2014). With regards to BMPR-II signalling within the pulmonary vasculature, Id1 and Id3 are demonstrated to be important targets (Yang et al., 2013b). Id3 has a crucial role in regulating PASMCs cell cycle progression (Yang et al., 2013b). Regulation of SMAD signalling occurs via SMAD ubiquitination and regulatory factors (SMURFs) which serve to repress SMAD activity (Shi et al., 2004).

The mechanisms of how BMPR-II mutations lead to manifestation of PAH remain poorly understood. BMPR-II receptors are broadly expressed with high distribution in PAECs (Atkinson et al., 2002). BMPR-II mutations are present within the germline i.e. the mutation is present within every cell type in the body yet curiously only the pulmonary vasculature appears to be consequently affected. Even still loss of function BMPR-II mutations cause detriment in a cell specific manner as PAECs display an increased risk of
apoIosis whereas on the contrary PASMCs display a heightened proliferative state (Teichert-Kuliszewska et al., 2006; Yang et al., 2011b). It has been shown that in patients with BMPR-II mutations, BMPR-II protein expression is reduced in PASMCs and PAECs along with reduced expression of downstream SMAD signalling proteins (Atkinson et al., 2002; Yang et al., 2005). This expression profile is replicated in animal models of PAH including; hypoxia, monocrotaline and Sugen-Hypoxia (SU-HX) (Takahashi et al., 2006; Morty et al., 2007; Mair et al., 2014). Recently it has emerged that females may be predisposed to reduced BMPR-II signalling as PASMCs from female non-PAH patients exhibit reduced expression of BMPR-II, SMAD1, Id1 and Id3 compared to male non-PAH patients (Mair et al., 2015). Interestingly mice heterozygous for BMPR-II (BMPR-II<sup>R899X</sup>) do not spontaneously develop a PH phenotype at baseline but do after exogenous administration of the highly potent vasoconstrictor and mitogen serotonin (Long et al., 2006). More recently a knock-in BMPR-II mutation (BMPR-II<sup>R899X</sup>) specifically induced in SMCs demonstrated increased RVSP and pulmonary vascular pruning (West et al., 2008). Similarly PASMCs extracted from mice expressing global BMPR-II<sup>R899X+/−</sup> exhibited an enhanced proliferative phenotype and dysfunctional SMAD mediated signalling (Long et al., 2011). In PASMCs from patients with BMPR-II mutations, a reduction in SMAD-dependent signalling i.e. anti-proliferative Id signalling, can lead to unopposed SMAD-independent signalling i.e. pro-proliferative ERK1/2 (extracellular-signal-regulated kinases 1/2) signalling (Yang et al., 2008). It was also observed that BMPR-II<sup>R899X+/−</sup> mice developed a mild PH phenotype at 6 months of age. This converging evidence demonstrates the importance of BMPR-II signalling in PAH disease development and restoration of the BMPR-II signalling pathway represents a novel therapeutic strategy. Rescuing the pathway with FK506 prevents a PH phenotype in mice deficient in BMPR-II and the SU-HX rat model (Spiekerkoetter et al., 2013).
Figure 1-3 Canonical BMPR-II signalling pathway

A schematic overview of canonical BMPR-II signalling pathway in a pulmonary artery smooth muscle cell. Upon BMP ligand binding BMPR-II forms a heterodimer complex with BMPR-I which activates SMAD proteins (1/5/8). SMAD1/5/8 forms a complex with SMAD4 which translocates to the nucleus where it can exert control of target gene transcription. The Id proteins are gene targets which function to inhibit proliferation. BMPR= bone morphogenetic protein receptor, BMP= bone morphogenetic protein, SMAD= Sma and MAD (mothers against decapentaplegic), Id= inhibitor of DNA binding.
1.3.2 ALK-1 Mutations

Novel mutations within the ALK-1 gene were discovered by Harrison and colleagues and found to be associated with hemorrhagic telangiectasia type 2 (HHT) and HPAH patients (Harrison et al., 2003). ALK-1 is a TGF-β type I receptor and is a serine-threonine receptor kinase similar to BMPR-I and BMPR-II. The mutations identified in ALK-1 were missense mutations again leading to a loss of function of the receptor. The ALK-1 receptor functions in the same signalling pathway as BMPR-II further emphasising the fundamental importance a complete TGF-β signalling pathway has in pulmonary vasculature homeostasis. With regards to PAH, mutations in ALK-1 are even more detrimental than BMPR-II mutations (Girerd et al., 2010).

1.3.3 KCNK3 Mutations

Recently, whole-exome sequencing has identified six novel missense variants in the KCNK3 gene in both HPAH (those not already harbouring a known mutation) and IPAH patients (Ma et al., 2013). The mutation is thought to have incomplete penetrance and affect both female and male patients. KCNK3 also known as TASK-1 (TWK-related acid sensitive potassium channel) is a member of the two-pore-domain channel superfamily involved in $K^+$ efflux and plays a critical role in maintaining the resting membrane potential therefore in PASMCs it holds a fundamental role in vascular tone. The missense variant identified by Ma and colleagues was found within the region responsible for the channel gate function which suggests the loss of function mutation could have disruptive impact on normal operating function. This was highlighted by the reduction in $K^+$ current of mutant KCNK3 (Ma et al., 2013). In the setting of PASMCs the loss of function of KCNK3 could potentially lead to an increase in vascular tone. It is of interest that KCNK3 is sensitive to $O_2$ and hypoxia can reduce its function (Osipenko et al., 1997). The identification of KCNK3 mutations has provided a new therapeutic target. Current prostacyclin based treatments including treprostinil may even mediate their beneficial properties through activation of KCNK3 (Olschewski et al., 2006).

1.4 Serotonin

1.4.1 Serotonin Biosynthesis and Metabolism

Serotonin is a central and peripheral neurotransmitter which can also function as a local hormone within the vasculature. Serotonin was first isolated from blood serum and identified as a potent vasoconstrictor in 1948 (Rapport et al., 1948). The majority of
serotonin’s synthesis (80%) and distribution is within enterochromaffin cells in the intestinal wall but it is also present in high concentrations in platelets and regions of the brain. As blood passes through the intestinal circulation the active uptake of serotonin into platelets occurs via the serotonin transporter (SERT) generating a circulating serotonin store (Berger et al., 2009).

Serotonin is known chemically as 5-hydroxytryptamine (5-HT) and is synthesised from the primary precursor amino acid L-tryptophan. Within the peripheral system tryptophan is converted to 5-Hydroxytryptophan by the rate-limiting enzyme tryptophan hydroxylase (TPH) before being converted to 5-HT by L-aromatic acid decarboxylase. There are two recognised isoforms of TPH, TPH1 and TPH2 which share 71% sequence homology (Walther et al., 2003). TPH1 is located mainly within the intestinal tract and responsible for peripheral 5-HT synthesis (Darmon et al., 1988) whereas TPH2 is exclusively distributed within the CNS (Walther et al., 2003). Although the majority of peripheral 5-HT synthesis occurs in the gut there is convincing evidence supporting local synthesis of 5-HT within the pulmonary and systemic vasculature specifically the arterial ECs (Eddahibi et al., 2006; Ni et al., 2008). In IPAH patient lungs and PAECs it was demonstrated that there is increased expression of TPH1 generating a heightened 5-HT microenvironment within the pulmonary vasculature (Eddahibi et al., 2006).

5-HT is chiefly metabolised in the liver via monoamine oxidase-A (MOA) by the process of oxidative deamination to produce 5-hydroxyindol-aldehyde before being further oxidised to 5-hydroxyindoleacetic acid (5-HIIA). The 5-HIIA metabolite is excreted by the kidneys in urine and can serve as a biomarker of 5-HT production. Consistent with its synthesis, 5-HT is also locally degraded within the pulmonary vasculature. The actions mediated by 5-HT are vast and varying due to the number of distinct 5-HT receptors and diverse signalling pathways.

1.4.2 Serotonin Signalling

1.4.2.1 The 5-HT Receptors

Current pharmacological classification highlights there are 14 distinct 5-HT receptors arranged into 7 individual classes (5-HT1-7) depending on criteria such as receptor type, location and downstream second messenger systems (Hoyer et al., 1994). All 5-HT receptors are membrane-bound and most belong to the heptahelical guanine nucleotide-
binding (G)-protein coupled receptor (GPCR) superfamily with the exception of 5-HT\textsubscript{3}. 5-HT\textsubscript{3} is a ligand-gated cation channel evoking excitatory actions within the nervous system.

Structurally GPCRs comprise of seven transmembrane α-helices, an extracellular N-terminal domain, and an intracellular C-terminal domain and are coupled to their intracellular signalling mechanisms via a G-protein. G-proteins exist as trimers consisting of α, β and γ subunits with the α subunit containing bound guanosine 5’-diphosphate (GDP) and the β and γ subunits forming a βγ complex. Upon 5-HT binding of the extracellular domain, a conformational change occurs with the associated G-protein being activated and initiating the cell’s effector response. On the G-protein’s α subunit, bound GDP is exchanged for GTP and dissociation from the βγ complex occurs. This allows both the α-GTP and βγ-complex to perform individual downstream signalling. The effector pathway activated by the α-GTP complex is solely determined by the specific G protein involved. There are four G proteins; G\textsubscript{s}, G\textsubscript{i/0}, G\textsubscript{q} and G\textsubscript{12/13}, each governing somewhat divergent intracellular signalling. The fate of GPCR activation and therefore fate of a cell can depend greatly on the coupled G-protein. The majority of the GPCR 5-HT receptors mediate their downstream signalling transduction through G\textsubscript{i/0} thus decreasing cyclic adenosine monophosphate (cAMP) levels apart from receptors of the 5-HT\textsubscript{2} subtype which couple to G\textsubscript{q} and therefore activate phospholipase C (MacLean, 1999).

5-HT receptors are found throughout the central and peripheral nervous system plus the intestinal tract and cardiovascular tissues. This implicates their involvement within several disorders and diseases including depression, schizophrenia, migraine, systemic hypertension, pulmonary hypertension and irritable bowel syndrome, amongst others. Regarding the pulmonary circulation a heterogeneous population of 5-HT receptors have been reported. Messenger RNA (mRNA) expression of the 5-HT\textsubscript{1B}, 5-HT\textsubscript{2A}, 5-HT\textsubscript{2B} and 5-HT\textsubscript{7} has been documented within smooth muscle cells and endothelial cells (Ullmer \textit{et al.}, 1995).

1.4.2.1.1 5-HT\textsubscript{1} Receptors

The 5-HT\textsubscript{1} receptor family has five distinct isoforms, 5-HT\textsubscript{1A}, 5-HT\textsubscript{1B}, 5-HT\textsubscript{1D}, 5-ht\textsubscript{1E} and 5-ht\textsubscript{1F} all sharing 40-63% sequence homology indicating a common evolutionary origin. Preferentially 5-HT\textsubscript{1} receptors couple with the G\textsubscript{i/0} protein thus upon receptor activation will inhibit adenylate cyclase and reduce the production of cAMP from adenosine triphosphosphate (ATP). 5-HT\textsubscript{1A} and 5-HT\textsubscript{1B} are widely expressed in the central nervous
system (CNS) with roles pre-synaptically acting as auto-receptors inhibiting neuronal firing and hetero-receptors initiating the release of other neurotransmitters (Riad et al., 2000). 5-HT$_{1A}$ receptors are also found to harbour inhibitory actions within the intestinal tract. 5-HT$_{1B}$ receptors are expressed in a variety of vascular beds including cerebral and pulmonary arteries. Within human pulmonary arteries the 5-HT$_{1B}$ receptor is the most abundantly expressed 5-HT receptor and mediates the majority of 5-HT induced signalling making it a favourable target for pulmonary artery specificity (Morecroft et al., 1999). In pulmonary arteries 5-HT$_{1B}$ receptor activation triggers not only SMC contraction but smooth muscle cell proliferation and therefore can contribute majorly to pulmonary artery vasoconstriction and pulmonary artery remodelling, two distinguishable hallmarks of PAH (Keegan et al., 2001; Lawrie et al., 2005). The 5-HT$_{1D}$ receptor is less abundantly expressed compared to the 5-HT$_{1B}$ receptor yet still provides important function as the non-selective 5-HT$_{1B/D}$ receptor agonist, sumatriptan has proved successful as an anti-migraine therapy. Definitive physiological roles have yet to be fully established for the 5-HT$_{1E}$ and 5-HT$_{1F}$ receptors therefore they are denoted in lower case.

1.4.2.1.2 5-HT$_2$ Receptors

The 5-HT$_2$ receptor family comprises of the 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptor subtypes which share 46-50% sequence homology. Distinct from the 5-HT$_1$ receptor family the 5-HT$_2$ receptors are coupled primarily to the G$_{q/11}$ protein activating phospholipase C (PLC) leading to increased levels of 1, 2-diacylglycerol (DAG) and inositol-1, 4, 5 triphosphate (IP3). The 5-HT$_{2A}$ receptor mediates contractile responses within a variety of smooth muscle including vascular, bronchial and uterine. With respect to vascular smooth muscle, 5-HT$_{2A}$ receptors are thought to elicit vasoconstriction in the systemic circulation. In accordance with this an anti-hypertensive therapy was established utilising a 5-HT$_{2A}$ selective antagonist ketanserin for use in systemic hypertension (Frishman et al., 1995). 5-HT$_{2A}$ receptors are thought to mediate pulmonary artery vasoconstriction in some animal models (Cogolludo et al., 2006) but their involvement in human pulmonary arteries was disputed when ketanserin had minimal effect on pulmonary vascular resistance (McGoon et al., 1987). Furthermore 5-HT$_{2A}$ exerts function in platelet aggregation causing thrombosis which can contribute to PH pathobiology (Berger et al., 2009; Bampalis et al., 2011).
1.4.2.2 The Serotonin Transporter

The human SERT was cloned in 1993 and was found to share 88% and 71% homology with mouse and rat SERT respectively (Blakely et al., 1991; Ramamoorthy et al., 1993; Chang et al., 1996). SERT is encoded by a single gene located on chromosome 17q11.2 and can be transcriptionally regulated by two alleles for the promoter region. A 44-bp insertion or deletion confers an l or s allele respectively. The short or s-allele confers lower transcriptional efficiency and the long or l-allele promotes higher transcriptional efficiency of the gene (Lesch et al., 1996). Therefore individuals homozygous for the l/l allele will have increased SERT expression and activity compared to those homozygous for s/s. Indeed PASMCs from l/l carriers present with a 2-3 fold increase in SERT mRNA expression compared to s/s carriers (Eddahibi et al., 2001). In a small cohort of IPAH patients the SERT l/l polymorphism was detected in 65% of patients compared to 27% of non-PAH controls suggesting SERT over-expression could be linked to the development of PAH (Eddahibi et al., 2001).

Located on the cell membrane of neurons, platelets, ECs and vascular SMCs the principle role of SERT is to uptake extracellular serotonin for either metabolism or vesicular storing in the intracellular portion therefore terminating its action. SERT is a sodium dependent transporter belonging to the wider Sodium/chloride (Na⁺/Cl⁻) transporter family and therefore utilises Na⁺ influx for serotonin entry into the cell. Firstly Na⁺ binds to the SERT protein followed by serotonin and Cl⁻. A conformational change proceeds allowing intracellular release of serotonin. To drive the SERT back to its original state a K⁺ ion is released.
Figure 1-4 Serotonin signalling

A schematic overview of serotonin (5-HT) signalling in the pulmonary vasculature. Serotonin is synthesised within pulmonary arterial endothelial cells via the TPH-1 enzyme. Acting in a paracrine manner, serotonin can activate 5-HT receptors and serotonin transporter on underlying pulmonary arterial smooth muscle cells. Activation of 5-HT₁ and 5-HT₂ receptors leads to the decrease in cAMP levels and the increase in 1,2-Diacylglycerol and inositol-1,4,5 triphosphate respectively. This along with activation of the serotonin transporter can ultimately lead to contraction and proliferation of pulmonary artery smooth muscle cells. TPH-1= tryptophan hydroxylase-1, cAMP= cyclic adenosine monophosphate.
1.4.2.3 5-HT Signalling Pathways

As briefly mentioned two prominent pathological mechanisms mediated by serotonin are potent vasoconstriction and aberrant proliferation of PASMCs of the small pulmonary arteries. It is well recognised that contractile properties of serotonin exist mainly through adenylate cyclase activity and ultimately via intracellular Ca\(^{2+}\) handling however, the intricacies of serotonin mediated downstream signalling regarding proliferation remains relatively elusive. The literature supports a role for the mitogen-activated protein kinase (MAPK) pathway, generation of ROS and involvement of small G proteins e.g. RhoA and Ras amongst others.

Several studies implicate a critical role for MAPK in the mitogenic effects evoked by serotonin. MAPKs are serine/threonine (Ser/Thr) protein kinases meaning they phosphorylate the hydroxide (OH) group of the serine or threonine. In total there are 14 evolutionary conserved MAPKs identified which transmit extracellular stimuli into diverse intracellular signalling pathways. Classic MAPK signalling transpires through extracellular-signal-regulated kinases 1/2 (ERK1/2), c-Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3) and p38.

Of the conventional MAPK signalling mediators ERK1/2 is the most explored. ERK1 and 2 were the first MAPK to be cloned and share ~83% homology. Their ubiquitous expression confers important fundamental function in various cellular processes. Activation of ERK1/2 can occur in response to a plethora of stimuli e.g. growth factors, cytokines, activation of cell surface receptors such as receptor tyrosine kinases and GPCRs. The ERK1/2 module consists of a series of sequentially acting kinases beginning with the small GTP-ase, Ras activating the primary kinase Raf. Activated Raf subsequently phosphorylates and activates MAPK kinase (MEK1/2) which finally binds and phosphorylates ERK1/2. Upon activation ERK1/2 translocates from the cytoplasm through nuclear pores and into the nucleus where it can phosphorylate various transcription factors thus controlling transcription of multiple genes e.g. Elk-1, GATA-4 and cyclin D1 and mediating a vast range of physiological responses accordingly. Proliferation is a major cellular process under regulation of ERK1/2 with ERK1/2 activation required for G1 to S phase cell cycle progression. Therefore sustained activation of this signalling cascade could trigger aberrant cellular proliferation akin to many diseases e.g. cancer and PAH. Indeed Lee and colleagues show that 1µM of serotonin is sufficient to initiate rapid phosphorylation and activation of downstream ERK1/2 pathway in PASMCs (Lee et al.,...
This was accompanied by an increase in proliferative response to serotonin which could be consequently blocked by not only a MAPKK inhibitor but also the antioxidant Tiron thus suggesting the pathway activated by serotonin stimulation is critically involved in the formation of ROS and activation of ERK1/2. ERK1/2 mediated proliferation is a signalling component downstream of the BMP signalling pathway. In BMPR-II deficiency, the loss of anti-proliferative SMAD-mediated signalling leads to unopposed pro-proliferative ERK1/2 signalling (Yang et al., 2008).

Another small GTPase activated by serotonin signalling is Rho A. The effector of Rho A in PASMCs is Rho kinase (ROCK) which functions to signal the translocation of ERK1/2 to the nucleus. Thus again promoting the transcription of proliferative genes e.g. GATA-4 and cyclin D1. A role for ROCK in pulmonary artery vasoconstriction has also been demonstrated as an acute vasodilatory effect in catheterised PH rats was highlighted after the administration of the ROCK inhibitor fasudil (Oka et al., 2007). Analysis of the ROCK signalling pathway has highlighted ROCK is critical in serotonin mediated SMC proliferation as serotonin induced proliferation and subsequent induction of the cyclin D1 gene was diminished by the ROCK inhibitor Y27632. Y27632 and other ROCK inhibitors have been utilised in a number of experimental PH models in vivo where they have shown efficacy in reducing PH indices e.g. RVSP, RVH and muscularisation of small pulmonary arteries as well as associated ROCK activity (Abe et al., 2004; Abe et al., 2006; Yasuda et al., 2011; Chou et al., 2013). In vitro evidence suggests serotonin mediated signalling of ROCK occurs through activation of the 5-HT$_{1B}$ receptor as Y27632 was able to inhibit ERK1/2 activation through effects of the selective 5-HT$_{1B}$ agonist but not the selective 5-HT$_{2A}$ agonist (Mair et al., 2008). Likewise the 5-HT$_{1B}$ receptor antagonist GR55562 blocked the serotonin mediated ROCK activation and induction of the pro-proliferative gene cyclin D1 (Liu et al., 2004).

1.4.3 Serotonin and Pulmonary Arterial Hypertension

In the 1960’s and 1980’s a subset of patients treated with anorexigenic drugs e.g. aminorex and dexfenfluramine (DFEN) developed PAH. The incidence of PAH within this subset of patients was 23-fold higher than aged- and sex-matched controls (Abenhaim et al., 1996). Anorexigenic drugs function by indirectly acting as serotinergic agonists at the SERT causing the efflux of serotonin from platelet stores and preventing its re-uptake thus increasing the plasma concentration of “free” serotonin. This PAH “epidemic” subsequently led to a plethora of clinical and experimental research implicating the
pathogenic role of serotonin in the development of PAH and formed the basis of the term coined the “serotonin hypothesis of PAH”. Serotonin can be implicated in PAH disease initiation and progression via its synthesis, bioavailability and intracellular signalling and therefore can implicate TPH1, SERT and 5-HT receptors respectively. These 5-HT-candidate genes and their associated processes provide potential therapeutic targets to exploit that could interfere with a network of downstream effects having a wide impact on disease pathobiology.

Concerning the cardiovascular system in normal physiology, serotonin is stored in platelets owing to low levels of “free” circulating serotonin in plasma. However, in PAH patients increased plasma serotonin levels have been documented (Herve et al., 1995). This is thought to be partially due to abnormal platelet function which could increase extracellular serotonin. Indeed in patients with platelet storage disorders PAH is a risk (Herve et al., 1990) and experimentally Fawn-hooded rats (which exhibit platelet storage disorder) spontaneously develop PH (Sato et al., 1992). Platelet storage disorder in fawn-hooded rats is associated with increased SERT expression in lung tissue (Morecroft et al., 2005). Additionally, use of selective serotonin re-uptake inhibitors known as SSRI’s in late pregnancy are thought to correlate with up to a 6-fold increase in incidence of PPHN (Chambers et al., 2006; Kieler et al., 2012; Grigoriadis et al., 2014). PPHN is a severe condition which occurs when the resistance in the pulmonary vasculature fails to decrease at birth therefore limiting pulmonary blood flow. SSRI’s increase extracellular serotonin levels (Ceglia et al., 2004) and have been shown to cross the placental barrier accumulating to up to 70% in foetal cord blood (Rampono et al., 2004). Therefore in pregnant mothers taking SSRI’s an increase in “free” serotonin could contribute to disease pathology in the newborn. In support of this, evidence from experimental PPHN demonstrates in vivo infusion of SSRI’s results in a substantial increase in pulmonary vascular resistance and reduction in pulmonary blood flow (Delaney et al., 2011). Additionally, post-natal examination of foetuses from SSRI dosed rats presented increased RVH and pulmonary artery remodelling (Fornaro et al., 2007). This indicates that increasing the bioavailability of serotonin, by prohibiting its uptake, may be sufficient to trigger detrimental consequences on the pulmonary vasculature. Crucially the potential harmful effect of pregnant mothers taking SSRI’s on foetal development of the pulmonary circulation is currently unknown. Increased de-novo serotonin synthesis in the pulmonary vasculature could also be accountable for heightened “free” serotonin levels. Via the TPH1 enzyme serotonin synthesis can occur locally within PAECs and act in a paracrine fashion
to underlying PASMCs to exert vasoconstrictive and mitogenic properties. TPH1 expression is augmented in PAECs from IPAH patients in turn increasing the synthesis rate of serotonin (Eddahibi et al., 2006). Additional evidence highlighting the importance of peripheral serotonin in PAH development stems from TPH1/-/- mice. These mice have depleted peripheral serotonin and are protected from a hypoxia and DFEN induced PH phenotype (Izikki et al., 2007; Morecroft et al., 2007; Dempsie et al., 2008). Moreover targeted PAEC knockdown of TPH1 attenuates hypoxia induced PAH in rats supporting the critical role local pulmonary serotonin synthesis plays (Morecroft et al., 2012). Chronic administration of the non-specific TPH inhibitor, p-chlorophenylalanine (PCPA) or LP533401 also reduces a PH phenotype induced by hypoxia or over-expression of SERT in SMC (Izikki et al., 2007; Abid et al., 2012).

Serotonin is considered a chief vasoconstrictor and mitogen within the pulmonary circulation and possesses fundamental involvement in increased vascular tone and pulmonary artery remodelling in response to a hypoxic environment. This is thought to be due to effects mediated by both the 5-HT receptors and SERT. It was originally thought it was compulsory for serotonin to be internalised by SERT to evoke mitogenic effects but activation of 5-HT1B and 5-HT2A/B receptors also relay proliferative responses. In a rat model of hypoxia-induced PH, serotonin exposure enhances the phenotype (Eddahibi et al., 1997). Serotonin exhibits contractile responses in all three orders of pulmonary arteries within rats i.e. main pulmonary artery, first pulmonary artery branch and pulmonary resistance artery. However, serotonin is most potent in the small pulmonary resistance arteries (MacLean et al., 1996b). In isolated human pulmonary arteries from PAH patients, serotonin augments contractile response (BRINK C., 1988). Exogenous administration of serotonin can also uncover a PH phenotype in BMPR2 heterozygous mice (BMPR2+/−) (Long et al., 2006). Here the authors show at baseline BMPR2+/− have a similar phenotype to wild-type (WT) littermates, however, upon chronic infusion of serotonin the RVSP, RVH and pulmonary arterial remodelling is significantly augmented. Additionally, pulmonary arteries excised from BMPR2+/− mice show enhanced contractile responses to serotonin vs. WT and isolated PASMCs exhibit heightened proliferation to serotonin vs. WT. This again reiterates BMPR-II mutations do not fully confer disease susceptibility and that secondary factors e.g. serotonin are potentially required for disease onset.

Of the 14 5-HT receptors mainly the 5-HT1B, 5-HT2A and 5-HT2B have been defined with regards to PH. In 1996 the 5-HT1 receptor was identified as the predominant 5-HT
receptor mediating 5-HT induced signalling within pulmonary arteries in humans as 5-HT and 5-carboxamidotryptamine (5-CT) an unselective 5-HT\textsubscript{1} receptor agonist, were equipotent at triggering contractile responses. In addition the 5-HT\textsubscript{1B/D} selective antagonist GR55562 inhibited 5-HT induced contractions whereas the 5-HT\textsubscript{2A} selective antagonist ketanserin only inhibited 5-HT induced contractions at non-physiological concentrations (MacLean et al., 1996a; Morecroft et al., 1999). In pulmonary arteries the 5-HT\textsubscript{1B} receptor exhibits important roles in vascular contraction and smooth muscle cell proliferation, two fundamental contributing characteristics of PAH disease pathogenesis. Specifically, \textit{in vitro} examination has confirmed the 5-HT\textsubscript{1B} receptor mediates vasoconstriction in isolated human pulmonary arteries as the contractile response to the selective 5-HT\textsubscript{1B/D} agonist sumatriptan was inhibited by the 5-HT\textsubscript{1B} selective receptor antagonist SB224289 and unaffected by the 5-HT\textsubscript{1D} selective antagonist BRL15572 (Morecroft et al., 1999). Furthermore, it has been proven that within isolated human PASMCs it is the 5-HT\textsubscript{1B} receptor which partially regulates the calcium binding protein S100A4/Mts1 (MTS1) induced proliferation (Lawrie et al., 2005). Accordingly the 5-HT\textsubscript{1B} receptor is more highly expressed in remodelled pulmonary arteries from human PH patients than non-patients (Launay et al., 2002). In agreement with this, increased expression of the 5-HT\textsubscript{1B} receptor is observed in experimental models of PAH e.g. fawn-hooded rat model (Morecroft et al., 2005), hypoxia rat (Heeley R.P., 1998) and piglet shunt-induced PH (Rondelet et al., 2003) (Launay et al., 2002). \textit{In vivo} analysis highlights inactivation of the 5-HT\textsubscript{1B} receptor by either knockdown or receptor antagonism is sufficient to reduce the development of hypoxia-induced vascular remodelling, pulmonary artery contraction and overall PAH phenotype (Keegan et al., 2001). Proving a critical role for 5-HT\textsubscript{1B} in hypoxia induced PH phenotype.

Within the pulmonary vasculature the 5-HT\textsubscript{2A} and 5-HT\textsubscript{2B} receptors are also localised to the PASMCs (Dumitrascu et al., 2011). The 5-HT\textsubscript{2A} receptor is thought to play a role in vasoconstriction of both pulmonary and systemic arteries. Ketanserin, a 5-HT\textsubscript{2A} selective antagonist, has proven successful as a systemic anti-hypertensive therapy (Frishman et al., 1995) however has displayed insignificance in treating PAH. The unsuccessfulness of ketanserin treatment in PAH and the inability for a 5-HT\textsubscript{2A} selective antagonist to distinguish between systemic and pulmonary vasculature has curtailed the attractiveness of targeting this receptor subtype in PAH therapy. Nonetheless some experimental animal data has presented evidence in support of a role for the 5-HT\textsubscript{2A} receptor. Respectively the 5-HT\textsubscript{2A} receptor is increased and mediates serotonin induced vasoconstriction in a model
of PPHN. Ketanserin was able to increase pulmonary blood flow and reduce pulmonary vascular resistance in this foetal model further implicating the importance of 5-HT$_{2A}$ receptor. Serotonin triggers platelet aggregation which is a pathological trait in PH (Berger et al., 2009). This is mediated through the 5-HT$_{2A}$ receptor (Bampalis et al., 2011).

Additional contribution of the involvement of more than one 5-HT$_2$ receptor in PAH emerges as 5-HT$_{2B}$ / mice and mice dosed with a 5-HT$_{2B}$ selective antagonist RS-127445 exhibit a reduced hypoxia induced PH phenotype (Launay et al., 2002). Activity and function of the 5-HT$_{2B}$ receptor is also implicated in remodelling processes of pulmonary arteries as terguride, a 5-HT$_{2A/B}$ antagonist, diminished proliferation rat PASMCs and chronic administration reduced medial wall thickness in the rat monocrotaline model of PH (Dumitrascu et al., 2011). Regarding human PAH, increased expression of the 5-HT$_{2B}$ receptor was demonstrated in pulmonary arteries from IPAH patients (Dumitrascu et al., 2011). However, contradictory evidence showing 5-HT$_{2B}$ receptors mediate relaxation in pig pulmonary arteries limits the promise of utilising 5-HT$_{2B}$ antagonists in PAH therapy (Glusa et al., 2000).

Higher expression of SERT is recorded in the lungs compared to the brain (Ramamoorthy et al., 1993), more specifically SERT is found within PASMCs within the pulmonary vasculature (Fanburg et al., 1997; Eddahibi et al., 2001). Upon stimulation with serotonin aberrant hyperplasia of PASMCs is demonstrated in cells derived from PAH patients but not control patients. This difference is thought to be partly underwritten by an increase in SERT expression and activity as proliferation was diminished in the presence of fluoxetine and citalopram, two specific SERT inhibitors. Indeed this was the case as lung tissue from PAH patients exhibit increased SERT expression where it was predominantly confined to PASMCs from muscularised pulmonary arteries (Eddahibi et al., 2001). More specifically 65% of a small PAH cohort (89 patients) presented homozygous for the l SERT polymorphism which corresponded to not only an increase in SERT expression in PASMCs but an increase in the PASMCs proliferative response to serotonin and serum compared to those with the l/s or s/s polymorphism (Eddahibi et al., 2001). This result suggests that the l/l variant of the SERT gene may predispose to PAH or be partially involved in pathological processes. However, upon further examination SERT polymorphisms were not confirmed in a larger and wider population of PAH patients suggesting that the l/l allele may contribute to disease characteristics but may not be enough to confer disease susceptibility (Machado et al., 2006b; Willers et al., 2006; Baloira et al., 2012).
Global over-expression of SERT in mice (SERT+) confers spontaneous development of PH in mice aged 5-6 months (MacLean et al., 2004). Pulmonary arterial remodelling and RVSP were both increased significantly compared to WT littermates. In agreement with this observation, mice over-expressing SERT specifically within smooth muscle cells (SM22-5-HTT+) also develop PH although disease is present at 2 months of age in this model (Guignabert et al., 2006). Interestingly in both these models the systemic haemodynamics are unchanged indicating a pulmonary specific consequence for SERT over-expression. Serotonin uptake, via SERT, into platelets was unaffected in the SM22-5-HTT+ mice indicating physiological effects were specific to SERT over-expression in SMCs. Whole blood serotonin levels were not reported in the SERT+ mice therefore it is uncertain if increased SERT expression in platelets lead to an increased storage of serotonin. Guignabert and colleagues also found the SM22-5-HTT+ mice had markedly diminished expression of potassium channels Kv1.5 and Kv2.1 in lung homogenate as a direct consequence of the SMC specific SERT over-expression. This reduction in potassium channels was restored by treatment with the SSRI’s citalopram and fluoxetine in PASMCs (Guignabert et al., 2006). Intriguingly it was subsequently found that only female SERT+ mice developed a PH phenotype suggesting interplay with the estrogen system (White et al., 2011a). This estrogen-orientated effect was confirmed when a PH phenotype was abolished in ovariectomised female SERT+ mice (White et al., 2011a). The phenotype was restored upon re-administration of the main circulating oestrogen 17β-estradiol (E2). In the chronic hypoxic rat model an increase in SERT expression and activity is observed within PASMCs (Eddahibi et al., 1999).

Clinically the therapeutic use of SSRIs in PH patients has shown tentative attenuation of PH development and reduction in mortality (Kawut et al., 2006; Shah et al., 2009). This is mirrored by experimental data that demonstrates depletion of SERT expression and function in either SERT knockout mice or SSRI treated mice reduces a hypoxia induced phenotype (Eddahibi et al., 2000; Marcos et al., 2003; Guignabert et al., 2005). SSRI treatment is also effective in diminishing monocrotaline induced PH in rats (Guignabert et al., 2005). The therapeutic effect of SERT inhibition is in complete contrast to the maternal use of SSRI’s resulting in PPHN as discussed earlier. Ultimately the main difference is adult vs. foetal exposure of SSRI’s on the pulmonary vasculature. This highlights divergent effects of SSRI’s could be due to differences in the pharmacology of the developing pulmonary vasculature compared to the mature pulmonary vasculature. Indeed this was demonstrated by in vitro analysis of pulmonary arteries from adult and foetal rats (Fornaro
et al., 2007). The SSRI fluoxetine generated increased pulmonary artery contraction and remodelling in foetal pulmonary arteries and not adult. Other clinical studies have however re-iterated harmful effects of SSRIs as reports show an association with SSRI use and increased mortality and disease worsening in PAH patients (Sadoughi et al., 2013). On the whole this provides convincing evidence implicating a fundamental role for SERT’s involvement in pathological hallmarks of PAH development.

1.5 The Influence of Sex on Pulmonary Arterial Hypertension

Mostly all classifications of PH including PAH exhibit an enhanced female susceptibility with their being up to 4 female patients for every one male patient (Rich et al., 1987b; Humbert et al., 2006; Badesch et al., 2010; Ling et al., 2012). This increased female to male ratio in PAH prevalence strongly suggests there is an influence of sex in the development of PAH. Dresdale noted an increase in frequency of female patients as early as the 1950s (Dresdale et al., 1951) yet to date there are still no PAH treatments that incorporate this factor. To add to the complexity female PAH patients appear to survive longer than males and tend to develop PAH to a lesser degree than males (Humbert et al., 2010a; Humbert et al., 2010b). Elucidating explanation for these differences is the focus of much current PAH research. One fundamental difference between females and males is the presence or absence of specific sex hormones. Experimentally some animal models mirror the sex bias in PAH. In some models of PH the female sex hormone estrogen is thought to be protective (Lahm et al., 2012) whereas other models show it to be causative i.e. the hypoxic model produces a more severe phenotype in males but the SERT+ model exhibits female susceptibility (Rabinovitch et al., 1981; White et al., 2011a). Altogether this conflicting role of estrogen in PAH prevalence and development led to the concept of the “estrogen paradox”.

1.5.1 Estrogen Biosynthesis and Metabolism

Estrogens are the primary female sex hormone functioning considerably in development and maintenance of the female reproductive system. In recent years evidence has demonstrated a significant role for estrogens in other systems including the cardiovascular system (Knowlton et al., 2012). Estrogens are mainly synthesised in the ovaries in females and to a lesser extent the testis in males. Under neural control estrogens are secreted from the sex organs as a result of activation of the hypothalamic-pituitary-gonadal (HPG) axis. Estrogens are a collective of steroid molecules consisting of estrone (E1), 17β-estradiol (E2) and estriol (E3). Within pre-menopausal females E2 is perceived as the most potent
estrogen and therefore considered the principle circulating estrogen. E1 is considered important after the menopause and E3 is fundamental in maintaining a healthy uterine lining during pregnancy (Khosla et al., 1997; Stricker et al., 2006).

As estrogens are steroid molecules their ultimate precursor molecule is cholesterol. Cholesterol is a lipid molecule present in every cell however it will only participate in the steroidogenesis pathway depending on the bioavailability of specific enzymes and/or control from the HPG axis. Therefore estrogen synthesis is limited to specific areas within the body. As previously mentioned estrogen biosynthesis primarily occurs within the ovarian follicles however, secondary extra-gonadal sites are also recognised e.g. breast tissue, adipose cells and liver (Labrie et al., 1998; Simpson et al., 2001). Additionally local estrogen synthesis has been highlighted in both ECs and SMCs of the systemic and pulmonary vasculature implicating a fundamental role in vascular homeostasis (Harada et al., 1999; Sasano et al., 1999; Mukherjee et al., 2002). The majority of gonadal derived estrogens are released into the bloodstream acting in an endocrine manner however extra-gonadal derived estrogens are thought to act at the site of synthesis in a paracrine manner (Labrie et al., 1998). Pregnenolone is converted from cholesterol by the cytochrome P450 (CYP) 11A1 enzyme, which is subsequently converted to progesterone by 3β-hydroxysteroid dehydrogenase (HSD) before being hydroxylased and lyased by CYP17A1 to form androstenedione. Androstenedione has the ability to produce E2 through using either testosterone or E1 as intermediates (Figure 1.5). The CYP19A1 enzyme (aromatase) is crucial in both intermediate pathways in production of E2.

The aromatase gene is present in gonadal and extra-gonadal tissues with its site-specific transcription being driven by different signalling pathways at alternative sites in the promoter region. For example in gonadal tissue aromatase gene expression is controlled by follicle stimulating hormone (FSH) and cAMP associated signalling at the proximal promoter (Jenkins et al., 1993) whereas in adipose tissue aromatase gene expression is controlled by glucocorticoids and the Janus kinase/signal transducers and activators of transcription (Jak/STAT) pathway at the distal promoter (Zhao et al., 1995). Over-expression of the aromatase gene has been associated with a number of estrogen-positive diseases including breast cancer and endometriosis (Noble et al., 1996; Harada, 1997). Inhibition of the aromatase enzyme by anastrozole has shown great promise in breast cancer patients and is now widely recognised for use in the clinic (Arimidex et al., 2008). Most aromatase inhibitors act in a global manner thus can affect normal aromatase
function such as maintenance of bone growth (Sasano et al., 1997). It is of interest then to modulate aromatase expression in a targeted therapy approach. Specifically the distinct tissue specific promoters of the aromatase gene could potentially be exploited to target a tissue of interest to alter the expression of aromatase and minimise side effects.

Expression of aromatase has also been defined within lung tissue giving rise to the presence of a local pulmonary vasculature estrogen microenvironment. In non-small cell lung cancer (NSCLC) patients, aromatase expression is increased within NSCLC cells (Marquez-Garban et al., 2009) and has the ability to predict survival in females with NSCLC (Mah et al., 2007). These findings implicate a pathological role for aromatase expression and also increased E2 levels in the lung. In addition, a single nucleotide polymorphism (SNP) in the aromatase gene has been identified in patients with portopulmonary hypertension (PPHTN) (Roberts et al., 2009). The SNP identified in PPHTN also correlated with increased plasma levels of E2 further associating a role for aromatase and E2 levels in disease. Currently an ongoing phase II clinical trial is examining the safety and efficacy of the aromatase inhibitor, anastrozole, in PAH where data will be documented on the ability to reduce E2 levels and the effect on right ventricle function (ClinicalTrials.gov Identifier: NCT01545336).

Elimination of estrogens from the body occurs initially via oxidative metabolism. The majority of this metabolism exists within the liver and involves a cohort of CYP enzymes. The conversion of estrogens to less active water soluble metabolites allows for their excretion by the kidneys within the urine. Just as the synthesis of estrogens, extra-hepatic regions of metabolism have been identified including breast tissue, brain, lung and vasculature. These regions are most likely to correlate with the areas of synthesis (co-localisation) suggesting that it may not just be estrogens eliciting effect but also their metabolites could act as local signalling mediators.

CYP1A1, CYP1A2 and CYP3A4 are predominantly involved in the metabolism of E2 to 2-Hydroxyestradiol (2-OHE2) whereas CYP1B1 favours metabolism of E2 to 4-Hydroxyestradiol (4-OHE2) (Lee et al., 2003a). Additional CYP enzymes involved in the metabolism of E2 lead to the formation of further hydroxylated metabolites including 6α, 6β, 7α, 12β, 15α, 15β, 16α and 16β. CYP enzymes involved in E2 metabolism have region selective expression therefore expression of metabolites can vary between areas in the body. For example, breast tissue expresses high quantities of CYP1B1 promoting the predominant formation of 4-OHE2 (Wen et al., 2007). Increased expression of CYP1B1 is
positively correlated with breast cancer suggesting 4-OHE2 mediates malignant processes (Wen et al., 2007). Indeed 4-OHE2 has been shown to produce free radicals that can lead to DNA damage and is thought to be a more potent mitogen than E2 itself (Nutter et al., 1991; Mosli et al., 2013). Tobacco smoke has shown to increase CYP1B1 and its E2 derived metabolites within the lung and expression is more prominent in females than males (Peng et al., 2013). In addition, CYP1B1 expression is localised in smooth muscle cells and endothelial cells of the vasculature indicating a role in vascular homeostasis (Kerzee et al., 2001; Conway et al., 2009). In a regulatory feedback mechanism, E2 can promote its own breakdown by driving the transcription of the human CYP1B1 gene via an estrogen response element in the promoter region (Tsuchiya et al., 2005). Gain-of-function polymorphisms within the human CYP1B1 gene have been identified in and contribute to the development of estrogens-positive cancers (Bailey et al., 1998; Hanna et al., 2000). Therefore, inhibition of CYP1B1 has potential therapeutic value in estrogen associated cancers. Further metabolism of 2-OHE2 and 4-OHE2 can occur via catechol-O-methyltransferase (COMT) enzyme activity to produce 2- and 4-methoxyestradiol respectively. This detoxification action is thought to act beneficially by preventing the formation and action of pathological free radicals from the hydroxyestradiols (Salama et al., 2008).
Figure 1-5 Estrogen synthesis and metabolism pathway

Cholesterol is converted to androstenedione via the CYP11A1, 3β-HSD and CYP17A1 enzymes. From androstenedione, 17β-estradiol can be converted via either estrone or testosterone by the 17β-HSD and CYP19A1 enzymes. 17β-estradiol can be metabolised to either 2-, 4- or 16-hydroxyestradiol via a variety of CYP enzymes including CYP1B1. The 2- and the 4-hydroxyestradiols can be further metabolised to 2- and 4-methoxyestradiol respectively by the COMT enzyme. CYP= cytochrome P450, HSD= hydroxysteroid dehydrogenase, COMT= catechol-O-methyltransferase.
1.5.2 Estrogen Signalling

Estrogens were once solely thought of as hormone regulators of reproductive function signalling only within the endocrine system. However, a greater understanding has highlighted their wider physiological role. The recognition of extra-gonadal synthesis re-defined the importance of paracrine estrogen signalling within further biological systems, including the cardiovascular. As steroid molecules, estrogens are lipophilic and can easily enter into the cell’s cytosol via diffusion to mediate their effector response. Intracellular estrogens mediate their response through both direct and indirect genomic signalling. Genomic estrogen signalling involves the activation of nuclear receptors estrogen receptor-α (ERα) and estrogen receptor-β (ERβ). In addition, estrogens also exhibit rapid signalling which suggests their involvement in non-genomic signalling i.e. receptor mediated signalling cascade. This is strengthened by effects observed from estrogens in non-nucleated cells (Moro et al., 2005). Thus membrane bound estrogen receptors were identified. Evidence suggests ERα and ERβ can additionally act as membrane bound receptors but there is also recognition for a third estrogen receptor named G protein-coupled estrogen receptor 1 (GPER).

1.5.2.1 Nuclear estrogen receptor signalling

ERα and ERβ are encoded by the ESR1 and ESR2 genes respectively and are regarded as ligand-activated transcription factors. Located within the cytoplasm ligand binding promotes receptor dimerisation and mobilisation. Classical direct signalling involves translocation of the receptors to the nucleus where they are able to modulate gene transcription through binding to estrogen-response-elements (EREs) within the promoter region of multiple target genes. Therefore their activation can exert a multitude of effects. Most nuclear receptors share structural commonality with an N-terminal domain, a core DNA binding domain, a hinge region, a ligand binding domain and a C-terminal domain. The N-terminal domain exhibits the main heterogeneity within the receptor family and harbours the activation function 1 (AF1) site. The AF1 site binds other transcription factors that can act as either co-activators or co-repressors thus regulate the activity of the receptor and consequently affect the transcription function. The core DNA binding domain is the most highly conserved region and is responsible for recognising and binding to the ERE on the target gene. The hinge region is important in receptor dimerisation and the ligand binding domain contains a highly conserved activation function 2 (AF2) site fundamental for activation by the specific estrogen ligand. The ligand binding domain also harbours sites for heat shock protein (HSP) binding which aids with the process of nuclear
translocation in a chaperone manner. The EREs can either be positive or negative and enhance or repress gene transcription respectively. For example, recruitment of the co-regulator p300 to the transcription complex of ERα (not ERβ) results in activation of gene transcription (Hanstein et al., 1996), whereas recruitment of short heterodimer partner (SHP) leads to inhibition of ERα and ERβ mediated transcription (Johansson et al., 1999). The milieu of co-regulators can vary between cell types and ERα and ERβ can tether different co-regulators (Misiti et al., 1998; Suen et al., 1998). Thus cell type and receptor expression will coordinate different co-regulators and impact on the ability to either activate or repress distinct gene transcription. Consequently, this alters translation of proteins and ultimately the fate of the cell therefore allows both receptors to exhibit divergent effects within the same cell type i.e. in ovarian cancer cells ERα mediates proliferative effects of E2 whereas ERβ mediates anti-proliferative effects of E2 (Chan et al., 2014). ERs can additionally function in genomic signalling indirectly through alternatively interacting with other transcription factors. For example, the cyclin-D1 gene and GATA-1 gene contain no ERE however E2 is thought to modulate their expression through interaction with cAMP response element (CRE) and direct protein-protein contact respectively (Blobel et al., 1995; Sabbah et al., 1999).

1.5.2.2 Membrane estrogen receptor signalling

The discovery of an acute E2 mediated effector response indicated there was participation of ERs not involved in transcriptional processes. Transcriptional effects are normally initiated after a period of time thus the rapid non-genomic effects of E2 have been delineated. In addition to their cytoplasmic localisation ERα and ERβ are also known to be expressed on the cell membrane. Here they are recognised to engage in downstream signalling cascades including ERK/MAPK (Filardo et al., 2000), PI3K and cAMP pathways. Furthermore a new membrane bound ER was identified in 2005 termed G protein-coupled estrogen receptor 1 (GPER) (Filardo et al., 2000; Filardo et al., 2005). GPER is a heptahelical GPCR and is coupled to the G proteins Gs (Filardo et al., 2002) and Gi/0 (Broselid et al., 2014). GPER activation stimulates and inhibits adenylyl cyclase via the Gs and Gi protein respectively therefore regulates intracellular cAMP levels and in turn cAMP can mediate ERK activity (Filardo et al., 2000; Filardo et al., 2002). As well as mediating rapid and transient effects of E2, GPER is additionally involved in the transcription of genes independently of binding EREs. For example, evidence shows stimulation by the selective GPER agonist G-1 up-regulates the expression of c-fos in ovarian cancer cells (Albanito et al., 2007). The distribution of GPER is vast with
expression identified in the stomach, adrenal gland, spinal cord, heart, lung and kidney tissue (Otto et al., 2009). At a cellular level GPER has been identified in smooth muscle and endothelial cells within the vasculature (Lindsey et al., 2011). Along with ERα and ERβ, evidence demonstrates a pathological role for GPER in malignancies such as lung cancer (Jala et al., 2012). A carcinogenic role for GPER is attributed to the highly proliferative effects mediated by E2 (Albanito et al., 2007).

1.5.2.3 Estrogen Signalling within the Cardiovascular System

Within the cardiovascular system ERs are expressed in both cardiac tissue and vascular beds and thought to mediate protective control from multiple cardiovascular pathologies. This is in complete contrast to specific cancers where E2 mediated effects through ERs are highly pathologic. It is well recognised that pre-menopausal women are at less risk from cardiovascular disease (CVD) compared to men suggesting a fundamental protective role for E2 and ERs signalling (Maas et al., 2010). This is strengthened by evidence highlighting women are more at risk of CVD following the menopause when E2 levels substantially decline (Hu et al., 1999). ERα, ERβ and GPER are present within cardiomyocytes and cardiac fibroblasts (Grohe et al., 1997; Deschamps et al., 2009) and function to control the expression of cardiac-related genes i.e. L-type Ca\(^{2+}\) channel, K\(^{+}\) channels (Drici et al., 1996; Johnson et al., 1997). Transgenic mice over-expressing ERα specifically within cardiomyocytes are protected against pathological cardiac remodelling post myocardial infarction (MI). This protective effect is partly mediated by ERα inducing the expression of VEGF gene and promoting angiogenesis (Mahmoodzadeh et al., 2014). In agreement with this ERα is increased in human hearts from patients with dilated cardiomyopathy in a bid to compensate the loss of normal cardiac function (Mahmoodzadeh et al., 2006). Further cardio-protective effects of E2 are mediated through ERβ. Pedram and colleagues demonstrated ERβ activation inhibited synthesis of key proteins e.g. collagen and fibronectin involved in fibrosis and prevented hypertrophic phenotype in angiotensin-II (ANG-II) treated cardiac fibroblasts (Pedram et al., 2008; Pedram et al., 2010). In addition, ERβ\(^{-}\) mice have heightened cardiac fibrosis and hypertrophy compared to WT mice conferring a protective cardiac role for ERβ (Fliegner et al., 2010). GPER also participates in protective cardiac mechanisms as the GPER selective agonist G-1 reduces cardiac contractile dysfunction post-ischemia in ex vivo mouse heart preparations (Deschamps et al., 2009).
E2 signalling through ERs is also considered beneficial within the vasculature. ERs are located within both ECs and SMCs of the systemic and pulmonary vasculature (Karas et al., 1994; Venkov et al., 1996; Register et al., 1998; Hodges et al., 2000; Wright et al., 2015). E2 has shown to promote the release of the potent vasodilators NO and prostacyclin through up-regulation of eNOS and PGIS genes in systemic arterial endothelial cells (Hishikawa et al., 1995; Mikkola et al., 1995; Ospina et al., 2002). In addition, E2 exerts a protective role in atherosclerosis by elevating apolipoprotein-E (APO-E) via ERα (Srivastava et al., 1997). A further athero-protective function for ERα has been described in a mouse model of vascular injury. Here the authors highlighted E2 reduced proliferation of vascular SMCs and medial thickening in a model of vascular injury but this effect from E2 was abolished in ERα knockout (KO) mice implicating protective mechanisms are mediated via ERα (Pare et al., 2002). ERβ signalling has been shown to be more potent at mediating the inhibitory action of E2 on vascular smooth muscle cell (VSMC) proliferation than ERα. Watanabe and colleagues demonstrated that VSMCs over-expressing ERβ were more capable than those over-expressing ERα to inhibit VSMC proliferation as a result of E2 stimulation (Watanabe et al., 2003). ERβ/ mice develop age-dependent systemic hypertension indicating ERβ may play an additional role in vasodilation of systemic arteries (Zhu et al., 2002). Indeed a vasodilatory function of ERβ has been documented as rapid E2 induction of vasodilation in carotid and femoral arteries is abolished in ERβ/- mice (Guo et al., 2005). This relaxation effect of E2 was also absent in ERα/- mice showing a fundamental role for both receptors. The rapid response observed from E2 suggests that membrane bound ERα and ERβ were responsible for mediating vasodilation. Inhibition of ERK/MAP kinase and PI3K also attenuated the E2 induced vasodilation implicating the membrane bound ERs were modulating the rapid effects through kinase pathways (Guo et al., 2005). Activation of the membrane bound GPER is thought to trigger rapid vasodilation through nitric oxide dependent mechanisms (Meyer et al., 2010). The involvement of GPER in the regulation of vascular tone is also emphasised by data highlighting vasodilation evoked from G-1 is absent in GPER knockout mice (Haas et al., 2009). Consistent with this female GPER knockout mice develop age-dependent systemic hypertension due to increased peripheral vascular resistance (Martensson et al., 2009).

It is evident that within the cardiovascular system E2 promotes favourable physiological conditions with regards to anti-hypertrophic, anti-fibrotic, anti-proliferation and vasodilation. However, E2 does exhibit potential pathological qualities. Patients receiving hormone replacement therapy (HRT) with E2 show elevations in plasma prothrombotic
fragment 1 and 2 (F1 and F2) indicative of coagulation activity suggesting estrogen has prothrombotic properties (Teede et al., 2000). Thrombosis is a major risk factor for many CVD (Kannel et al., 1987) and also is the cause of the PH classification chronic thromboembolic pulmonary hypertension (CTEPH) (Jenkins et al., 2012). This in conjunction with the detrimental effects of excessive E2 in estrogen-related cancers conveys that effects mediated by E2 signalling must be regarded with caution.

1.5.3 Estrogen Pathway and Pulmonary Arterial Hypertension

As previously, mentioned females have a higher prevalence of PAH compared to males and therefore sex represents a major risk factor in PAH development. Regarding this, extensive PAH research has examined the pathways and interactions of the main female pre-menopausal estrogen, E2. Within the pulmonary circulation and more specifically regarding PAH, E2 presents a complex and ambiguous role. As a predominantly female hormone, E2 is assumed detrimental in generating the high prevalence of female PAH patients. In agreement with this, heightened exposure to exogenous E2 through both use of oral contraceptives and HRT is associated with PAH (Masi, 1976; Morse et al., 1999). On the contrary, there is evidence to suggest HRT can prevent the development of PH in patients with systemic sclerosis (Beretta et al., 2006). Elevated endogenous levels of E2 can occur through increasing the estrogen biosynthesis pathway. The bioavailability of the enzyme aromatase is a major factor in E2 synthesis. Increased expression of aromatase is observed in both the clinical and experimental setting of PH. For example, patients with PPHTN present with an SNP in the aromatase gene which is associated with increased plasma levels of E2 (Roberts et al., 2009). However the expression of aromatase is unchanged in female patient vs. female non-patient isolated PASMCs (Mair et al., 2014). Although this is not to suggest that the activity of aromatase is also unchanged and warrants further investigation. Aromatase expression is increased in the hypoxic mouse and SU-HX rat models of PH which also exhibit correlating increases in E2 levels. Indeed, in the SU-HX rat model E2 levels positively correlated with disease severity including RVH and muscularisation of pulmonary arteries indicating a pathogenic role for E2 (Mair et al., 2014). Within the pulmonary circulation E2 is highly mitogenic causing concentration dependent increases in proliferation of PASMCs (White et al., 2011a). This estrogenic effect is unique to E2 as other estrogens E1 and E3 do not evoke a proliferative response in PASMCs (White et al., 2011a). Endogenous E2 plays a role in several female susceptible PH models including SERT+ and DFEN as ovariectomy of these animals demonstrates a reversal and prevention of a PH phenotype (White et al., 2011a; Dempsie et
As these models demonstrate female susceptibility their findings are perhaps more clinically relevant. In addition, E2 is involved in up-regulation of several genes including TPH1, SERT and 5-HT1B in PASMCs which are known mediators of PAH pathogenesis (White et al., 2011a). Inhibition of E2 synthesis appears an attractive therapeutic target to combat the detrimental effects of E2 within the pulmonary circulation. Indeed an inhibitor of the aromatase enzyme, anastrozole, is able to reverse the PH phenotype established in hypoxic mouse and SU-HX rat models. Intriguingly the beneficial effect from anastrozole treatment was only observed in female mice and rats (Mair et al., 2014). In agreement with this, ovariectomy of female SU-HX rats alleviates high RVSP present within this model (Tofovic et al., 2013).

Conflicting views surrounding the effects modulated by E2 are strengthened by data which demonstrates E2 treatment can attenuate increases in RVSP, RVH and pulmonary arterial remodelling in male rats from chronic hypoxic models of PH (Lahm et al., 2008). In addition, ovariectomy increases the disease severity in monocrotaline (MCT) rat model and administration of E2 can rescue an established PH phenotype in MCT treated rats promoting the protective effects of E2 (Ahn et al., 2003; Umar et al., 2011). The controversial role surrounding E2 has complicated the understanding of its function in PAH.

Regarding ERs in PAH, Rajkumar and colleagues demonstrated increased expression of ERα in lungs of female PAH patients compared to female non-patients (Rajkumar et al., 2010). Consistent with this, ERα expression is increased in pulmonary arteries from hypoxic female mice compared to normoxic however there are no observed changes in male tissue (Mair et al., 2014). Interestingly, evidence shows an ERα binding site in the promoter region of the BMPR-II gene and ERα activation can decrease the expression of BMPR2 and its downstream signalling pathway (Austin et al., 2012). As BMPR-II is a fundamental aspect of PAH pathogenesis, down-regulation by E2 stimulation may contribute to disease development in females. In addition, inhibition of ERα prevents the development of a hypoxic phenotype in female mice (Mair et al., 2014) further implicating the unfavourable nature of ERα signalling in PAH. ERβ is found to be decreased within pulmonary arteries from hypoxic female mice compared to normoxic controls suggesting the loss of this receptor may be involved in disease processes (Mair et al., 2014). Indeed, a reduction of ERβ is also observed within lung and right ventricle tissue from MCT rat model of PH (Matori et al., 2012). This is in line with the inability of E2 to rescue MCT
induced PH in the presence of an ERβ antagonist (Umar et al., 2011). Altogether this evidence suggests that E2 may exert detrimental effects through ERα and mediate beneficial/protective effects through ERβ. Therefore the divergent effects mediated by E2 and its complex function in PAH may be dependent on receptor distribution and expression.

Data suggests that it might not be E2 per se initiating detrimental effects in the onset and development of PAH. As E2 is rapidly metabolised, it may be the pool of metabolites which independently influence PAH pathogenesis. As discussed earlier, E2 can be metabolised to toxic and non-toxic metabolites by a variety of CYP enzymes. Evidence suggests the major E2 metabolising enzyme CYP1B1 is pathologically associated with PAH as increased expression is observed in PASMCs from IPAH patients and also pulmonary arteries from SERT+ mice, hypoxic mice and SU-HX rats (White et al., 2011b; White et al., 2012). Recent data shows inhibition of CYP1B1 reduces and reverses the PH phenotype in hypoxic and SU-HX models respectively (White et al., 2012). Furthermore, CYP1B1−/− mice produce a less severe PH phenotype after exposure to hypoxia compared to WT mice (White et al., 2012). CYP1B1 mediated metabolism of E2 favours the production of 4-OHE2 but can also be involved in the metabolism to 2-OHE2 and 16α-OHE1 to a lesser extent (Lee et al., 2003a). 16α-OHE1 is a potent mitogen in human PASMCs and levels are increased by hypoxia whereas 2-OHE2 and 4-OHE2 do not initiate proliferation (White et al., 2012). In fact 2-OHE2 promotes cell cycle arrest and causes a decrease in proliferation within endometrial cancer cells (Gong et al., 2011). Regarding the pulmonary vasculature, administration of 2-OHE2 reduced the PH phenotype induced by MCT rats (Tofovic et al., 2005). When administered in vivo the proliferative metabolite 16αOHE1 can produce increases in RVSP, RVH and remodelling of pulmonary arteries consistent with PH (White et al., 2012). In addition, HPAH patients exhibit a lower ratio of 2-OHE1/16α-OHE1 suggesting preferential metabolism to the proliferative metabolite 16α-OHE1 is associated with disease pathogenesis (Austin et al., 2009a). Altogether, the diversity of metabolites produced could create either a pro- or anti-proliferative environment within the pulmonary vasculature and ultimately influence the disease manifestation.

1.6 microRNAs

Around 75% of the human genome is transcribed however only 2% actually encodes protein sequences (International Human Genome Sequencing, 2004; Djebali et al., 2012).
Thus there is a proportion of non-coding ribonucleic acid (ncRNA) within our cells. ncRNA was once thought to serve no functional purpose as it did not contribute to the proteome. However research over the past decades has led to an understanding that the large quantity of ncRNA is responsible for complex and critical shaping of gene networks and can ultimately govern the cellular fate (Santosh et al., 2015). Therefore the repertoire of ncRNAs is implicated in biological processes concerned not only in health but also disease. The nomenclature of ncRNAs is deciphered either from their known function i.e. transfer RNA (tRNA) or nucleotide length i.e. microRNA (miRNA) and long non-coding RNA (lncRNA).

The first miRNA was first discovered in 1993 by the Ambrose and Ruvkun laboratory (Lee et al., 1993; Wightman et al., 1993). Then termed “lin-4” the ncRNA was identified in caenorhabditis elegans with its temporal expression found to negatively correlate the expression of the LIN-14 protein. By definition miRNAs can direct the expression of genes in a post-transcriptional manner through either degradation of target mRNA or silencing of mRNA translation. Following this, almost 2000 miRNAs have since been identified in humans (www.mirbase.org 2014) and their regulatory ability empowers them in cellular responses and thus wider physiological consequences.

1.6.1 The Biogenesis of MicroRNAs

Genes encoding miRNAs can be located either in intronic, exonic or intergenic regions. Intergenic miRNAs have their own promoter site whereas intronic miRNAs are encoded within the intron regions of a host gene transcript therefore can be coupled to the same transcription start site and transcribed simultaneously with the host gene before being spliced. Most miRNAs exist exclusively in a single gene, however, multiple miRNAs can arise from a single gene also i.e. exist in a cluster. 36% of miRNAs are found in clusters within the human genome (Griffiths-Jones et al., 2008). For a miRNA to be categorised into a cluster it must be located within 10 kilobases (kb) from another miRNA or arise from a polycistronic primary miRNA transcript. Typically miRNAs within a cluster are not only expressed in close proximity of each other but also can be simultaneously expressed i.e. during a developmental stage, can regulate the same mRNA or multiple mRNA involved within a close biological network/pathway.

In the canonical pathway miRNAs are synthesised and processed through a series of cleavage steps (Figure 1.6). Firstly, within the nucleus large primary miRNA (pri-miRNA)
transcripts ~2kb in length are generated via activity of the RNA polymerase II enzyme initiating transcription at an upstream promoter site. Pri-miRNAs consist of a double stranded hairpin loop with long single stranded sequences at both 5’ and 3’ end. The 5’ end harbours a 7-methylguanosine cap and the 3’ end a polyadenylated tail (Cai et al., 2004). For authentic pri-miRNAs to be distinguished from other hairpin loop RNAs in humans they contain specific basal stem motif elements i.e. UG and CNNC motifs (Auyeung et al., 2013). This enables them to be recognised and cleaved by a nuclear RNase III superfamily enzyme called Drosha and ensure efficient processing (Lee et al., 2003b). This cleavage step directs the formation of a ~80 nucleotide (nt) hairpin loop structure with a 2 nt overhang at the 3’ end and is named the precursor miRNA (pre-miRNA). In addition, in humans the co-factor DiGeorge syndrome critical region gene 8 (DGCR8) is required to work in conjunction with Drosha. The Drosha-DGCR8 complex termed the microprocessor is crucial for this cleavage step as genetic silencing of Drosha or DGCR8 can curtail this initial processing stage with downstream consequences evident by the reduction in pre-miRNA and mature miRNA (Lee et al., 2003b; Landthaler et al., 2004). Pre-miRNAs are then transported out of the nucleus and into the cytoplasm by Exportin 5 (Exp5) (Yi et al., 2003). Exp5 is a nucleocytoplasmic transport factor which is dependent on Ran-GTP. Once in the cytoplasm the long double-stranded RNA (dsRNA) pre-miRNA binds to the RNA-binding domain on a cytoplasmic RNase III superfamily enzyme named Dicer and undergoes further cleavage to form the 22 nt mature miRNA sequence (Bernstein et al., 2001). Analysis of the structure of Dicer by MacRae and colleagues revealed the length between the RNA-binding domain and the catalytic domain was respectfully ~22 nts in length thus Dicer acts as a molecular ruler ensuring the correct size of mature miRNAs are generated (Macrae et al., 2006). As with Drosha, the importance of Dicer’s involvement in miRNA processing is highlighted by the effects evident upon silencing it. Preventing the function of Dicer through either small interfering RNA (siRNA) (Grishok et al., 2001) or mutation (Ketting et al., 2001), leads to accumulation of the 80 nt pre-miRNA and reduction in formation of the 22 nt mature miRNA. This demonstrates cleavage by Dicer is a fundamental step in miRNA processing. Similarly to Drosha, functioning Dicer activity in humans requires the co-factors transactivating response dsRNA-binding protein (TRBP) and protein activator of PKR (PACT) (Chendrimada et al., 2005; Lee et al., 2006). Cleavage activity by Dicer removes the loop region of the pre-miRNA and forms an intermediate miRNA duplex containing the mature and the passenger or antisense miRNA. The two strands are also termed 5p or 3p to denote the 5’ or 3’ origin respectively. The miRNA duplex is subsequently bound with argonaute
2 (Ago2) and becomes part of the RNA-induced silencing complex (RISC) (Hammond et al., 2001). Ago 2 is a member of the argonaute protein family which specialise in small RNA guided gene regulation. *In vitro* knockdown of Ago2 prevents miRNA guided mRNA cleavage (Meister et al., 2004). Upon binding with the miRNA duplex, Ago2 initiates the duplex to unwind leaving two separate miRNA strands (Kwak et al., 2012). The selection between the two strands for mature or passenger strand is dictated by which strand has the lowest thermodynamic stability at the 5’ end (Khvorova et al., 2003; Schwarz et al., 2003). It is generally thought that only the mature miRNA is preferentially retained within RISC and acquires function while the passenger strand is degraded. However evidence suggests prominent roles can exist for the passenger strand also (Okamura et al., 2008; Yang et al., 2011a). The mature miRNA still bound to Ago2 and encompassed within RISC is now able to facilitate in post-transcriptional regulation of mRNA.
Figure 1-6 Biosynthesis of microRNAs

Primary miRNA (pri-miRNA) transcripts are processed by the RNase III superfamily enzyme DROSHA in the nucleus to form a ~80 nucleotide precursor miRNA (pre-miRNA). Pre-miRNAs are then exported out of the nucleus and into the cytoplasm by the nucleocytoplasmic transport factor Exportin 5. The pre-miRNA undergoes further cleavage by the cytoplasmic RNase III superfamily enzyme Dicer to generate a miRNA duplex containing the ~22 nucleotide mature miRNA and passenger strand. The mature miRNA is incorporated into RISC where it can now facilitate in post-transcriptional regulation of mRNA i.e. repression of mRNA translation or degradation of mRNA.
1.6.2 The Function of MicroRNAs

The RISC complex utilises the mature miRNA to direct the Ago2 protein to the target mRNA strand. Ago2 is responsible for the endonuclease activity and is a requirement for mediating mRNA cleavage (Meister et al., 2004). The 5’ end of miRNAs contain a seed sequence at nt 2-8 which follow base pair complementation with the 3’ untranslated region (UTR) of mRNA (Lewis et al., 2003). Most miRNA seed sequences are generally well conserved depicting fundamental biological importance. The basis of miRNA-mRNA complementation has allowed in silico predictive programmes to generate an abundance of putative miRNA targets. Evidence shows miRNAs are not limited/confined to “seed match” at the 3’UTR but can also bind to the 5’UTR and coding sequence regions (Tay et al., 2008; Zhou et al., 2014). This has added to the complexity of predicting potential miRNA targets. Generally complete complementation between miRNA and mRNA results in Ago2 mediated cleavage of the mRNA strand whereas incomplete complementation between miRNA and mRNA is thought to lead to repression of mRNA translation rather than mRNA cleavage and degradation (Zeng et al., 2003). Incomplete “seed matching” can prevent the initiation step of mRNA translation from occurring (Humphreys et al., 2005). The miRNA can also cause inhibition after mRNA translation activity has already been initiated (Nottrott et al., 2006). Incomplete complementation has also shown to initiate degradation pathways. For example several studies have highlighted miRNA induced mRNA decay is mediated by deadenylation and decapping mechanisms (Wu et al., 2006) (Behm-Ansmant et al., 2006). RISC is thought to deliver the mRNA, miRNA and Ago2 to cytoplasmic processing bodies (p-bodies) where they are targeted for degradation (Liu et al., 2005).

Ultimately through either repression of target mRNA translation or degradation of target mRNA, miRNA regulate the expression of genes in a post-transcriptional manner. In total the population of miRNAs are thought to act on ~60% of coding mRNA (Esteller, 2011). This enables miRNAs to negatively regulate protein expression and interact with a diversity of cellular systems. Mutation within the 3’UTR of the mRNA strand and/or in the seed sequence of the miRNA transcript can lead to miRNA-mRNA seed mismatch and a disruption of target gene regulation (Chen et al., 2006). Specifically, SNPs within the 3’UTR of the amyloid precursor protein, which are associated with Alzheimer disease, prevent the binding of miRNA-147 and lead to an accumulation of amyloid precursor protein which correlates to neuronal death (Delay et al., 2011). Likewise SNPs in miRNA seed sequences can affect their biosynthesis and also their functionality i.e. a point
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mutation identified in the miRNA-96 seed region impairs processing to its mature form and its ability to recognise targets and ultimately is responsible for hearing loss (Mencia et al., 2009). Although small in size, miRNAs are thought to be able to exert large impact as one miRNA can repress an abundance of different genes. In similar context multiple different miRNAs can target the same gene.

1.6.3 MicroRNAs in Disease

Since their discovery there has been a plethora of research implicating the role of miRNAs in disease. From cancer, cardiovascular disease, neurodegenerative disease and autoimmune disorders, miRNAs exert key control of cellular events including proliferation, apoptosis, differentiation and metabolism (Lu et al., 2005; Lukiw, 2007; De Santis et al., 2010; Small et al., 2011). Understandably a mis-expression of miRNAs or an imbalance in miRNA-regulated events could have consequences that may lead to a disease phenotype i.e. a change in miRNA expression could have the potential to switch cells from a normal to a malignant state. Therefore the expression pattern of miRNAs could be disease dependent. Regarding this, it has been documented that specific miRNA profiles can identify and distinguish between certain cancers i.e. down-regulation of miRNA-15 and miRNA-16 are observed in chronic lymphocytic leukaemia whereas over-expression of miRNA-21 is documented in glioblastoma (Chan et al., 2005; Cimmino et al., 2005). It is also apparent that changes within clusters of individual stem loops can contribute to disease pathology. For example, the members of the miRNA-29 cluster, (miRNA-29a, b1/2 and c) are down-regulated after myocardial infarction (MI) and are implicated in maladaptive cardiac remodelling and fibrosis (van Rooij et al., 2008). Studies have also revealed that the extent of change within a miRNA’s expression does not necessarily correlate with its ability to trigger a disease-related process as anything from a twofold-tenfold expression alteration has shown to be pathological (Calin et al., 2004; He et al., 2005). This indicates that even a small change in miRNA expression has the ability to generate detrimental consequences. The understanding of unique miRNA expression signatures correlating with disease led to the speculation that miRNAs could potentially act as disease biomarkers. This would allow miRNAs to be used in screening methods of an at risk population for early detection of potential disease and/or allow treatment initiation before a disease has had the ability to fully manifest (i.e. early intervention). miRNAs are stable and easily detectable within bio-fluids e.g. plasma, serum and urine (Weber et al., 2010). For example, miRNA-1, miRNA-133a and miRNA-208a were all significantly increased in patients complaining of chest pain before the onset of acute MI (AMI) (Wang
et al., 2010). In addition, a further study documented increased expression of miRNA-208b and miRNA-499-5p after AMI was associated with a greater risk of heart failure and mortality (Gidlof et al., 2013). Thus utilising miRNA signatures as biomarkers could be useful for diagnosis and prognosis of certain diseases. As aforementioned, SNP’s can exist within both the miRNA seed sequence and target mRNA 3’UTR which could prerequisite disease therefore screening for such mutations in at risk patients could also be employed as biomarkers (Chen et al., 2006; Mencia et al., 2009).

1.6.4 MicroRNAs as Therapeutics

As miRNAs are involved in the fine tuning of gene networks and can be implicated in disease processes it is crucial that we recognise their importance and potential in advancing medical therapies. Endogenous expression of miRNA can either be attenuated by anti-miRNA-oligonucleotides (AMO) treatment or augmented by miRNA-mimics. Experimentally the use of exogenous miRNA manipulation has proven successful in a variety of in vivo animal models of disease. For example, administration of anti-miRNA-214 prevents against the development of a mouse model of renal tubulointerstitial fibrosis (Denby et al., 2014) and administration of a miRNA-29b mimic reverses pulmonary fibrosis in the mouse (Montgomery et al., 2014). As with all exogenous drug administration targeting tissue specificity where the miRNA imbalance is observed can prove difficult. Reinstating the balance globally within all cells and tissues is not ideal. Introducing a miRNA into a tissue or cell where it wasn’t dysregulated or even present before could promote adverse unwanted side effects. Thus delivery to target tissue or cell specificity poses a major difficulty in the use of miRNA based therapies. Utilising different routes of administration could help overcome this obstacle i.e. inhalational administration for bronchial based therapies (Mattes et al., 2009). In addition, the use of tissue-specific viral vectors to aid and direct miRNA delivery in a targeted manner could limit the desired effect to disease associated cells (Robson et al., 2003).

Applying the potential use of miRNA based therapies in a clinical setting is now being investigated. Currently there are two miRNA-targeted therapies under examination in human clinical trials, miravirsen and MRX-34. Miravirsen is a locked-nucleic-acid (LNA)-modified antisense oligonucleotide against miRNA-122 which functions to reduce endogenous activity/levels of miRNA-122 (Janssen et al., 2013). miRNA-122 is exclusively expressed within the liver and has a fundamental role in protecting the hepatitis C viral (HCV) from nucleolytic degradation thus allowing it to initiate liver disease
Miravirsen hybridises to the endogenous miRNA-122 thus preventing miRNA-122 interaction with HCV which is then free to be targeted for degradation. In phase II clinical trials patients receiving Miravirsen exhibited successful reduction in HCV levels in a dose dependent manner (Janssen et al., 2013). MRX-34 is a liposomal formulated mimic of miRNA-34 which acts to increase endogenous expression of miRNA-34. Pre-clinical studies demonstrated inhibition of cancer cell growth and induction of apoptosis in cancer cells in a variety of mouse models (He et al., 2007; Liu et al., 2011; Hu et al., 2013). Now in phase I clinical trials, the safety and tolerability of MRX-34 is being investigated in patients with primary and secondary liver cancer (ClinicalTrials.gov Identifier: NCT01829971).

### 1.7 MicroRNAs in Pulmonary Arterial Hypertension

MicroRNAs were first implicated in PAH pathogenesis in 2010 by Caruso and colleagues (Caruso et al., 2010). Here it was documented that miRNA expression was dynamic with expression changing throughout disease progression. Since then further evidence has highlighted their crucial role in disease onset and development. Dysregulated miRNAs have been identified regarding diseases processes such as remodelling of the pulmonary vasculature, more specifically PAEC apoptosis and PASMC proliferation, and hypertrophy of the right ventricle.

#### 1.7.1 MicroRNA-17/92

The polycistronic miRNA cluster 17/92 encompasses 6 individual mature miRNAs including miRNA-17, miRNA-18a, miRNA-19a, miRNA-19b, miRNA-20a and miRNA-92a (www.mirbase.org 2014). *In silico* analysis revealed the gene encoding BMPR-II was a putative target of the miRNA-17/92 cluster. A reduction in BMPR-II expression and function is a major hallmark of HPAH and isolated cases of IPAH. It was of interest that over-expression of miRNA-17/92 cluster *in vitro* reduced the protein expression of BMPR-II in PAECs (Brock et al., 2009). Furthermore the miRNA-17/92 cluster is induced by interleukin-6 (IL-6) a cytokine which has previously been implicated in PAH pathogenesis (Brock et al., 2009). Increased levels of IL-6 are present in PAH patients and a mouse model over-expressing IL-6 spontaneously develops a PH phenotype (Humbert et al., 1995; Steiner et al., 2009). This evidence suggests the increased IL-6 expression in PAH could promote an increase in miRNA-17/92 which consequently decreases expression of BMPR-II. This modulation was found to be signal transducer and activator of transcription 3 (STAT-3) dependent and further evaluation revealed a STAT3 binding site in the...
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The promoter region of the miRNA-17/92 cluster (Brock et al., 2009). Individual miRNAs from this cluster have also been associated with PAH in particular miRNA-17 which targets the cyclin-dependent kinase inhibitor 1A (p21). Over-expression of miRNA-17 reduced the expression of p21 and was also associated with an increase in proliferation in PASMCs (Doebele et al., 2010; Pullamsetti et al., 2012). Inhibition of miRNA-17 in vivo by administration of an antagonir to miRNA-17 reversed the PH phenotype in both the hypoxic mouse model and rat MCT model (Pullamsetti et al., 2012).

1.7.2 MicroRNA-21

Extensive research into miRNA-21 has highlighted a prominent yet ambiguous role in PAH. Generally miRNA-21 expression is induced in PAH proposing a detrimental role. Hypoxia is a major mediator in PAH and has been shown to up-regulate miRNA-21 expression in human PASMCs and PAECS (Sarkar et al., 2010; Parikh et al., 2012) and distal pulmonary arteries in lungs from mice exposed to hypoxia (Pullamsetti et al., 2012; Yang et al., 2012). Increasing the expression of miRNA-21 using a miRNA-21 mimic increases the proliferative and migratory phenotype of PASMCs. This pathological effect is mediated through reduction of the target genes programmed cell death 4 (PDCD4) and BMPR-II (Sarkar et al., 2010; Yang et al., 2012). PDCD4 is a tumour suppressor factor with pro-apoptotic function. A decrease in expression of PDCD4 reduces the pro-apoptotic function and promotes proliferation (Sarkar et al., 2010). Treatment with an anti-miRNA-21 diminishes PASMC proliferation and reduces the PH phenotype in the hypoxic mouse model (Sarkar et al., 2010; Yang et al., 2012). Conversely, evidence from miRNA-21/− mice revealed an exaggerated PH phenotype compared to WT controls in response to SU-HX insult (Parikh et al., 2012). This could be due to increased expression of the miRNA-21 target RhoB. In the pulmonary vasculature RhoB enhances vasoconstriction and remodelling of pulmonary arteries and genetic deletion of RhoB reduces the PH phenotype in hypoxic mouse model (Wojciak-Stothard et al., 2012). This data confers a protective role for miRNA-21. Accordingly, further data from miRNA-21/− mice show a PH phenotype mediated by an increase in PAEC apoptosis induced by activation of a PDCD4/caspase-3 axis (White 2014). In agreement, mice over-expressing miRNA-21 show a reduction in PDCD4 activation and a concomitant decrease in SU-HX induced PH phenotype (White et al., 2014). Additionally, lung samples from IPAH patients showed a down-regulation in miRNA-21 expression (Caruso et al., 2010). MiRNA-21 expression is also decreased in lung homogenates in MCT rat model of PH (Caruso et al., 2010).


1.7.3 **MicroRNA-145**

MiRNA-145 is part of the bicistronic miRNA-143/145 cluster with both mature miRNAs playing fundamental roles in SMC physiology. The abundant and exclusive expression of miRNA-145 in adult SMCs allows it to be utilised as a phenotypic marker and further emphasises its significance in vascular homeostasis (Ji et al., 2007). Multiple studies have implicated a protective role for miRNA-145 within the systemic circulation as evidence shows a reduction in expression of miRNA-145 in carotid arteries with neointimal formation and over-expression of miRNA-145 can reduce neointima formation in balloon injured carotid arteries (Cheng et al., 2009b). In addition, SMCs isolated from miRNA-145/− mice are more susceptible to proliferation and migration than those from WT mice (Elia et al., 2009). However, an opposing role for miRNA-145 is indicated within the pulmonary circulation. Caruso et al. highlighted miRNA-145 expression was increased in tissue samples from PAH patients including PASMCs, pulmonary arteries and whole lung (Caruso et al., 2012). Similarly enhanced expression of miRNA-145 was demonstrated in lung and RV tissue from mice exposed to hypoxia (Caruso et al., 2012). Furthermore, reduction in endogenous miRNA-145 expression by both genetic knockout and inhibition using anti-miRNA-145 reduced the PH phenotype in the hypoxic mouse model (Caruso et al., 2012). Interestingly, ligands of the TGF-β signalling pathway e.g. BMP-4 are known to mediate the transcription of miRNA-145 (Davis-Dusenbery et al., 2011). Silencing of BMPR-II has also shown to increase miRNA-145 in PASMCs suggesting miRNA-145 is downstream of BMPR-II signalling (Caruso et al., 2012). MiRNA-145 targets inhibitory mediators of WNT signalling (Caruso et al., 2012). A reduction in miRNA-145 leads to a reduction in WNT signalling and WNT related genes e.g. cyclin D1 (CCND1) which can consequently decrease proliferation (Baldin et al., 1993; Caruso et al., 2012).

1.7.4 **MicroRNA-206**

MiRNA-206 has recently been implicated in the proliferative phenotype of PASMCs associated with PAH pathogenesis. In the hypoxic rat model of PH, miRNA-206 is down-regulated in lung samples (Yue et al., 2013). Likewise PASMCs isolated from mice exposed to hypoxia show a reduction in expression of miRNA-206 expression and a concomitant increase in Notch-3 (Jalali et al., 2012). Notch-3 is a member of the Notch family receptors and is specifically expressed in vascular SMCs. Notch-3 is fundamental to SMC survival and proliferation and has been demonstrated to be up-regulated in PASMCs from PAH patients (Li et al., 2009). Silencing of Notch-3 via genetic knockout prevents the obliteration of distal pulmonary arteries and protects mice from a hypoxic induced PH
phenotype (Li et al., 2009). A reduction in miRNA-206 is coupled with an increase in proliferation and decrease in apoptosis of human PASMCs with a reverse observed upon miRNA-206 over-expression (Jalali et al., 2012; Yue et al., 2013).

### 1.7.5 MicroRNA-424/503

MiRNA-424 and miRNA-503 are part of a polycistronic cluster separated by only 250 nucleotides on the genome (www.mirbase.org 2014). Both miRNA-424 and miRNA-503 can be transcribed together and their transcription induced by apelin in PAECs (Kim et al., 2013). Apelin is a peptide and activation of its receptors can promote the release of nitric oxide (Tatemoto et al., 2001). Apelin is highly abundant in PAECs and acts as a paracrine signalling molecule with neighbouring PASMCs cementing its crucial role in vascular homeostasis (Sheikh et al., 2008; Kim et al., 2013). In PAECs isolated from IPAH and HPAH patients apelin levels are down-regulated (Chandra et al., 2011). Down-regulation of apelin reduces the induction of miRNA-424/503 and therefore increases their targets fibroblast growth factor 2 (FGF2) and fibroblast growth factor receptor 1 (FGFR1) (Kim et al., 2013). Subsequently, an increase in proliferation of PAECs is observed (Kim et al., 2013). FGF2 levels are increased in PAH patients and associated with disease (Benisty et al., 2004). In conjunction with this evidence, mice devoid of apelin (apelin-/-) exhibit an exacerbated PH phenotype induced by hypoxia (Chandra et al., 2011). Altogether this evidence suggests a protective effect of miRNA-424/503 within the pulmonary circulation. In agreement with this over-expression of miRNA-424/503 reduces proliferation of PAECs via a decrease in pERK1/2 (Kim et al., 2013). Indeed in an in vivo mouse model of PH targeted over-expression of miRNA-424/503 in the lung decreases the disease phenotype including the remodelling of pulmonary arteries (Kim et al., 2013).

### 1.8 Sex and MicroRNAs

#### 1.8.1 Sex Differences in MicroRNAs

The female and male genome shares ~99% content similarity with variation arising from the presence of specific genes on the Y sex chromosome. However, it has long been recognised that the expression of genes between females and males is substantially different implicating that transcriptional and post-transcriptional regulatory modifications may play a fundamental role in shaping the architecture of female and male gene expression (Ranz et al., 2003; Yang et al., 2006; Reinius et al., 2008). As previously discussed, miRNAs are a fundamental part of a cells post-transcriptional regulatory
machinery playing a significant role in shaping genetic networks in both physiological and pathological processes. The expression of miRNAs has been demonstrated to be different in females compared to males highlighting that miRNAs are expressed or regulated in a sex-dependent manner (Marco et al., 2013; Wu et al., 2013). Thus sex could be an important factor in miRNA-mediated regulation of genetic networks. Online databases document the X chromosome encompasses 118 miRNAs whereas the Y chromosome only encodes 2 (www.miRBase.org 2014). Sexual dimorphism of miRNA expression is apparent in multiple processes including embryological development. For example, within the developing foetal lung, sex differences in miRNA expression coincide with important developmental stages. These miRNA target genes involved in lung maturation and vascularisation suggesting that sex differences in their expression could potentially led to structural and functional differences between sexes post-natal (Mujahid et al., 2013). In addition, an enrichment of the miRNA-302 cluster was discovered in differentiating male embryonic stem cells but not female (Ciaudo et al., 2009). Furthermore, an increase in expression of miRNA-21 is observed within male mouse hearts compared to females where it is thought to regulate fibrotic-related genes (Queiros et al., 2013).

Certain diseases have a sex bias in incidence, pathogenesis and treatment response i.e. PAH, liver fibrosis, systemic lupus erythematosus (SLE) and specific cancers (Rigamonti et al., 2005; Brenner et al., 2007; Shapiro et al., 2012; Ohta et al., 2013). Therefore there is rational to speculate/anticipate sex-dependent miRNAs may play a role in development and progression of sex bias pathologies. Indeed in a mouse model of SLE which replicates/mirrors the sex bias observed in human patients, there was recognition of sex-specific miRNAs after disease onset (Dai et al., 2013). Likewise male mice are more susceptible to experimentally induced liver disease which correlated with reduced liver levels of miRNA-29a and miRNA-29b compared to females (Zhang et al., 2012).

1.8.2 Estrogen Regulated MicroRNAs

Sex hormones signal their effector response through binding and activating nuclear receptors. A ligand bound nuclear receptor can directly bind to and interact with DNA at promoter regions and thus exert control over transcriptional regulation of genes through recruitment of either co-activators or co-repressors. Like most protein coding genes, those encoding miRNAs, pri-miRNA, are also transcribed through interaction at their promoter sites. It has been demonstrated that the sex hormones E2, progesterone and testosterone all influence miRNA levels (Bhat-Nakshatri et al., 2009; Waltering et al., 2011; Bae et al.,
Therefore sex specific expression of miRNAs could be attributed to by the presence or absence of sex hormones. This is demonstrated by evidence showing inhibition of E2 synthesis, via the aromatase enzyme inhibitor formestane, altered the miRNA expression profile in the neonatal mouse brain (Morgan et al., 2011). The main female circulating hormone E2 can govern miRNA expression directly through estrogen-response-elements in the promoter element of the pri-miRNA gene (Di Leva et al., 2010) or indirectly through transcription of estrogen related genes e.g. c-MYC which can in turn interact with transcription of pri-miRNA gene (Castellano et al., 2009). E2-related miRNAs could play a fundamental role in estrogen positive/orientated diseases e.g. breast cancer, SLE and PAH. As previously mentioned sex-specific miRNAs were associated with the sex bias of SLE development. In particular, the SLE associated miRNA-182-96-183 cluster and miRNA-379 were up-regulated in female diseased mice and not males. It was also highlighted that exogenous treatment of estrogen exacerbated the increase in females (Dai et al., 2013). Estrogen administration correlates with an exacerbation of SLE pathogenesis (Roubinian et al., 1979). Thus estrogen could affect disease development through directing expression of specific miRNAs. Furthermore, induction of miRNA-29a and miRNA-29b by estrogen is thought to play a role in protecting female mice from experimental liver injury (Zhang et al., 2012).

Estrogen is also thought to exert control of miRNA expression through interaction with miRNA processing machinery. For example there is an ERα binding site in the promoter region of the Dicer gene and accordingly the expression of Dicer can be increased with estrogen stimulation (Bhat-Nakshatri et al., 2009). In the same study estrogen stimulation increased the expression of several miRNAs suggesting that miRNA biogenesis could be increased by estrogen through an increase in the processing gene Dicer. However, in contrast, a similar study showed expression of the Dicer gene was down-regulated by stimulation of estrogen (Lin et al., 2007). Differential expression of Dicer has been highlighted in ER+ vs. ER- breast cancers (Cheng et al., 2009a). Therefore the discrepancy in the two previous studies could be due to the estrogen status of the cells investigated.

1.9 **Hypothesis and Aims**

In summary, PAH is a progressive and presently incurable vasculopathy which affects the pulmonary arteries. PAH is characterised by intense irreversible remodelling of the pulmonary arteries, high pulmonary arterial pressure and right heart failure. Regression of these attributes are the main focus of current and future PAH therapies.
One unexplained factor of PAH is an observed high female susceptibility with their being up to 4 female patients for every one male patient (Rich et al., 1987b; Humbert et al., 2006; Badesch et al., 2010; Ling et al., 2012). This increased female to male ratio in PAH prevalence strongly suggests there is an influence of sex or sexual dimorphism in the development of PAH. In particular there is strong evidence suggesting a causative role for female sex hormones in PAH pathogenesis (White et al., 2012; Mair et al., 2014; Wright et al., 2015). Anomalous estrogen synthesis, signalling and metabolism have all been linked to the remodelling of pulmonary arteries. Many experimental animal models which mirror the enhanced female susceptibility are associated with the peripheral serotonin system indicating that there is interplay between the estrogen and serotonin pathways (Dempsie et al., 2011; White et al., 2011a; Dempsie et al., 2013). Serotonin plays a pathological role in pulmonary artery remodelling via the 5-HT₁B receptor making it a favourable gene to therapeutically target (Keegan et al., 2001; Morecroft et al., 2005).

Building evidence also implicates a role for microRNAs (miRNAs) in PAH (Caruso et al., 2010). It is recognised that sex differences exist in miRNA expression and therefore miRNAs which are influenced by sex may shape the architecture of female and male gene expression differently (Marco et al., 2013; Wu et al., 2013). Sexual dimorphism of miRNAs is thought to play a role in the development of sex bias diseases such as SLE with expression potentially regulated by the presence or absence of sex hormones (Dai et al., 2013; Ohta et al., 2013). With regards to PAH, it is currently unknown whether female and male differences in miRNA expression exist and could underlie female susceptibility in disease onset and development. Little is also known surrounding the interplay between estrogens and miRNAs in PAH. Examining the sexual dymorphism in miRNAs with regards to PAH disease may provide insight into the sex bias observed in PAH.

The hypothesis of this thesis was that sex differences in miRNA expression could underpin differences in gene expression that may confer female susceptibility in PAH.

The central research focus of this thesis was to investigate the influence of sex on miRNA expression, primarily in PASMCs, from both experimental and clinical PAH samples and understand how this could potentially underpin the sex bias in PAH. This was achieved by the following research aims:

1. In vitro identification of female and male sex differences in miRNA expression from PASMCs.
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2. *In vitro* characterisation and validation of a target for a sex specific miRNA.
3. *In vitro* examination of the role of estrogen in regulation of a sex specific miRNA.
4. *In vivo* evaluation of miRNA-target regulation.
5. *In vivo* investigation of the effect of miRNA modulation on prevention and reversal of a PH phenotype in female mice.
Chapter Two

2 Materials and Methods
2.1 **Chemicals and Reagents**

All chemicals and reagents were supplied by Sigma-Aldrich (Dorset, United Kingdom) or Fisher Scientific (Loughborough, United Kingdom) unless otherwise stated. Materials for RNA and protein analysis were obtained from Qiagen (United Kingdom) and Life Technologies (Paisley, United Kingdom). All experimental procedures involving RNA were carried out using certified nuclease-free reagents and plastics and supplied by Ambion, Life Technologies (Paisley, United Kingdom). All cell culture plastics were obtained from Corning, Fisher Scientific (Loughborough, United Kingdom).

2.2 **Ethical Approval**

All experimental procedures utilising animals conformed with the United Kingdom Animal Procedures Act (1986) and with the “Guide for the Care and Use of Laboratory Animals” published by the United States National Institutes of Health (publication No.85-23, revised 8th edition 2001). All *in vivo* procedures were performed under project license 60/4404 held by Professor Margaret R. MacLean, University of Glasgow, and personal license 60/12915 held by Emma Wallace, University of Glasgow. Ethical approval was granted by the University of Glasgow Ethics Committee. All experimental procedures utilising primary human pulmonary artery smooth muscle cells conformed with the principles outlined in the Declaration of Helsinki and were approved by Cambridgeshire 1 Research Ethics Committee (REC reference: 08/H0304/56+5).

2.3 **Animals**

All animals were housed either at the Central Research Facility (CRF), University of Glasgow, or the licensed facility located in the West Medical Building, University of Glasgow. All animals were maintained in a continuous 12 hour light/dark cycle with access to food and water *ad libitum*. All genetically modified animals were ear clipped by staff at the CRF and genetic background confirmed by genotyping. Where necessary, wild-type (WT) littermates were studied as controls. Where appropriate, all animals were randomly allocated to experimental groups and studied in a blinded manner. Experimental groups were un-blinded upon completion of analysis.
2.3.1 Wild-type Mice

Inbred wild-type (WT) mice aged 7 weeks were obtained from Charles River (United Kingdom). Mice were housed at the CRF, University of Glasgow for a 1 week acclimatisation and monitoring period before use in any experimental procedures.

2.3.2 BMPR-II^{R899X+/−} Mice

Mice harbouring a heterozygous knock-in mutation within the BMPR-II gene (R899X, BMPR-II^{R899X+/−}) were kindly supplied by Professor Nicholas W. Morrell, University of Cambridge. Briefly, the BMPR-II gene was cloned and the R899X substitution mutation was introduced using site-directed mutagenesis. The vector containing the mutated BMPR-II was transfected into 129S3 mouse embryonic stem cells by electroporation. The modified embryonic stem cells were then injected into blastocytes and implanted into a pseudopregnant C57Bl/6 female mouse. Chimeric male mice were then bred with female C57Bl/6 mice to produce heterozygous R899X offspring which were back-crossed to C57Bl/6 for 5 generations. BMPR-II^{R899X+/−} mice have previously been shown to exhibit a mild PH phenotype at 6 months of age (Long et al., 2011; Long et al., 2015). For experimental procedures subsequently mentioned, the BMPR-II^{R899X+/−} mice were utilised at 6 months of age and WT littermates studied as controls.

2.4 Lung Histology

2.4.1 Fixation, Paraffin Embedding and Sectioning of Lung Tissue

At sacrifice the left lung of the mouse was gently flushed with sterile phosphate buffered saline (PBS) and inflated with 10% (v/v) neutral buffered formalin (NBF). The lung was then dissected free of the mouse cadaver, submerged in 10% NBF and kept overnight at room temperature on a shaker with slight agitation. Lungs were then processed through a series of dehydration steps and paraffin embedded (Table 2.1) using a Citadel 1000 tissue processor (Thermo Scientific, United Kingdom).

Paraffin embedded lung blocks were stored at room temperature, but were briefly placed at -20°C prior to sectioning to aid with the cutting process. 5 μm frontal plane lung sections were cut using a microtome (Leica RM2125, Leica Microsystems, Milton Keynes, United Kingdom) and mounted onto polylysine coated glass microscope slides. Mounted lung sections were stored at room temperature.
### Table 2.1 Fixation and paraffin embedding of mouse lung tissue

<table>
<thead>
<tr>
<th>Processing Step</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% NBF</td>
<td>Minimum of 4 hours</td>
</tr>
<tr>
<td>70% (v/v) Ethanol in H₂O</td>
<td>15 minutes</td>
</tr>
<tr>
<td>80% (v/v) Ethanol in H₂O</td>
<td>15 minutes</td>
</tr>
<tr>
<td>95% (v/v) Ethanol in H₂O</td>
<td>25 minutes</td>
</tr>
<tr>
<td>95% (v/v) Ethanol in H₂O</td>
<td>25 minutes</td>
</tr>
<tr>
<td>100% (v/v) Ethanol</td>
<td>15 minutes</td>
</tr>
<tr>
<td>100% (v/v) Ethanol</td>
<td>15 minutes</td>
</tr>
<tr>
<td>100% (v/v) Ethanol</td>
<td>15 minutes</td>
</tr>
<tr>
<td>Histoclear</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Histoclear</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Paraffin</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Paraffin</td>
<td>30 minutes</td>
</tr>
</tbody>
</table>

#### 2.4.2 Immunohistochemistry

Lung sections were de-waxed in xylene for 30 minutes and rehydrated through an alcohol/water gradient consisting of 100% ethanol for 10 minutes, 100% ethanol for 5 minutes, 90% ethanol for 2 minutes, 70% ethanol for 2 minutes and finally running tap water for 10 minutes. Rehydrated lung sections were then immersed in 10 mM citric acid buffer (Sigma Aldrich, Dorset, United Kingdom) at pH 6 and boiled in a microwave for 4x 5 minutes in a process called heat-induced-epitope-retrieval. This step is also known as antigen retrieval and aids unmasking of antigen sites by breaking down protein cross-links which are formed during the fixation process. Lung sections were left to cool in the citric acid buffer at room temperature for 20 minutes before being washed in running tap water for 10 minutes. As most tissue contains endogenous peroxidase, addition of a horse-radish-peroxidase conjugated antibody may result in non-specific background staining. To block endogenous peroxidase, lung sections were submerged in a solution of methanol containing 3% (v/v) hydrogen peroxide (Sigma Aldrich, Dorset, United Kingdom) for 30 minutes before being washed in running tap water for 10 minutes. Following this, non-specific blocking was carried out by blocking the lung sections with 2.5% (v/v) horse serum (ImmPRESS kit, Vector Labs, Peterborough, United Kingdom) for 1 hour in a
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Materials and Methods

humidified chamber at room temperature. The α-smooth muscle actin (α-sma), proliferating cell nuclear antigen (PCNA) or von williebrand factor (vWF) primary antibody (diluted in 1% (w/v) bovine serum albumin (BSA) in 10mM phosphate buffered saline (PBS)) (Table 2.3) was then applied to the lung sections overnight in a humidified chamber at 4°C. An immunoglobulin G (IgG) negative control was used on duplicate lung sections at the same concentration to observe any non-specific binding. Next, lung sections were washed twice in tris-buffered saline (TBS) for 10 minutes and then incubated with anti-rabbit Ig peroxidase polymer secondary antibody (ImmPRESS kit, Vector Labs, Peterborough, United Kingdom) for 1 hour in a humidified chamber at room temperature. The secondary antibody should selectively bind to the primary antibody. Lung sections were then washed twice in TBS for 10 minutes. Immunolocalisation of α-smooth muscle actin was visualised via a 2-5 minute incubation with 3,3’-diaminobenzidine (DAB) and nickel substrate kit (Vector Labs, Peterborough, United Kingdom). A dark brown staining was indicative of positive immunolocalisation. To terminate the reaction lung sections were immersed in running tap water for 10 minutes. Lung sections were then counterstained with haemotoxylin and eosin, which stains nuclear and cytoplasmic portions, respectively, for 5 minutes before a final wash in running tap water for 5 minutes. Lung sections were then rapidly dehydrated through a water/alcohol gradient consisting of 70% ethanol for 1 minute, 90% ethanol for 1 minute, twice in 100% ethanol for 5 minutes and finally twice in histo-clear for 5 minutes. Cover slips were mounted onto the lung sections using Tissue-Tek (Sakura Finetek, Netherlands) and staining visualised on a Zeiss Imager M.1 AX10 and axiovision Rel. 4.8 (Carl Zeiss Microscopy Ltd, Cambridge, United Kingdom).

2.4.3 Elastin Pico-Sirius Red Staining

A modified Miller’s Elastin staining protocol was utilised protocol (Miller, 1971). Briefly, lung sections were de-waxed in histo-clear for 20 minutes and rehydrated through an alcohol/water gradient consisting of 100% ethanol for 5 minutes, 90% ethanol for 5 minutes, 70% ethanol for 5 minutes and finally running tap water for 5 minutes. Rehydrated lung sections were then submerged into 0.5% (w/v) potassium permanganate solution for 5 minutes. This oxidises the lung tissue and enhances subsequent staining by eliminating the formalin pigment. Lung sections were then rinsed in running tap water for 5 minutes before being placed in 1% (w/v) oxalic acid for 2-3 minutes. This decolourises the potassium permanganate from the sections. The lung sections were further rinsed in tap water and 95% ethanol. Sections were then submerged in Millers Elastin Stain (Thermo
Scientific, United Kingdom) for 2 hours. To remove excess staining, lung sections were rinsed in 95% ethanol and then running tap water. The lung sections were then very briefly counterstained with pico-sirius red and finally rinsed in running tap water before being rapidly dehydrated through a water/alcohol gradient consisting of 70% ethanol for 1 minute, 90% ethanol for 1 minute, twice in 100% ethanol for 5 minutes and finally twice in histo-clear for 5 minutes. Cover slips were mounted onto the lung sections and staining visualised on a light microscope (Zeiss Imager M.1 AX10) as aforementioned. Elastin pico-sirius red staining highlights elastic fibres in black and collagen in red.

2.4.4 In situ Hybridisation

In situ hybridisation was performed as previously described (Pena et al., 2009). Briefly, lung sections were de-waxed in xylene for 30 minutes and rehydrated through an alcohol/water gradient consisting of 100% ethanol for 5 minutes, 100% ethanol for 5 minutes, 95% ethanol for 5 minutes, 95% ethanol for 5 minutes, 70% ethanol for 5 minutes and finally RNAase free H2O for 10 minutes. Deproteinisation was then performed on rehydrated lung sections which consisted of each lung section being treated with pre-warmed proteinase K (20µg/ml, Life Technologies, Paisley, United Kingdom) for 20 minutes at 37°C in a humidified chamber. This step aids permeabilisation of the tissue and unmasking of hybridisation sites by breaking down protein cross-links which are formed during the fixation process specifically those associated with nucleic acids. To inactivate the proteinase K and preserve morphology of the lung sections were first washed in 0.1M glycine in TBS for 5 minutes at room temperature before being fixed with 4% para-formaldehyde for 10 minutes at room temperature. Next, lung sections were washed in 0.1M glycine in TBS for 5 minutes at room temperature then incubated in imidazole buffer (300mM NaCl, 0.1M 1-methylimidazole, pH 8) for 10 minutes at room temperature. The lung sections were washed once more in 0.1M glycine in TBS before a final fixation in EDC solution (300mM NaCl, 0.1M 1-methylimidazole, 0.1M N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride) for 2 hours at room temperature in a humidified chamber. The lung sections were then treated with acetylation buffer (1% (v/v) triethanolamine, 0.5% (v/v) acetic anhydride in RNAase free H2O) for 30 minutes at room temperature before washing in TBS for 5 minutes at room temperature. The acetylation reaction eliminates any remaining positive charges in the lung sections and reduces the background signal. Lung sections were then pre-hybridised by applying 500µl of hybridisation solution (#H7782 Sigma Aldrich, Dorset, United Kingdom) to each section for 1 hour at probe annealing temperature before overnight probe annealing in
hybridisation oven. Double DIG-labelled (3’ and 5’) mercury LNA hsa-miR-96 probe (50nmol/L) and mercury LNA scramble-miR probe (negative control) were used at hybridisation temperature 52°C and 57°C respectively. To remove any unbound probe, post-hybridisation stringency washes were carried out on the lung sections. This involved a series of sodium citrate washes with increasing salt stringency. Lung sections were then washed in TBS before beginning immunodetection with the DIG nucleic acid detection kit (ROCHE #11175041910, Roche Life Sciences, Burgess Hill, United Kingdom) as per manufacturer’s instructions. Positive staining was evident by a purple colour. The reaction was terminated by immersing lung sections in H2O and cover slips were mounted onto the lung sections using VectaMount AQ aqueous mounting medium (Vector Laboratories, Peterborough, United Kingdom) and staining visualised on a Zeiss Imager M.1 AX10 and axiovision Rel. 4.8 (Carl Zeiss Microscopy Ltd, Cambridge, United Kingdom).

2.5 Cell Culture

All cell culture experiments were performed under sterile conditions in a class II laminar flow cabinet. When in culture all cells were maintained in a humidified incubator at 37°C with continuous supply of 5% CO₂ and 95% air.

2.5.1 Mouse Pulmonary Artery Smooth Muscle Cell Culture

Mouse pulmonary artery smooth muscle cells (PASMCs) were generated and kindly supplied by Professor Nicholas W. Morrell, University of Cambridge. Mouse PASMCs were derived from pulmonary arteries of BMPR-II R899X+/− and WT female and male mice. The SMC phenotype of the mouse PASMCs was confirmed by their general cell morphology. Mouse PASMCs were maintained in 75cm³ culture flasks containing 20% (v/v) foetal bovine serum (FBS, Sera Laboratories International, West Sussex, United Kingdom), 1% (v/v) antibiotic solution (10,000 units penicillin and 10mg streptomycin, Sigma Aldrich, Dorset, United Kingdom) in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies, Paisley, United Kingdom) supplemented with smooth muscle cell growth factor kit (0.5ng/ml Epidermal Growth Factor, 2ng/ml Basic Fibroblast Growth Factor, 5µg/ml Insulin, Promocell, United Kingdom). DMEM was routinely replenished every 48 hours. Mouse PASMCs were passaged when they reached ~90% confluency (~90% monolayer coverage of culture flask). This involved aspirating the DMEM off the PASMCs, washing twice with sterile PBS warmed to 37°C and addition of 2ml of trypsin/EDTA solution (0.1% (w/v) EDTA in PBS, Life Technologies, Paisley United Kingdom) warmed to 37°C. The culture flask was then returned to the incubator for 1
minute to aid the trypsinisation process. Trypsin is a proteolytic enzyme and dissociates the adherent PASMCs from the culture flask. Once detached the PASMCs were immediately re-suspended in 8mls of 20% FBS DMEM. This action neutralises the trypsin and terminates the trypsinisation process. The new PASMC suspension is then further sub-cultured accordingly. Mouse PASMCs were seeded at a density of 10,000 per cm$^3$. DMEM was always replenished 24 hours (maximum) after a newly generated sub-culture. Mouse PASMCs were utilised for experiments between passages 4-8.

2.5.2 Human Pulmonary Artery Smooth Muscle Cell Culture

Human pulmonary artery smooth muscle cells (hPASMCs) were generated and kindly supplied by Professor Nicholas W. Morrell, University of Cambridge. HPASMCs were derived from distal pulmonary arteries (~1mm external diameter) of both female and male PAH patients and non-PAH patients (Table 2.2) as previously described (Wharton et al., 2000). PAH patients were a mix of heritable PAH and idiopathic PAH and hPASMCs were explanted at lung transplantation. Non-PAH patients had no incidence of PAH disease and the lungs were macroscopically normal. Pathology reports were examined for the non-PAH patients and no evidence of pulmonary arterial remodelling was detailed. Non-PAH hPASMCs were explanted from donor tissue and studied as controls. The SMC phenotype of the hPASMCs was confirmed by their general cell morphology (Figure 2.1). HPASMCs were maintained in 75cm$^3$ culture flasks containing 10% (v/v) FBS (Sera Laboratories International, West Sussex, United Kingdom), 1% (v/v) antibiotic antymycotic solution (10,000 units penicillin, 10mg streptomycin and 25µg amphotericin B, Sigma Aldrich, Dorset, United Kingdom) in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies, Paisley, United Kingdom). HPASMC culture was carried out as described in 2.5.1 except 10% FBS DMEM was used. HPASMCs were utilised for experiments between passages 3-8.

2.5.3 HeLa Cells

HeLa cells are an immortalized cell line which was initially derived from cervical cancer cells. HeLa cells were obtained commercially and were maintained in 150cm$^3$ culture flasks containing 10% (v/v) FBS (Sera Laboratories International, West Sussex, United Kingdom), 1% (v/v) antibiotic antymycotic solution (10,000 units penicillin, 10mg streptomycin and 25µg amphotericin B, Sigma Aldrich, Dorset, United Kingdom) in DMEM (Life Technologies, Paisley, United Kingdom). HeLa cell culture was carried out as described in 2.5.1 except 10% FBS DMEM was used.
**Figure 2-1 Morphology of distal pulmonary arterial smooth muscle cells**

Microphotograph of human distal pulmonary artery smooth muscle cells grown in a T75 culture flask.
Table 2.2 Patient information for human pulmonary arterial smooth muscle cells

<table>
<thead>
<tr>
<th>Patient I.D.</th>
<th>Sex</th>
<th>Age</th>
<th>Disease Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>9MP</td>
<td>Male</td>
<td>72</td>
<td>N/A</td>
</tr>
<tr>
<td>23MP</td>
<td>Male</td>
<td>43</td>
<td>APAH (Eisenmengers Syndrome)</td>
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<td>32MP</td>
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<td>58</td>
<td>Mild Emphysema</td>
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<tr>
<td>34MP</td>
<td>Male</td>
<td>62</td>
<td>Emphysema</td>
</tr>
<tr>
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<td>Female</td>
<td>N/A</td>
<td>HPAH (N903S)</td>
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<tr>
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<td>33</td>
<td>IPAH</td>
</tr>
<tr>
<td>37MP</td>
<td>Female</td>
<td>24</td>
<td>IPAH</td>
</tr>
<tr>
<td>38MP</td>
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<td>PAH</td>
</tr>
<tr>
<td>56MP</td>
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<td>HPAH (C347R)</td>
</tr>
<tr>
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<td>17</td>
<td>HPAH (W9X)</td>
</tr>
<tr>
<td>73MP</td>
<td>Female</td>
<td>30</td>
<td>HPAH (R899X)</td>
</tr>
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<tr>
<td>75MP</td>
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<td>Metastatic disease</td>
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<tr>
<td>77MP</td>
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</tr>
<tr>
<td>78MP</td>
<td>Male</td>
<td>68</td>
<td>Lung Carcinoma</td>
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<td>Squamous cell carcinoma</td>
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<tr>
<td>106MP</td>
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</tr>
<tr>
<td>115MP</td>
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<td>IPAH</td>
</tr>
</tbody>
</table>
2.6  **Pulmonary Artery Smooth Muscle Cell Proliferation Assay**

2.6.1  **Haemocytometer Cell Counting**

HPASMCs were counted manually using a haemocytometer to evaluate cell proliferation. For proliferation assays hPASMCs were seeded in 24-well plates at a density of 10,000 cells per well and maintained in 10% FBS DMEM until 60% confluency was reached. HPASMCs were then quiesced in 0.2% (v/v) FBS DMEM for 24 hours. Quiescing is necessary to synchronise cell cycle stage before subsequent stimulation. HPASMCs were stimulated with the appropriate agonist for a further 72 hours in the presence of 2.5% (v/v) FBS DMEM. A stimulation of 2.5% and 10% FBS DMEM was studied as a negative and positive control, respectively. Where necessary, hPASMCs were incubated with the appropriate antagonist for 1 hour prior to agonist stimulation. Each experimental condition was performed in triplicate. Upon completion of the 72 hour stimulation time point, DMEM was aspirated from the hPASMCs and cells were washed twice with 1ml of sterile PBS warmed to 37°. Next 150µl of trypsin/EDTA solution (Life Technologies, Paisley, United Kingdom) was added to each well. The 24-well plate was then returned to the incubator for 1 minute to aid the trypsinisation process. Once detached, the hPASMCs were immediately re-suspended in 500µls of 10% FBS DMEM per well and gently pipetted up and down. The total content of each well was then transferred to a 1.5ml eppendorf and spun in a pre-cooled centrifuge at 4°C at 3500xg for 10 minutes. The supernatant was then carefully aspirated off to ensure a cell pellet remained intact. The cell pellet was then re-suspended in 200µl of 10% FBS DMEM and vortexed briefly to obtain an even suspension. With the haemocytometer, 10µl of the cell suspension was pipetted into the square grid. Using a hand tally counter, the number of cells in each 16-squared red-outlined corner was counted (Figure 2.2). The average of the 4 corners was used to calculate the number of cells. The haemocytometer is designed so that the number of cells in one 16-square corner is equivalent to the number of cells x 10⁴/ml. As this provides a number per ml, the cell number was divided by 5 to obtain a number per 200µl (accounting for the dilution factor at re-suspension). Duplicate 24-well plates were studied to obtain RNA or protein lysates for further experimental analysis.
Figure 2-2 Haemocytometer counting grid

A schematic representation of the haemocytometer counting grid. Pulmonary arterial smooth muscle cells were counted in each 16-square corner outlined in red and an average cell number count was obtained.

2.6.2 5-bromo-2-deoxyuridine Incorporation

PASMCs were also counted via the amount of 5-bromo-2-deoxyuridine (BrdU) incorporation into DNA. BrdU is a derivative of uridine and is a structural analogue of thymidine. When added to actively proliferating cells, it can mimic thymidine and be incorporated into newly synthesised DNA during the synthesis phase (S-phase) of the cell cycle. The BrdU incorporation assay is a common method to measure proliferation. As the BrdU assay is non-isotopic it is generally considered a safer alternative of proliferation assay than those involving tritiated substances. For assessment of proliferation the Calbiochem® BrdU Cell Proliferation Assay was utilised with the adherent cell protocol (Calbiochem, Merck Millipore, Germany). This is an enzyme immunoassay kit and uses colorimetric detection. Briefly, hPASMCs were seeded at 2,500 cells per well in a 96-well plate and maintained in 10% FBS DMEM until 60% confluency was reached. Two separate control conditions were utilised consisting of 10% FBS DMEM (blank control)
and no BrdU label (background control). HPASMCs were then quiesced in 0.2% (v/v) FBS DMEM for 24 hours. HPASMCs were stimulated with the appropriate agonist for a further 72 hours in the presence of 2.5% (v/v) FBS DMEM. A stimulation of 2.5% and 10% FBS DMEM was studied as a negative and positive control respectively. Where necessary, hPASMCs were incubated with the appropriate antagonist for 1 hour prior to agonist stimulation. Each experimental condition was performed in quadruplicate. The BrdU label was prepared at a working stock of 1µl BrdU label in 2ml of 10% FBS DMEM and in the last 24 hour time period of the experiment 20µl of the working BrdU stock was added to each well (except for the background control wells). Following this, the contents of the 96-well plate was removed by inverting over the sink. The 96-well plate was then carefully blotted dry with paper towels before addition of 200µl of fixative/denaturing solution to each well. This solution acts to fix, permeabilise and denature the DNA of the cells for the incorporated BrdU to be subsequently detected. After a 30 minute incubation period the contents of the 96-well plate was removed by inverting over the sink and carefully blotted dry. The 100x anti-BrdU antibody was diluted 1:100 in the antibody dilution buffer and 100µl of the final solution was added to each well for one hour incubation at room temperature. The anti-BrdU monoclonal antibody binds to the incorporated BrdU in the cells. A working solution of 1x wash buffer was prepared by diluting the 20x stock 1:20 in distilled H₂O (dH₂O). Each well was washed 3 times with 200µl 1x wash buffer and then carefully blotted dry. This allows removal of unbound antibody and prevents unwanted background labelling. The peroxidase goat anti-mouse IgG horse radish peroxidase (HRP) conjugate was prepared by diluting in the conjugate diluent and syringe filtered through a 0.2µm filter. Next, 100µl of the diluted conjugate was added to each well and incubated at room temperature for 30 minutes. The HRP conjugate binds to the anti-BrdU antibody in the cells. Following this, each well washed a further 3 times with 200µl 1x wash buffer. The 96-well plate was then completely submerged in dH₂O to flood each well and then carefully blotted dry. After this, 100µl of substrate solution was added to each well and incubated at room temperature in the dark for 15 minutes. The substrate solution contains the chromogenic substrate tetra-methylbenzidine (TMB) which is a colourless solution. The HRP enzyme of the conjugate catalyses the conversion of TMB to a blue solution of which the intensity is directly proportional to amount of BrdU incorporated into the cells. To terminate the reaction, 100µl of stop solution was added to each well. The absorbance was measured using a spectrophotometric plate reader at dual wavelength of 450-540nm within 30 minutes of adding the stop solution.
2.7 Transient Transfection of Pulmonary Artery Smooth Muscle Cells

To assess the effect of miRNA-96 over-expression on 5-HT$_{1B}$ expression and 5-HT-induced proliferation, HPASMCs were transiently transfected with small RNA molecules. Briefly, HPASMCs were seeded in 6-well and 24-well plates at equal density in 10% (v/v) FBS DMEM. At 60% confluency cells underwent transient transfection with 1nM pre-miR-96 (Ambion, PM10422) or 1nM pre-miR-negative control (Ambion, AM17110 #1) using Lipofectamine 2000 (Invitrogen) and optimem (Invitrogen) as per manufacturer’s instructions for a total of 6 hours before the media was replaced with 10% (v/v) FBS DMEM. For expression analysis each condition was performed in duplicate to allow RNA harvest 48 hours and protein harvest 72 hours post-transfection for Taqman and western blot analysis respectively. For proliferation analysis each condition was performed in triplicate and PASMCs were subsequently quiesced in 0.2% FBS and the proliferation protocol followed as of section 2.6.1.

2.8 Western Blotting

2.8.1 Protein Extraction and Preparation

For PASMCs protein was extracted from either 6-well plates or T-75 flasks. Briefly, at experimental end point the culture dish containing PASMCs was immediately placed on ice. The media was aspirated off and cells washed twice with either 1ml or 5ml of cold PBS per well or flask, respectively. Following this 120µl (per well) or 300µl (per flask) of ice-cold radio immuno precipitation assay (RIPA, Thermo Fisher Scientific, Loughborough, United Kingdom) buffer supplemented with protease inhibitors (0.1mmol/L PMSF, 1µg/ml soybean trypsin inhibitor and 1µg/ml benzamidine) was added to the culture dish. RIPA buffer lyses whole cells and allows proteins to be released and solubilised. The protease inhibitors help prevent proteolysis, dephosphorylation and denaturation of the protein sample. Keeping the culture dish on ice, the cells were scraped using a sterile plastic scraper and cell lysates collected in a pre-chilled 1.5ml eppendorf. Samples were then left on ice for 30 minutes to allow disassociation of protein complexes. Alternatively, for whole lung samples protein was prepared as following. Upon dissection from the mouse cadaver the right lung lobe was either immediately snap-frozen in liquid nitrogen and stored in a freezer at -80°C, or placed in a pre-chilled 2ml eppendorf containing 500µl of RIPA buffer supplemented with protease inhibitors. A 5mm stainless steel bead (Qiagen, United Kingdom) was added to each sample eppendorf which was then placed in a Tissue Lyser II (Qiagen, United Kingdom) for homogenisation. This process
involved high-speed shaking (frequency 25Hz) for 4x 30 second intervals. After this, samples were left on ice for 30 minutes and sonicated on ice for 2 minutes to allow disassociation of protein complexes. Following this, samples were then spun in a pre-chilled centrifuge at 4°C for 10 minutes at 8,500xg. After centrifugation the sample supernatant was transferred into a new pre-chilled eppendorf carefully avoiding the debris pellet and stored in a freezer at -80°C.

2.8.2 Bicinchoninic Acid Assay

To determine protein concentration a bicinchoninic acid (BCA) assay (Pierce, Thermo Fisher Scientific, Loughborough, United Kingdom) was performed. This assay involves protein induced reduction of Cu$^{2+}$ to Cu$^+$. Cu$^+$ and bicinchoninic acid forms a purple complex which is directly proportional to the concentration of protein present (Smith et al., 1985). To evaluate protein concentration within experimental samples, colorimetric detection was compared against a range of bovine serum albumin (BSA) standards (0-2mg/ml BSA solution diluted in RIPA buffer). Briefly, BSA standards and experimental samples in duplicate were pipetted into a 96-well plate. BCA assay solution was prepared by adding together 9.8ml of reagent A to 200µl of reagent B and mixed thoroughly. Immediately 200µl of this solution was added to each well and left on a micro-plate shaker with gentle agitation for 20 minutes at room temperature. The 96-well plate was then read by a POLARstar OPTIMA microplate reader (BMG Labtech, Germany) at a wavelength of 562nm. A standard curve (with R$^2$ of ≥0.99) was obtained from the BSA standards and sample protein concentration determined.

2.8.3 Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis

Protein samples were separated according to their molecular weight using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). 30µg of protein samples were prepared by adding NuPAGE reducing agent and NuPAGE sample buffer (containing SDS, Novex, Life Technologies, Paisley, United Kingdom) in a ratio of 10:2:4 respectively. Protein samples were then denatured and reduced by heating at 70°C for 10 minutes. This process is necessary to unfold the 3-dimensional conformation of the protein and enable antibody recognition. The SDS within the sample buffer binds to the protein and makes the protein negatively charged. As SDS binds to protein in a ratio of 1.4:1, the negative charge given to the protein by SDS is proportionate to its size (molecular weight). Samples were loaded into NuPAGE Novex 4-12% Bis-Tris Mini Gels along with the SeeBlue Plus2 (Invitrogen, Paisley, United Kingdom) pre-stained molecular weight
marker. The protein standard allowed observation of electrophoresis progression and determination of protein size. The gel was placed in NuPAGE MOPS SDS running buffer and run at 150Volts until adequate migration and separation of protein samples.

2.8.4 Protein Transfer and Visualisation

Proteins were transferred onto a polyvinylidene fluoride (PVDF) microporous membrane (Millipore Corporation, Massachusetts, USA) using a wet transfer method. To activate the PVDF membrane it was pre-treated in 100% methanol. The wet transfer tank was prepared containing 5% (v/v) NuPAGE transfer buffer, 20% (v/v) methanol in dH₂O and run at 30Volts for 2 hours. To check successful transfer, the membrane was immersed in 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid solution for 1 minute to visualise protein bands on the membrane. Following this, the membrane was rinsed in dH₂O twice to remove the Ponceau S stain. Before antibody detection, membranes were blocked to prevent non-specific background binding of both primary and secondary antibodies. A 5% (w/v) non-fat milk (marvel) solution in 0.1% (v/v) Tween 20 (Fisher Scientific, Loughborough, United Kingdom) in TBS (TBST) was prepared. The membrane was incubated in the blocking solution on a shaker for 1 hour at room temperature before being washed in TBST for 3x 10 minutes.

2.8.5 Immunoblotting

For specific protein analysis primary antibody was added to the membrane according to Table 2.3 diluted in 5% (w/v) marvel in TBST. Membranes were incubated overnight on a shaker at 4°C. The membrane was then washed in TBST for 3x 10 minutes. This allowed removal of any unbound primary antibody. Following this, membranes were incubated for 1 hour on a shaker at room temperature with secondary antibody according to Table 2.3 diluted in 5% (w/v) marvel in TBST. The membrane was then washed in TBST for 3x 10 minutes. The secondary antibody is conjugated to HRP which allowed detection by enhanced chemiluminescence (ECL) using Immobilon western chemiluminescence HRP substrate (Merck Millipore, Massachusetts, USA) or Pierce ECL (Thermo Scientific, United Kingdom). The working ECL solution contains the chemiluminescent substrate luminal. In the presence of the HRP enzyme on the secondary antibody, luminol is oxidised. Oxidised luminal emits light which can be captured by exposure to x-ray film and allows visualisation of the specific protein. Membranes were then washed in TBST for 10 minutes before being stripped using Restore western blot stripping buffer (Thermo Scientific, United Kingdom) for 15 minutes on a shaker at room temperature. To check
even protein loading, the membranes were re-probed with the loading control α-tubulin. Quantification of protein expression was carried out by densitometry analysis using TotalLab 1D gel analysis (TotalLab, United Kingdom) and the protein: α-tubulin ratio was calculated.

### Table 2.3 Antibody experimental conditions

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration Used</th>
<th>Supplier</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT₁B (goat polyclonal antibody)</td>
<td>2.5µg/ml</td>
<td>Abcam-52025</td>
<td>Western Blot</td>
</tr>
<tr>
<td>α-tubulin (mouse polyclonal antibody)</td>
<td>0.1µg/ml</td>
<td>Abcam-7291</td>
<td>Western Blot</td>
</tr>
<tr>
<td>Anti-goat</td>
<td>0.1µg/ml</td>
<td>Sigma-A4174</td>
<td>Western Blot</td>
</tr>
<tr>
<td>Anti-mouse</td>
<td>0.1µg/ml</td>
<td>Sigma-A9044</td>
<td>Western Blot</td>
</tr>
<tr>
<td>α-smooth muscle actin</td>
<td>0.4µg/ml</td>
<td>Abcam-5694</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>PCNA</td>
<td>0.2µg/ml</td>
<td>Abcam-2426</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>Von-Willebrand factor</td>
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<td>Dako A0082</td>
<td>Immunohistochemistry</td>
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</tbody>
</table>

### 2.9 RNA Analysis

#### 2.9.1 RNA Extraction

For PASMCs, RNA was extracted from either 6-well plates or T-75 flasks. Briefly, at experimental end point the culture dish containing PASMCs was immediately placed on ice. The media was aspirated off and cells washed twice with either 1ml or 5ml of cold PBS per well or flask respectively. Following this, 700µl QIAzol lysis reagent was added to each well or T75 flask. QIAzol is a phenol/guanidine-based lysis reagent which facilitates lysis of cells/tissues, inhibits RNases and removes DNA and proteins. Keeping the culture dish on ice, the cells were scraped using a sterile plastic scraper and cell lysates collected in a pre-chilled 1.5ml eppendorf. Alternatively, for whole tissue samples RNA was extracted via the following. Upon dissection from the mouse cadaver the right lung lobe was either immediately snap-frozen in liquid nitrogen and stored in a freezer at -80°C, or placed in a pre-chilled 2ml eppendorf containing 700µl of QIAzol. A 5mm stainless steel bead (Qiagen, United Kingdom) was added to each sample eppendorf which was then placed in a Tissue Lyser II (Qiagen, United Kingdom) for homogenisation for 4x 30 second intervals. Further extraction and purification of RNA was performed using the miRNEASY extraction kit according to manufacturer’s instructions (Qiagen, United Kingdom). This kit allows extraction of RNA species as small as 18 nucleotides in length. Briefly, after homogenisation samples were left on the bench top at room temperature for 5
minutes to help promote dissociation of nucleoprotein complexes. Following this 140µl of chloroform was added to each sample and vigorously shaken for 15 seconds to aid subsequent phase separation. Each sample was left for a further 3 minutes on the bench top at room temperature before being centrifuged at 12,000xg at 4°C for 15 minutes. This step separates the sample into 3 phases; an upper colourless aqueous phase, a middle white interphase and a lower red organic phase. The RNA is contained within the upper phase which is carefully transferred into a new eppendorf. To precipitate the RNA phase, 1.5 volumes of 100% ethanol was added to each sample and mixed thoroughly by pipetting. Each sample was then loaded into an RNeasy Mini spin column in a 2ml collection tube and centrifuged at 8,500xg at room temperature for 15 seconds. The flow through in the collection tube was discarded. The RNA is captured in the mini spin column and is washed with 350µl of RWT buffer. The mini spin column in the collection tube is then centrifuged at 8,500xg at room temperature for 15 seconds. The flow through in the collection tube was discarded. To remove any residual amounts of DNA within the mini spin column, an on-column DNase digestion was performed on each sample. The DNase digestion solution was prepared by adding DNase I stock solution to buffer RDD in a ratio of 1:7. The working solution was mixed by gently inverting and 80µl was pipetted directly onto each mini spin column membrane to ensure complete digestion. Each sample was left to incubate at room temperature for 20 minutes before adding 350µl of RWT buffer to the mini spin column and centrifuging at 8,500xg at room temperature for 15 seconds. The flow through was discarded and 500µl of RPE buffer was added to each mini spin column. The sample was centrifuged at 8,500xg at room temperature for 15 seconds and again the flow through was discarded. This step was repeated but centrifuged for 2 minutes. The mini spin column was placed in a new 2ml collection tube and centrifuged at 8,500xg at room temperature for 1 minute to remove any residual RPE buffer. The mini spin column was then transferred into a new 1.5ml eppendorf and 30µl RNase free H₂O pipetted directly onto each mini spin column membrane. Each sample was then centrifuged at 8,500xg at room temperature for 1 minute to elute the RNA before being immediately transferred to ice or storage at -80°C.

### 2.9.2 Quantification of RNA

The quality and concentration of RNA within each sample was determined by a NanoDrop, ND-1000 spectrophotometer (Thermo Scientific, United Kingdom). The spectrophotometer measured the absorbance of each sample at 260nm (absorbance of RNA) and 280nm (absorbance of protein). The quality of RNA was analysed by the
260nm/280nm ratio. Samples with a ratio ~2 were accepted to have a good purity of RNA. The concentration of RNA was analysed using a modified Beer-Lambert equation and an extinction coefficient of 40ng·cm/µl as follows:

\[
\text{Concentration of RNA (ng/µl)} = \frac{\text{260nm Absorbance} \times \text{extinction coefficient}}{\text{pathlength in cm}}
\]

2.9.3 Reverse Transcription

For mRNA detection RNA samples were first reverse transcribed to complementary DNA (cDNA) using Taqman® reverse transcription reagents (Life Technologies, Paisley, United Kingdom) as per manufacturers guide. Briefly, 1µg of RNA template was prepared in a 96-well plate with a reaction mix containing RT-buffer [10x], magnesium chloride [25mM], deoxynucleotide triphosphates (dNTPs) [2.5mM each], random hexamers [50µM], RNase inhibitor [20U/µl] and multiscribe [50U/µl]. The 96-well plate was briefly vortexed and centrifuged to ensure adequate mixing. Reverse transcription (RT) was performed using the Veriti® Thermal Cycler (Life Technologies, Paisley, United Kingdom) with the following temperature programme: 25°C for 10 minutes, 48°C for 30 minutes and 95°C for 5 minutes. These conditions allow for RNA template annealing, reverse transcription and transcription inactivation, respectively. For miRNA detection RNA samples were first reversed transcribed using Taqman® microRNA reverse transcription kit (Life Technologies, Paisley, United Kingdom) as per manufacturers guide. Briefly, 5ng of RNA template was prepared in a 96-well plate with a reaction mix containing RT-buffer [10x], dNTPs [2.5mM each], RNase inhibitor [20U/µl], multiscribe [50U/µl], RNase free H₂O and miRNA primer [5x] (Table 2.4). The temperature programme for miRNA RT was as follows: 16°C for 30 minutes, 42°C for 30 minutes and 85°C for 5 minutes.

2.9.4 Quantitative Real Time-Polymerase Chain Reaction

For mRNA and miRNA detection Taqman® universal PCR master mix and fluorescently tagged Taqman® primers (Life Technologies, Paisley, United Kingdom) were used according to Table 2.4. The ViiA7™ Real-Time PCR System (Life Technologies, Paisley, United Kingdom) was used for measuring fluorescence of the quantitative real time-polymerase chain reaction (qRT-PCR). The fluorescence measured after each amplification cycle was proportional to level of PCR product. For mRNA expression the temperature cycle conditions were 50°C for 2 minutes, 95°C for 10 minutes and 50 cycles of 95°C for 15 seconds, 60°C for 1 minute. For miRNA expression the temperature cycle conditions
were 95°C for 10 minutes and 50 cycles of 95°C for 15 seconds, 60°C for 1 minute. Each sample was performed in triplicate and expression analysis determined relative to housekeeping control primers as listed in Table 2.4. For quantification analysis the comparative cycle threshold (CT) method was used. CT is the number of cycles in the PCR reaction required for the accumulated fluorescence to surpass a threshold level of fluorescence. The CT number is inversely proportional to the measured mRNA or miRNA level. A ΔCT value is obtained from the difference between the CT of the RNA of interest and the CT of the housekeeper RNA. Further to this a ΔΔCT value is obtained from the difference in ΔCT of test sample and the ΔCT of control sample. A relative quantification (RQ) was calculated from $2^{-\Delta\Delta CT}$. This method allows the control samples (i.e. non-patient data) to be expressed as 1 with test samples (i.e. patient data) expressed as a fold change.
Table 2.4 List of miRNA/mRNA Taqman primers

<table>
<thead>
<tr>
<th>miRNA/mRNA name</th>
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</thead>
<tbody>
<tr>
<td>miRNA-16</td>
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<tr>
<td>miRNA-18a</td>
<td>002422</td>
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<tr>
<td>miRNA-19a</td>
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<td>miRNA-21</td>
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<tr>
<td>miRNA-22</td>
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<td>miRNA-96</td>
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<td>002536</td>
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<tr>
<td>U6</td>
<td>001973</td>
</tr>
<tr>
<td>RNU48</td>
<td>001006</td>
</tr>
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<tr>
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<td>Mm004321134</td>
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<tr>
<td>Id3</td>
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<tr>
<td>GAPDH</td>
<td>Hs03929097, Mm03302249</td>
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</table>
2.10 Molecular Cloning

2.10.1 3’UTR PCR

A 456 base pair (bp) length of the 5-HT₁B 3’un-translated region (UTR) was PCR-amplified from mouse genomic DNA (gDNA) using forward and reverse primers that introduced XhoI and NotI restriction endonuclease sites. The forward primer was 5’-GGGTACCGCTCGAGGCTCTGAGGGAGAAGTCTTGTG and the reverse primer was 5’-ATAAGAATGCCGCGCAGCAACCCACGCCTATCCTCT. The underlined regions denote the recognition sites for XhoI and NotI restriction endonucleases respectively. The different restriction sites allow for directional cloning. Briefly, in a 50µl reaction 100ng template gDNA was prepared with KOD buffer [1x], betaine [1M], MgSO₄ [1mM], dNTPs [0.2mM], KOD polymerase [0.01U/µl], (x) of H₂O, forward primer [0.3µM] and reverse primer [0.3µM]. The following PCR cycling conditions were used: 98°C for 1 minute, 30 cycles of 98°C for 10 seconds, 58°C for 30 seconds, 72°C for 30 seconds and a final 72°C for 10 minutes. To purify the PCR fragment from excess primers, nucleotides and polymerase a cleanup reaction was performed using the QIAquick PCR Purification Kit with microcentrifuge protocol as per manufacturer’s instructions. Briefly, 5 volumes of buffer PB were added to 1 volume of the PCR sample and mixed before being transferred into a QIAquick spin column in a 2ml collection tube. The sample was centrifuged for 1 minute at room temperature to allow the DNA to bind to the silica membrane. The flow through was discarded and the sample washed with 750µl of buffer PE before being centrifuged again for 1 minute at room temperature. The flow through was discarded and the sample centrifuged again for 1 minute at room temperature to remove residual ethanol from the wash buffer. The QIAquick spin column was then transferred into a new eppendorf tube and 30µl of nuclease-free H₂O was pipetted directly onto the spin column membrane. This was incubated for 1 minute before being centrifuged at 8,500xg for 1 minute at room temperature to elute the DNA. Samples were then immediately transferred to ice or storage at -20°C.

2.10.2 Agarose Gel Electrophoresis

To separate and identify the size of DNA fragments agarose gel electrophoresis was utilised. This technique uses an electrical current and a porous agarose gel matrix to migrate negatively charged DNA towards a positive electrode. The migration of DNA is dependent on several factors including molecular size of DNA and concentration of agarose gel. DNA migrates through the agarose gel inversely proportional to its molecular
size i.e. small molecules will migrate faster than large molecules. The concentration of agarose gels utilised were either 0.8 or 1.5\% (w/v) agarose (Invitrogen, Paisley, United Kingdom) in 1xTris-borate EDTA (TBE, 10mM Tris, 10mM boric acid, 10mM EDTA, pH 8) solution depending on the size of DNA fragments being examined. Agarose gels with low concentration provide large pore sizes within the gel and were used to separate DNA fragments at >1kb size. Agarose gels with high concentration (1.5\%) provide small pore sizes within the gel and were used to separate DNA fragments <1kb. Agarose gel electrophoresis was performed to ensure successful 3’UTR PCR and restriction endonuclease digest reactions. Briefly, samples were prepared with 6x blue/green loading dye (Invitrogen, Paisley, United Kingdom) and loaded into pre-cast wells on an agarose gel of appropriate concentration containing 10ng/ml ethidium bromide (Sigma Aldrich). Ethidium bromide binds to DNA and allows visualisation of DNA bands under ultraviolet light. A 100bp or 1kb DNA marker ladder was also loaded to allow standardisation of DNA bands. Gels were run constantly at 100V in a 1xTBE running buffer until loading dye had reached near the end of the gel. DNA bands were visualised on the gel by trans-ultraviolet illumination on a ChemiDoc XRS+ Imaging System (Bio-Rad Laboratories, Hemel Hempstead, United Kingdom).

2.10.3 Isolation of DNA Fragment from Agarose Gel

To recover DNA from agarose gels the QIAquick® Gel Extraction Kit (Qiagen) was employed using the microcentrifuge protocol as per manufacturer’s instructions. Briefly, following agarose gel electrophoresis, DNA fragments were visualised using ultraviolet illumination. Using a clean, sharp scalpel the DNA gel fragment was carefully excised and placed in an eppendorf tube containing 3 volumes of buffer QG to 1 volume of gel fragment. Next, the eppendorf tube was incubated at 50°C for 10 minutes with vortexing every 2 minutes until the gel fragment had completely dissolved. Isopropanol was then added and mixed to the eppendorf at a volume equivalent to 1 volume of gel fragment. The sample was then loaded into a QIAquick spin column in a 2ml collection tube and centrifuged for 1 minute at room temperature. This step enables the DNA to bind to the silica membrane within the QIAquick spin column. The flow through was discarded and 500\µl of buffer QG added to QIAquick spin column before being centrifuged for 1 minute at room temperature. This ensures complete removal of agarose within the sample. The sample was then washed with 700\µl of buffer PE by addition to the QIAquick spin column and centrifugation for 1 minute at room temperature. The flow through was discarded and the QIAquick spin column further centrifuged for 1 minute at room temperature to remove
any residual ethanol from the wash buffer. The QIAquick spin column was then transferred into a new eppendorf tube and 30µl of nuclease-free H₂O was pipetted directly onto the spin column membrane. This was incubated for 1 minute before being centrifuged at 8,500xg for 1 minute at room temperature to elute the DNA. Samples were then immediately transferred to ice or storage at -20°C.

2.10.4 Restriction Endonuclease Digestion

Restriction endonuclease digestion is a technique utilised to cut double stranded DNA in preparation for cloning. The digestion produces compatible “sticky” ends of both the insert (PCR fragment) and vector (psi-check-2) which can then be successfully ligated together. Using two different restriction endonucleases allows for directional insertion of the PCR fragment into the psi-check-2 vector. Briefly, in a 20µl reaction 1µg psi-check-2 vector was digested with XhoI [1U/µl] and NotI [1U/µl] restriction endonucleases (New England Biolabs) in buffer 3 [1x] (New England Biolabs), BSA [1x] and (x) of H₂O for 5 hours at 37°C. Diagnostic digest of the psi-check-2 was also carried out using the restriction endonucleases BamHI and NotI (New England Biolabs). Analysis of successful digestion was carried out by agarose gel electrophoresis as of 2.9.2 and correctly digested DNA was extracted as of 2.9.3.

2.10.5 Ligation of PCR fragment into Psi-Check-2 Vector

To create a recombinant DNA molecule the PCR fragment was inserted into the psi-check-2 vector via a ligation reaction. This was performed using T4 ligase (New England Biolabs) as per manufacturer’s instructions. Briefly, ligation reactions were performed using varying molar ratios of vector: insert i.e. 3:1, 1:1, 1:3 using the following formula:

\[
\text{ng of insert} = \left(\frac{\text{ng of vector} \times (\text{kb size of insert})}{\text{kb size of vector}}\right) \times \text{molar ratio}
\]

In a 20µl reaction 50ng of vector along with appropriate ratio of insert (PCR fragment) was added to DNA ligase buffer [1x], T4 DNA ligase [20U/µl] and made to volume with H₂O. The reaction was incubated at 16°C overnight before being heat inactivated at 65°C for 10 minutes. The eppendorf was then immediately placed on ice before proceeding to the transformation stage.
2.10.6 Transformation of Competent Bacteria

To generate large quantities of recombinant DNA, the ligated vector and insert were transformed with Stellar™ Competent Cells (Clontech, France) as per manufacturer’s instructions. Stellar Competent Cells are an E.coli HST04 strain of bacteria which have been prepared to uptake foreign genetic material. Exogenous genetic material is incorporated into the bacteria host and replicated upon bacteria growth. Briefly, 50µl of competent cells were thawed on ice and mixed gently to ensure even distribution. The competent cells were then transferred into a pre-chilled 14ml round-bottom tube and 5ng of ligated DNA was added. The competent cells and DNA were incubated on ice for 30 minutes before being heat-shocked for precisely 45 seconds at 42°C. This step allows the DNA to enter into the cell. The tube was then placed back on ice for 2 minutes before addition of 450µl of pre-warmed (37°C) super optimal broth with catabolite repression (SOC, Clontech, France) medium. Transformation samples were then placed in a shaking incubator at 150xg for 1 hour at 37°C. Working under an ethanol burner varying volumes of transformation samples were then pipetted and spread onto luria broth (LB, 10g/L bactotryptone, 5g/L bactoyeast extract, 5g/L NaCl, 15g/L agar, pH7.5) agar plates containing 100µg/ml of ampicillin. Plates were then incubated overnight at 37°C and checked for growth of colonies the following day. A positive (pUC19 vector) and negative control was utilised alongside the experimental ligated vector and insert.

2.10.7 Plasmid DNA Purification

2.10.7.1 Mini-Prep Small Scale

Single bacterial colonies which had grown overnight were chosen from the above mentioned LB agar plates to inoculate starter cultures. The starter culture consisted of a pipette tip with the single colony on the end immersed in 10ml of LB containing 100µg/ml ampicillin in a 50ml culture tube with a loose lid for aeration. The starter culture was left to grow overnight in a shaking incubator (150xg) at 37°C. In this time larger amounts of recombinant plasmid DNA were obtained. For long-term storage and reference purposes glycerol stocks were made of each starter culture. Here, 800µl of culture was added to 800µl of 40% (w/v) glycerol (Sigma Aldrich, Dorset, United Kingdom) and mixed thoroughly. The resultant mix was then stored at -80°C. For diagnostic purposes and to ensure successful cloning 2ml of the culture was purified for use in restriction endonuclease digestion reactions and sequencing.
Small scale plasmid DNA purification was performed using the PureLink® Quick Plasmid Miniprep Kit (Invitrogen, Paisley, United Kingdom) as per manufacturer’s instructions. This kit lyses bacterial cells utilising an alkaline/SDS procedure. Briefly, a 2ml culture was harvested by centrifugation at 5,000xg for 10 minutes to achieve a pellet. The LB media supernatant was removed and the pellet re-suspended in 250µl of re-suspension buffer R3.

The solution was gently mixed until a homogenous suspension was obtained to ensure complete lyses in the following step. Next, 250µl of lysis buffer L7 was added and gently mixed by inverting until the mixture was homogenous. Here the alkaline and detergent lysis solution breaks down the bacterial cells releasing the plasmid DNA. This was then left to incubate on the bench-top for 5 minutes at room temperature. To precipitate the bacterial cells genomic DNA and proteins 350µl of precipitation buffer N4 was added and mixed immediately by inverting until the mixture was homogenous. The tube was then centrifuged for 10 minutes at 10,200xg at room temperature. To bind the DNA to the silica membrane the supernatant was then loaded into a spin column in a 2ml collection tube and centrifuged for 1 minute at 10,200xg at room temperature. The flow through was discarded and the spin column washed with 500µl of wash buffer W10. The spin column was left to incubate on the bench-top for 1 minute at room temperature before being centrifuged for 1 minute at 10,200xg at room temperature. The flow through was discarded and the spin column washed further with 700µl of wash buffer W9. The spin column was centrifuged for 1 minute at 10,200xg at room temperature and the flow through was discarded. To remove any residual ethanol from the wash buffer the spin column was further centrifuged for 1 minute at 10,200xg at room temperature. Next, the spin column was transferred into a new eppendorf and 50µl of nuclease-free H$_2$O was pipetted directly onto the spin column membrane. This was incubated for 1 minute before being centrifuged at 10,200xg for 2 minutes at room temperature to elute the plasmid DNA. Samples were then immediately transferred to ice or storage at -20°C. Analytical restriction endonuclease digestion of mini-prep samples were then carried out as of 2.9.4 and examined by agarose gel electrophoresis as of 2.9.2

2.10.7.2 Maxi-Prep Large Scale

After successful mini-prep diagnostics the remaining starter culture was transferred into 500ml of LB containing 100µg/ml ampicillin in a 1L conical flask and left to grow further overnight in a shaking incubator at 37°C. In this time larger amounts of recombinant plasmid DNA were obtained.
Large scale plasmid DNA purification was performed using the PureLink® HiPure Plasmid DNA Maxiprep Kit (Invitrogen, Paisley, United Kingdom) as per manufacturer’s instructions. This kit utilises the same alkaline/SDS lyses principals as of 2.9.7.1 but an anion-exchange resin is used to purify the plasmid DNA. Briefly, a 500ml culture was harvested by centrifugation at 5,000xg for 15 minutes at 4°C to achieve a pellet. The LB media supernatant was removed and the pellet re-suspended in 10ml of resuspension buffer R3 with RNase A. The solution was gently mixed until a homogenous suspension was obtained to ensure complete lyses in the following step. Next, 10ml of lysis buffer L7 was added and gently mixed by inverting until the mixture was homogenous. Here the alkaline and detergent lysis solution breaks down the bacterial cells releasing the DNA. This was then left to incubate on the bench-top for 5 minutes at room temperature. To precipitate the bacterial cells genomic DNA and proteins 10ml of precipitation buffer N3 was added and mixed immediately by inverting until the mixture was homogenous. The precipitated lysate was then transferred into an equilibrated HiPure filter maxi column and left to run through the filter by gravity. The flow through was discarded and the maxi column washed with 10ml wash buffer W8 by letting the buffer run through the filter by gravity. Immediately after the wash buffer had finished flowing through the inner filtration cartridge was removed and the maxi column was further washed with 50ml of wash buffer W8. The buffer was again left to flow through by gravity and the maxi column transferred to a new 50ml centrifuge tube. Next, to elute the DNA 15ml of elution buffer E4 was added to the maxi column and the solution again left to flow through by gravity. The maxi column was discarded and 10.5ml of isopropanol was added to the elution tube to precipitate the DNA and mixed well. The elution tube containing the precipitated DNA was centrifuged at 10,200xg for 30 minutes at 4°C to obtain a DNA pellet. The supernatant was carefully removed and discarded. Following this, the DNA pellet was re-suspended in 5ml of 70% (v/v) ethanol until homogenous. The sample was centrifuged at 10,200xg for 5 minutes at 4°C and again the supernatant was carefully discarded. The DNA pellet was then left to air dry for 10 minutes to ensure all residual ethanol was removed before being re-suspended in 200µl of nuclease-free H₂O. Samples were then immediately transferred to ice or storage at -20°C. Analytical restriction endonuclease digestion of mini-prep samples were then carried out as of 2.9.4 and examined by agarose gel electrophoresis as of 2.9.2

2.10.8 DNA Sequencing

To confirm the nucleotide sequence within the plasmid DNA the dideoxynucleotide sequencing method was utilised. This technique involves the use of fluorescently labelled
dideoxynucleotide triphosphates (ddNTPs) which instead of having a hydroxyl (OH) group attached to the 3’ end have hydrogen (H). As H molecules are unable to form phosphodiester bonds with a proceeding deoxynucleotide the elongation of the DNA sequence is terminated. This results in a series of DNA fragments which differ in length by a single nucleotide base with the last nucleotide in each fragment being fluorescently labelled. The DNA fragments are separated by size and the sequence analyser reads the fragments from small to large enabling the generation of the DNA sequence.

Briefly, in a 25µl reaction, 100ng of template plasmid DNA was combined with sequencing buffer [0.8x] (Applied Biosystems, Paisley, United Kingdom), ready reaction mix [1x] (Applied Biosystems, Paisley, United Kingdom), forward or reverse primer [1.2µM] and sterile (x) of H2O. The temperature cycle conditions were as follows: 25 cycles of 50 seconds at 96°C (for denaturation), 20 seconds at 50°C (for primer annealing) and 3 minutes at 60°C (for elongation). To purify the sequencing PCR reaction from excess primers and nucleotides a cleanup reaction was performed. This process utilises magnetic beads to purify the DNA extension fragments. Briefly, 10µl of cleanseq was added to each reaction well. Next, 62µl of 85% (v/v) ethanol was added and gently pipetted up and down before being briefly vortexed and centrifuged. The reaction plate was then placed on a magnetic base for 2 minutes to separate the magnetic beads (with attached DNA) and the ethanol. Keeping the plate on the magnetic base the plate was upturned to remove the ethanol. This process was repeated with 150µl of 85% (v/v) ethanol. The plate was left on the bench-top to air dry for 10 minutes before adding 40µl of nuclease free H2O to each reaction well. The sample was briefly pipetted up and down to mix and again placed on the magnetic base for 2 minutes. Taking care not to disrupt the magnetic beads, 20µl of the sample was transferred into a new sequencing plate. The sample was then analysed on ABI 3730 automated sequencer and SeqScape v2.0 software (Applied Biosystems, Paisley, United Kingdom).

2.10.9 Site-Directed-Mutagenesis

To further examine the miRNA-96/5-HT1B relationship a targeted change within the seed region of the 5-HT1B 3’UTR molecular DNA sequence was generated. This was performed by site-directed mutagenesis and created a single point substitution mutation in the 5-HT1B 3’UTR to assess miRNA-96 binding as a result of DNA manipulation. Briefly, the plasmid DNA was methylated by adding a methyl group using S-adenosylmethionine (SAM) and CpG methyltransferase (M.SssI). This allows for targeted degradation of the non-mutated
plasmid by DpnI endonuclease later on. In an eppendorf 14µl of nuclease free H\textsubscript{2}O was added to NEB buffer 2 [1x], SAM [160µM], 100ng of plasmid DNA and M.SssI [4U/µl]. The reaction was placed at 37°C for 1 hour for the methylation to take place before reaction termination by heating at 65°C for 20 minutes. Next, the mutagenesis PCR was performed using a forward mutagenic primer and a reverse non-mutagenic primer. The sequences of the forward and reverse primer were as follows: forward 5’-CATGATGTGTGCTAGTGCCTAAGTCTGCAG-3’ and reverse 5’-GGCACTAGCACACATCATGTTTTGAGTTGT-3’. Briefly, two PCR reactions were carried out in parallel, one using the forward mutagenic primer and one using the reverse primer. Each 25µl PCR reaction mix contained KOD buffer [1x], betaine [1M], MgSO\textsubscript{4} [1mM], dNTPs [0.2mM], 50ng methylated plasmid DNA, KOD polymerase [0.01U/µl] and (x) nuclease free water in addition to either forward or reverse mutagenic primer [0.3µM]. The two individual PCR reactions were run on a thermocycler as follows: 98°C for 30 seconds, 98°C for 10 seconds, 59°C for 30 seconds and 72°C for 6 minutes 30 seconds. Immediately after the PCR cycle was finished the two reactions were combined and a further PCR was run with the following cycle: 98°C for 30 seconds, then 19 cycles of 98°C for 10 seconds, 59°C for 30 seconds, 72°C for 6 minutes 30 seconds and a final 72°C for 10 minutes. This second PCR allowed for annealing and multiplying of mutagenic forward and reverse strands. To remove the methylated parental non-mutated plasmid DNA a DpnI digest was performed. This involved incubating the DNA with 1 unit of DpnI for 1 hour at 37°C. The reaction was then heat-inactivated at 80°C for 20 minutes. The resultant mutated plasmid DNA was then transformed, analysed and sequenced as per 2.10.6, 2.10.4 and 2.10.8 respectively.

2.11 Dual Luciferase Activity Reporter Assay

2.11.1 Transient Transfection of HeLa Cells

To compare the transcriptional activity of the cloned 5-HT\textsubscript{1B} 3’UTR in the absence and presence of pre-miR-96 a dual luciferase activity reporter assay was performed. Briefly, immortalised HeLa cells were plated and grown in 24-well tissue culture plates as of section 2.5.3. Once approximately 60% confluent, HeLa cells underwent a transient transfection protocol using Lipofectamine® 2000 (Invitrogen) as per manufacturer’s instructions. Initially the cells were washed twice with 500µl optimem (Invitrogen) before addition of 500µl of 10% FBS (v/v) DMEM with no antibiotic too each well. Next, 100µl of transfection mix was added to each well (Table 2.5) and the cells transferred back to the
humidified cell culture chamber. After 6 hours the transfection media was removed and replaced with 1ml of 10% FBS (v/v) DMEM with 1% (v/v) AA. Cells were then left to grow for a further 48 hours. Plates were carried out in duplicate to confirm successful pre-miR-96 transfection by qRT-PCR.

Table 2.5 Transfection conditions for transient transfection of HeLa Cells

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<tr>
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</tr>
<tr>
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<td>1.5µl</td>
</tr>
<tr>
<td>Psi-CHECK2 + pre-miRNA negative control 25nM</td>
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</tr>
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<td>5-HT1B</td>
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</tr>
<tr>
<td>5-HT1B + pre-miRNA-96 25nM</td>
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<td>5-HT1B + pre-miRNA negative control 25nM</td>
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<tr>
<td>pGL3-73</td>
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</tbody>
</table>

2.11.2 Lysate Preparation

Growth media was aspirated from each well and each well washed with 1xPBS. The PBS was removed and 100µl of 1x passive lysis buffer (PLB) was added into each well of the 24-well plate. Alternatively, for duplicate plates 250µl of QIAzol was added to each well for RNA extraction (section 2.8.1) and subsequent qRT-PCR analysis (2.8.4). The 24-well plate was transferred to -80°C for 5 minutes to flash freeze the lysates. Next the 24-well plate was placed on ice and gently agitated on a shaker for 10 minutes. Each well was then
scraped, contents placed in an eppendorf and centrifuged at 10,200xg for 5 minutes at 4°C. Next 7.5µl of supernatant was transferred into a single well of a white 96-well plate containing 12.5µl of 1xPLB per well.

2.11.3 Stop N Glo

To measure the luciferase activity of each sample the Dual-Glo Luciferase Assay System (Promega, Southampton, United Kingdom) was utilised as per manufacturer’s instructions. The procedure was performed and luminescence detected via a LUMIstar OPTIMA microplate reader (BMG Labtech). Briefly, the LUMIstar was set to pump 50µl of LARII reagent into each well and then measure firefly luciferase activity. Following this, 50µl of STOP N GLO was immediately pumped into each well and then the renilla luciferase activity was measured. The renilla luciferase activity was normalised to the internal firefly luciferase activity and data expressed as a percentage of the internal control. Each sample was performed in triplicate.

2.12 Models of Pulmonary Hypertension

2.12.1 Chronic Hypoxia

To induce a PH phenotype, the chronic hypoxic model was utilised. Eight-week old wild-type (WT) mice were continuously exposed to chronic hypoxia via a hypobaric hypoxic chamber at 550mbar for 14 days. The sustained reduction in pressure from atmospheric room pressure (1000mbar) was equivalent to a decrease in O₂ availability to 10% O₂. Low O₂ levels result in pulmonary vasoconstriction and the development of a robust PH phenotype (Mair et al., 2014). The temperature and relative humidity were constantly monitored and maintained within optimal ranges at all times. Food, water and bedding were replenished every 5 days. Eight-week old WT mice were also placed in normoxic conditions (1000mbar) to act as controls.

2.13 Administration of miRNA mimic

To assess whether miRNA-96 was involved in the pathology of pulmonary hypertension, the miRNA-96 mimic (Applied Biosystems, #MC10422) was administered intravenously via the tail vein on day 0 and day 7 using the MaxSuppressor™ In Vivo RNA-LANCEr II delivery method as previously described to facilitate delivery to the lung (Trang et al., 2011). The mimic was prepared under sterile conditions in the delivery reagent as per manufacturing instructions at a dose of 1.5mg/kg per injection i.e. ~30ug per mouse per
injection. This dose was based on a previously published delivery to lung (Trang et al., 2011). Negative miRNA mimic (Applied Biosystems, #1) and PBS dosed animals were utilised as controls. On day 14 of the experimental timeline (Figure 2.3), characteristics of PH were assessed.

**Figure 2-3 In vivo Experimental timeline**

(A) Eight week old C57Bl/J mice were subjected to a 14 day hypoxic period. On day 0 and day 7 mice were dosed with either 1.5mg/kg miRNA-96 mimic, negative control or PBS and PAH phenotype assessed on day 14. (B) Six month old BMPR2<sup>B899X+</sup><sup>−</sup> mice were subjected to dosing on day 0 and day 7 with either 1.5mg/kg miRNA-96 mimic, negative control and PAH phenotype assessed on day 14.
2.14 **Assessment of Pulmonary Hypertension**

2.14.1 **Anaesthetic Induction**

All mice were initially anaesthetised in an induction chamber containing 3% (v/v) isoflurane (Abbot Laboratories, Berkshire, United Kingdom) supplemented with O₂ (flow rate, 0.5L/min). Post-induction mice were immediately weighed before being transferred to a face mask providing 1.5% isoflurane (v/v) supplemented with O₂ (flow rate, 0.5L/min). The absence of a hind limb reflex was checked to ascertain appropriate level of anaesthesia. To ensure maintenance of anaesthesia the hind limb reflex was monitored repeatedly throughout the *in vivo* procedure along with breathing and heart rates.

2.14.2 **Systemic Arterial Pressure**

To allow continuous observation of systemic arterial pressure (SAP) the left common carotid artery was cannulated. Briefly, an incision was made in the ventral neck and the layers of smooth muscle carefully blunt dissected until the trachea was reached. Further blunt dissection to the left of the trachea was carried out to expose the left common carotid artery. The left common carotid artery was identified by its pulsation and the presence of the vagus nerve (white in appearance) running alongside it. Next the carotid artery was carefully isolated from the vagus nerve and cleaned of any connecting tissue. Surgical silk monofilament suture (size 5.0, Harvard Apparatus, Massachusetts, U.S.A.) was used to ligate the carotid artery at the proximal (head) end. A microsurgical artery clip (Fine Science Tools, Heidelberg, Germany, FST#18055-04) was carefully secured at the distal end of the carotid artery to temporarily occlude blood flow. A small incision was made in the artery above the suture tie and carefully held open with curved forceps. Next, a heparinised saline-filled micro-cannula (Harvard Apparatus, Massachusetts, U.S.A.) was inserted and advanced gently into the lumen of the carotid artery. The micro-cannula was then ligated in place with a 5.0 silk suture before removal of the artery clip. The micro-cannula was connected to a transducer and data acquisition system (Biopac Systems, California, U.S.A.) for SAP reading (Figure 2.4). For analysis measurement of the mean SAP (mSAP) was obtained.
Figure 2-4 Systemic arterial Pressure Measurement

A three second representative trace recording of a systemic arterial pressure in mouse. Y axis shown in mmHg.

2.14.3 Right Ventricular Systolic Pressure

Indirect assessment of pulmonary arterial pressure was obtained by catheterisation of the right ventricle of the heart to allow measurement of right ventricular pressure (RVP). This was done by a transdiaphragmatic approach, a well-documented technique (Morecroft et al., 2007; Dempsie et al., 2011; White et al., 2011a). Briefly, the ventral sternum of the mouse was exposed and a 25mm gauge needle centrally aligned. Using a micromanipulator (Warner Instruments, Connecticut, U.S.A.) the needle was moved 2mm to the right and then advanced underneath the sternum through the diaphragm (indicated by a negative pressure) and eventually to puncture the right ventricle wall into the right ventricle. A characteristic pressure trace was observed when the needle was successfully in the right ventricle (Figure 2.5). For analysis the right ventricular systolic pressure (RVSP) and heart rate (HR) was obtained from the pressure readings. The correct placement of the needle was additionally confirmed at whole heart dissection with a noticeable puncture in the right ventricle wall. After in vivo measurements were obtained mice were killed by anaesthetic overdose.
Figure 2-5 Right Ventricular Systolic Pressure Measurement

(A) A schematic representation of catheter placement to achieve right ventricular pressure measurement. (B) A three second representative trace recording of a right ventricular pressure in non-PH mouse. (C) A three second representative trace recording of a right ventricular pressure in PH mouse. Y axis shown in mmHg. RV= right ventricular.

2.14.4 Right Ventricular Hypertrophy

Upon termination of mice, the heart and lungs were gently flushed with ice-cold PBS by injection through the right ventricle. Whole hearts were then excised and cleared of adjoining fatty tissue and blood vessels. Both right and left atria were also removed. Measurement of right ventricular hypertrophy (RVH) is determined using Fulton’s index: the ratio of the free right ventricle (RV) wall weight and the left ventricle (LV) wall plus septum (S) weight (RV/LV+S) (Fulton et al., 1952) (Figure 2.6). The right ventricle was cut away, blotted dry and weighed. This was then repeated with the remaining left ventricle plus septum and the ratio calculated.
Figure 2-6 Diagrammatic illustration of right ventricular hypertrophy

A schematic transverse section of the heart showing the left ventricle wall, septum and thin walled right ventricle in a normal non-PH heart (A) and hypertrophied right ventricle in a PH heart (B).
2.14.5 Pulmonary Artery Remodelling

To assess the extent of remodelling in PH, 5µm frontal plane lung sections were prepared and stained with elastic pico-sirius red as per section 2.4.1 and section 2.4.3. Stained lung sections were then microscopically examined for non-remodelled and remodelled pulmonary arteries <80µm external diameter. Remodelled pulmonary arteries were determined by the presence of a double elastic lamina (Figure 2.7). The number of remodelled pulmonary arteries was expressed as a percentage of total number of pulmonary arteries (non-remodelled and remodelled) within the lung section. Assessment was carried out in a blinded fashion.

![Remodelling of a small pulmonary artery](image)

**Figure 2-7 Remodelling of a small pulmonary artery**

Representative images of small distal pulmonary arteries from non-PH lung section (A) and PH lung section (B). Note the presence of the double elastic lamina in panel (B). Scale bar 50µm.

2.15 Statistical Analysis

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

3 *In Vitro* Identification and Validation of a Role for miRNA-96 in Female Pulmonary Hypertension
3.1 Introduction

The majority (~75%) of patients with HPAH and 20% of patients with IPAH present with mutations within the gene encoding BMPR-II (Atkinson et al., 2002; Machado et al., 2006a; Hamid et al., 2009). BMPR-II mutations lead to a reduction in expression or reduction in function of the BMPR-II receptor resulting in reduced BMPR-II mediated signalling pathways (Atkinson et al., 2002; Yang et al., 2005). Within the pulmonary vasculature and more specifically the distal PASMCs a reduction in BMPR-II expression triggers aberrant proliferation (Teichert-Kuliszewska et al., 2006). Dysregulated PASMCs proliferation generates muscularisation and remodelling of small distal pulmonary arteries and initiates PAH development.

BMPR-II mutations occur with reduced disease penetrance i.e. only 20% of mutation carriers will develop PAH. This implies BMPR-II mutations do not fully confer PAH disease susceptibility and secondary genetic or environmental factors are required for disease onset. One secondary factor of major interest is the serotonin system which has a wide implication in PAH development. Within the PASMCs of the distal pulmonary arteries serotonin plays an important role in pulmonary artery remodelling and pulmonary artery vasoconstriction, two major hallmarks of PAH disease (MacLean et al., 1996b; Long et al., 2006). Research from both human samples and experimental animal models highlight the fundamental involvement of the 5-HT$_{1B}$ receptor in pathological processes making it a favourable gene to therapeutically target (Keegan et al., 2001; Launay et al., 2002; Morecroft et al., 2005).

Within HPAH a gender disparity exists with a 2.7:1 female to male ratio suggesting interplay of sex with disease prevalence (Loyd et al., 1995). This sex bias is also observed in other PAH categories where a ~4:1 female to male ratio is demonstrated (Ling et al., 2012). Investigations into the sex bias in PAH have suggested anomalous estrogen synthesis, signalling and/or metabolism may be pathogenic in PAH. For example an increase in the expression and function of aromatase (the estrogen synthesising enzyme) is associated with PAH and interestingly an aromatase inhibitor is effective in reducing a PH phenotype only in female mice and not males (Mair et al., 2014). Additionally, increased expression of ER$\alpha$ is demonstrated in female patient PAH and an ER$\alpha$ antagonist reduces the remodelling of pulmonary arteries in female mice (Rajkumar et al., 2010; Mair et al., 2014). Overall it appears estrogen may confer susceptibility to PAH by involvement in proliferative and remodelling processes of pulmonary arteries.
More recently, miRNAs have been implicated in PAH with regards to their involvement in cellular processes such as proliferation and apoptosis. Amongst those mentioned in section 1.7, a role for miRNA-21 and miRNA-145 show dysregulation in human PAH samples and hypoxic and monocrotaline animal models (Caruso et al., 2010; Caruso et al., 2012). It is recognised that sex differences exist in miRNA expression and therefore miRNAs which are influenced by sex may shape the architecture of female and male gene expression differently (Marco et al., 2013; Wu et al., 2013). Sexual dimorphism of miRNAs is thought to play a role in the development of sex bias diseases such as SLE with expression potentially regulated by the presence or absence of sex hormones (Dai et al., 2013; Ohta et al., 2013). With regards to PAH, it is currently unknown whether female and male differences in miRNA expression exist and could underlie female susceptibility in disease onset and development.

Here, we examine female and male differences in miRNA expression in PASMCs from a BMPR-II mutation mouse model and PASMCs from human patients. Our data demonstrates a down-regulation of miRNA-96 only in female experimental and clinical PAH samples. This is associated with a concomitant up-regulation of the miRNA-96 target 5-HT<sub>1B</sub> receptor. We also demonstrate estrogen may play a role in regulating miRNA-96 and consequently the 5-HT<sub>1B</sub> receptor. A dysregulated miRNA-96/5-HT<sub>1B</sub> axis may confer proliferation in female patient PASMCs and contribute to the female susceptibility in PAH.

Aims of this chapter:

1. To identify female and male sex differences in miRNA expression.

2. To functionally validate a target for a sex specific miRNA.

3. To examine if estrogen regulates the sex specific miRNA.
3.2 **Results**

3.2.1 **Female BMPR2^{R899X+/-} mice exhibit an increase in remodelled and proliferating distal pulmonary arteries**

In the initial genesis of disease, normal non-muscularised distal pulmonary arteries become severely muscularised when PASMCs undergo aberrant proliferation. To assess sex differences arising from BMPR-II mutation we examined pulmonary artery muscularisation and proliferation by immunohistochemistry. Here we observed pulmonary arteries from female BMPR2^{R889X+/-} mice had a significantly greater wall thickness: vessel diameter ratio than male BMPR2^{R889X+/-} mice vs. their respective WT control as indicated by the intense staining for α-smooth muscle actin (Figure 3.1). This indicates that female BMPR2^{R889X+/-} pulmonary arteries are more muscularised than male BMPR2^{R889X+/-} pulmonary arteries. In addition, there was intense positive staining for proliferating cell nuclear antigen (PCNA) in pulmonary arteries from female BMPR2^{R889X+/-} mice compared to female WT mice (Figure 3.2). This intense positive staining for PCNA was not observed in pulmonary arteries from male WT or BMPR2^{R889X+/-} mice with the level of PCNA staining in both, likened to that of pulmonary arteries from female WT mice. This suggests that in female BMPR2^{R889X+/-} mice more PASMCs are in the S-phase (DNA replication) of the cell cycle and more likely to be proliferating.
Figure 3-1 The degree of muscularisation of small distal pulmonary arteries is increased in female BMPR2<sup>R899X</sup>+/− mice

Representative immunolocalisation of α-smooth muscle actin (α-SMA), a marker for smooth muscle cell, in 5µm frontal plane lung sections from female and male wild-type (WT) and BMPR2<sup>R899X</sup>+/− mice. Positive staining for α-SMA was visualised by the dark orange/brown colour (A). Quantification of muscularisation was assessed by expressing the ratio of the vessel wall thickness (as observed by the α-SMA positive stain): total vessel diameter (B). n=4 for female and male WT, n=8 for female and male BMPR2<sup>R899X</sup>+/− mice per group, **p<0.01, ***p<0.001, One-way ANOVA followed by Tukey’s post-hoc test. Data is expressed as the mean ± SEM. Scale bar = 50µm. WT= wild-type
Figure 3-2 Immunolocalisation of PCNA in small distal pulmonary arteries highlights intense staining in female BMPR2 $^{R899X+/-}$ mice

Representative immunolocalisation of proliferating cell nuclear antigen (PCNA), a marker for S-phase cell division, in 5µm frontal plane lung sections from female and male WT and BMPR2 $^{R899X+/-}$ mice. Positive staining for PCNA was visualised by the dark orange/brown colour. Scale bar = 50µm.
3.2.2 Sex affects microRNA expression in BMPR2<sup>R889X+/-</sup> PASMCs

MiRNAs are known to be involved in proliferative processes, more specifically proliferation of PASMCs in PAH development. Sex differences are also known to exist between certain miRNAs yet it has not been investigated if sex differences in miRNA exist in PAH that could account for the sex bias/female susceptibility in disease. Thus we wished to examine whether sex differences in miRNA expression could underlie differences in proliferative/muscularisation phenotype in PASMCs from BMPR2<sup>R889X+/-</sup> mice. To do this we examined the expression profile of various miRNAs either previously associated with PAH development or which target genes are associated with PAH development (Table 3.1). From 18 miRNAs examined, sex and genotype did not significantly affect miRNA-16, miRNA-22, miRNA-27b, miRNA-98 and miRNA-224 expression (Figure 3.3 and Figure 3.4). Expression of miRNA-17, miRNA-18a, miRNA-19a, miRNA-21, miRNA-29b, miRNA-96, miRNA-424, miRNA-451 and miRNA-503 were significantly reduced in female BMPR2<sup>R889X+/-</sup> PASMCs compared to female WT PASMCs while remaining unchanged between male BMPR2<sup>R889X+/-</sup> and WT PASMCs. MiRNA-145 and miRNA-206 were significantly up-regulated in both female and male BMPR2<sup>R889X+/-</sup> PASMCs compared to respective WT PASMCs. A significant decrease in miRNA-203 expression was observed in male BMPR2<sup>R889X+/-</sup> PASMCs compared to male WT PASMCs while remaining unchanged between female BMPR2<sup>R889X+/-</sup> and WT PASMCs. MiRNA-155 was dysregulated in both female and male BMPR2<sup>R889X+/-</sup> PASMCs compared to WT with expression decreased in female BMPR2<sup>R889X+/-</sup> but increased in male BMPR2<sup>R889X+/-</sup> PASMCs. This data suggest sexual dimorphism with respect to miRNAs does exist within a PH setting.
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Pulmonary artery smooth muscle cells (PASMCs) were explanted and cultured from female and male WT and BMPR2<sup>R899X+</sup>/- mice. The expression of miRNA was assessed by Taqman quantitative Real Time-PCR. Results were normalised to the small nuclear RNA, U6. n=8 per group in triplicate, *p<0.05, **p<0.01, ***p<0.001, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the RQ±RQ<sub>max</sub>/RQ<sub>min</sub>. RQ= relative quantification, WT= wild type.
Figure 3-4 Sex and BMPR2^R899X/+ mutation alters the expression of miRNAs in PASMCs Continued

Pulmonary artery smooth muscle cells (PASMCs) were explanted and cultured from female and male WT and BMPR2^R899X/+ mice. The expression of miRNA was assessed by Taqman quantitative Real Time-PCR. Results were normalised to the small nuclear RNA, U6. n=8 per group in triplicate, *p<0.05, **p<0.01, ***p<0.001, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the RQ ± RQ^max/RQ^min. RQ= relative quantification, WT= wild type.
3.2.3 MiRNA-96

As miRNA-96 was differentially expressed between female PASMCs and not male PASMCs and was a novel miRNA implicated in the PAH setting, we selected this miRNA for further analysis and investigation. MiRNA-96 has previously been implicated in various cancer models/tissues and as cancer is a proliferative disease akin to PAH this enhanced our interest in examining this miRNA. The human miRNA-96 gene is located on chromosome 7 (mouse chromosome 6) and lies within an intergenic region in the genome. MiRNA-96 is also evolutionarily conserved between several species indicating important function (Figure 3.5). MiRNA-96 also appears in a cluster with miRNA-182 and miRNA-183 however each miRNA has its own distinct pri-miRNA meaning expression can arise under different promoters. To understand if the whole cluster is dysregulated between sexes in PASMCs from BMPR2R899X+/− mice, we examined the expression of mature miRNA-182/183 levels by Taqman analysis. Here we found that miRNA-182 was down-regulated in both female and male BMPR2R899X+/− PASMCs compared to WT PASMCs with less expression observed in male BMPR2R899X+/− PASMCs than female BMPR2R899X+/− PASMCs (Figure 3.6). MiRNA-183 was down-regulated in male BMPR2R899X+/− PASMCs compared to male WT PASMCs, however, was unchanged in female PASMCs. Expression of miRNA-183 was also significantly down-regulated in male BMPR2R899X+/− PASMCs vs. female BMPR2R899X+/− PASMCs (Figure 3.6).

Global tissue expression of miRNA-96 was assessed to understand whether miRNA-96 was ubiquitously expressed or potentially selective/specific to the pulmonary system. Taqman qRT-PCR analysis revealed miRNA-96 expression was significantly greater in the lung compared to the liver, spleen and brain (Figure 3.7). The kidney and uterus expression level of miRNA-96 was also decreased compared to the lung however here statistical significance was not reached. This suggests that of the tissues analysed the highest expression of miRNA-96 is observed in the lung tissue. In addition it could suggest that miRNA-96 could have more of a physiological role in the lung compared to the tissues where there is less expression. It would also suggest that potential targets may also be co-localised within the lung tissue.
Figure 3-5 The sequence of miRNA-96 is evolutionarily conserved

The miRNA-96 sequence is highly conserved amongst several species emphasising its important physiological role.

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Pulmonary artery smooth muscle cells (PASMCs) were explanted and cultured from female and male WT and BMPR2^{R899X+/−} mice. The expression of miRNA was assessed by Taqman quantitative Real Time-PCR. Results were normalised to the small nuclear RNA, U6. n=8 per group in triplicate, *p<0.05, **p<0.01, ***p<0.001 One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the RQ ± RQ^{max}/RQ^{min}. RQ= relative quantification.

Figure 3-6 Sex and BMPR2^{R899X+/−} mutation alters the expression of miRNA-182/183 in PASMCs
Figure 3-7 Global expression analysis highlights high expression of miRNA-96 in lung tissue

The expression of miRNA-96 was assessed by Taqman quantitative Real Time-PCR in various tissues from WT mice. Results were normalised to the small nuclear RNA, U6. n=5 per group in triplicate, *p<0.05, ***p<0.001 One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the RQ ± RQ_{max}/RQ_{min}. RQ= relative quantification.
3.2.4 MiRNA-96 is localised within the smooth muscle cell layer

To ascertain the cellular location of miRNA-96 within lung tissue we performed *in situ* hybridisation. Analysis of mouse lung sections highlighted positive staining for miRNA-96 within the pulmonary artery smooth muscle cell layer (Figure 3.8). Staining for miRNA-96 was not detected in the endothelial cell layer within the pulmonary artery.
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Figure 3-8 Localisation of miRNA-96 to smooth muscle cell layer in pulmonary artery in mouse lung sections

Representative *in situ* hybridisation and immunohistochemistry localisation of miRNA-96, von-williebrand factor (a marker for endothelial cells) and α-smooth muscle actin (a marker for smooth muscle cells) in 5µm frontal plane lung sections from mice. Positive staining for miRNA-96 was visualised by a purple colour, von-williebrand and α-smooth muscle actin was visualised by the dark orange/brown colour. Scale bar = 50µm.
3.2.5 MiRNA-96 is down-regulated in human female PAH

Having identified positive staining for miRNA-96 in the pulmonary artery smooth muscle cell layer of lung sections we wished to examine the relative expression of miRNA-96 within human PASMCs from non-PAH and PAH patients. Here we showed female PAH patient PASMCs exhibited significantly decreased expression of miRNA-96 compared to female non-PAH PASMCs (Figure 3.9). There was no significant differences between male PASMCs and their expression level of miRNA-96 was comparable to female non-PAH PASMCs. These results correlate with the expression of miRNA-96 within PASMCs from female and male BMPR2<sup>R899X+</sup>/− mice. This data further suggest that sex may underlie differences in miRNA-96 expression in the setting of PAH disease.
Figure 3-9 Expression of miRNA-96 is down-regulated in female PASMCs from PAH patients only

Pulmonary artery smooth muscle cells (PASMCs) were explanted and cultured from female and male PAH patient and non-patient donors. The expression of miRNA-96 was assessed by Taqman quantitative Real Time-PCR. Results were normalised to the small nuclear RNA, RNU48. n=4-6 human samples per group in triplicate, *p<0.05, **p<0.01, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the RQ ± RQ_{max}/RQ_{min}. RQ= relative quantification.
3.2.6 5-HT$_{1B}$ is a target of miRNA-96

We next focused on investigating a putative target of miRNA-96. Online in silico target prediction software (www.mirwalk.com) showed a seed match for miRNA-96 in the 3’UTR of the HTR1B gene (Figure 3.10) and also that miRNA-96 is the only predicted miRNA with a seed match in the 3’UTR of the HTR1B gene (www.mirwalk.com). The HTR1B gene encodes the 5-HT$_{1B}$ receptor which has previously been implicated and extensively characterised with regards to the development of PAH. To begin to validate a relationship between miRNA-96 and 5-HT$_{1B}$ we initially performed Taqman qRT-PCR and western blot protein expression analysis on PASMCs from both BMPR2$^{R899X+/-}$ mice and human samples. Here we found the mRNA and protein expression of the 5-HT$_{1B}$ receptor was significantly increased in female BMPR2$^{R899X+/-}$ PASMCs compared to female WT and unchanged amongst males (Figure 3.11 and Figure 3.12). Likewise, the 5-HT$_{1B}$ receptor expression was also significantly increased in PASMCs from female PAH patient compared to female non-PAH at both mRNA and protein level (Figure 3.13 and Figure 3.14). Again there was no change in 5-HT$_{1B}$ expression between male non-PAH and PAH PASMCs. These results showed us that miRNA-96 and the 5-HT$_{1B}$ receptor co-exist within PASMCs and more importantly that there is an inverse relationship between the two. This provides evidence to suggest 5-HT$_{1B}$ is a target of miRNA-96.

![Figure 3-10] The 5-HT$_{1B}$ mRNA contains a miRNA-96 binding site

Schematic overview of the miRNA-96:5-HT1B mRNA interaction. The area highlighted yellow indicates the nucleotide binding in seed region.
Pulmonary artery smooth muscle cells (PASMCs) were explanted and cultured from female and male wild-type (WT) and BMPR2$^{R899X+/-}$ mice. The expression of 5-HT$_{1B}$ mRNA was assessed by Taqman quantitative Real Time-PCR. Results were normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). n=6 per group in triplicate, *p<0.05, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the RQ ± RQ$_{max}$/RQ$_{min}$. RQ= relative quantification.
Figure 3-12 The expression of 5-HT_{1B} protein is selectively increased in PASMCs from female BMPR2^{R899X+/-} mice

Pulmonary artery smooth muscle cells (PASMCs) were explanted and cultured from female and male wild-type (WT) and BMPR2^{R899X+/-} mice. The expression of 5-HT_{1B} protein was assessed by western blot and analysed by densitometric analysis (A). Representative immunoblot (B). α-tubulin was used as the internal loading control. n=6 mice per group, *p<0.05, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the mean ± SEM.
Figure 3-13 The expression of 5-HT1B mRNA is selectively increased in PASMCs from female PAH patients

Pulmonary artery smooth muscle cells (PASMCs) were explanted and cultured from female and male PAH patient and non-patient donors. The expression of 5-HT1B mRNA was assessed by Taqman quantitative Real Time-PCR. Results were normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). n=4-6 samples per group in triplicate, *p<0.05, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the RQ ± RQ_{max}/RQ_{min}. RQ= relative quantification.
Pulmonary artery smooth muscle cells (PASMCs) were explanted and cultured from female and male PAH patient and non-patient donors. The expression of 5-HT_{1B} protein was assessed by western blot and analysed by densitometric analysis (A). Representative immunoblot (B). α-tubulin was used as the internal loading control. n=4-6 samples per group in triplicate, ***,p<0.001, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the mean ± SEM.
3.2.7 The 5-HT$_{1B}$ receptor is a direct target of miRNA-96

To validate the 5-HT$_{1B}$ receptor as a target of miRNA-96 a dual luciferase reporter assay was performed. Initially a concentration gradient determined that the optimal pre-miRNA-96 concentration to obtain a significant reduction in luciferase activity was 25nM (Figure 3.15). Therefore both pre-miRNA-96 and pre-miRNA-negative control were used at a concentration of 25nM for further luciferase reporter assay experiments. 48 hours post-transfection the luciferase activity of the construct containing the 5-HT$_{1B}$ 3’UTR was significantly reduced when co-transfected with pre-miRNA-96 but unaltered by co-transfection with pre-miRNA-negative control (Figure 3.16). The selectivity of this interaction was emphasised by the inability of pre-miRNA-96 to reduce luciferase activity when a single point mutation was introduced within the seed region of the 5-HT$_{1B}$ 3’UTR (5-HT$_{1B}$ mutated). In addition, pre-miRNA-96 had no effect on luciferase activity of the control psicheck-2 construct. To verify successful transfection, RNA was harvested from duplicate plates and expression of miRNA-96 analysed. Here, results confirmed transfection of pre-miRNA-96 significantly increased mature miRNA-96 levels within the cells compared to a non-transfected control (NTC) (Figure 3.17). Transfection of pre-miRNA-negative control had no significant effect on mature miRNA-96 expression. In summary these results indicate that miRNA-96 has the functional ability to bind to the seed match region within the 3’UTR of the 5-HT$_{1B}$ mRNA.
Figure 3-15 The luciferase activity of the 5-HT$_{1B}$ construct is dependent on the pre-miRNA-96 concentration

HeLa cells were co-transfected with either the 5-HT$_{1B}$ construct (A and B) or psicheck-2 construct (C and D) and varying concentrations of pre-miRNA-96 (A and C) or pre-miRNA negative control (B and D). Luciferase activity was measured 48 hours post-transfection and the renilla luciferase activity was normalised to the internal firefly luciferase activity. n=3 per group in duplicate, **p<0.01, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as a percentage of the internal control.
Figure 3-16 Transfection with 25nM miRNA-96 reduces the luciferase activity of the 5-HT$_{1B}$ construct

HeLa cells were co-transfected with either the psicheck-2 construct, the 5-HT$_{1B}$ construct or the 5-HT$_{1B}$ mutated construct and 25nM of pre-miRNA-96 or pre-miRNA negative control. Luciferase activity was measured 48 hours post-transfection and the renilla luciferase activity was normalised to the internal firefly luciferase activity. n=3 per group in duplicate, **p<0.01, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as a percentage of the internal control.
Figure 3-17 Expression of miRNA-96 is increased after transfection with pre-miRNA-96

HeLa cells were co-transfected with either the psicheck-2 construct, the 5-HT_1B construct or the 5-HT_1B mutated construct and 25nM of pre-miRNA-96 or pre-miRNA negative control. The expression of miRNA-96 was assessed 48 hours post-transfection by Taqman quantitative Real Time-PCR. Results were normalised to the small nuclear RNA, RNU48. n=3 per group in triplicate, ****p<0.0001, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the RQ ± RQ^{max}/RQ^{min}. RQ= relative quantification, NTC= non-transfected control.
3.2.8 Heightened 5-HT_{1B} mediated proliferation in female PAH PASMCs

As PASMCs from female PAH patient had a significantly greater expression of 5-HT_{1B} compared to female non-PAH and male we wished to examine whether this translated into a biological effect. To investigate any pathophysiological consequence of dysregulated 5-HT_{1B} expression we analysed serotonin (5-HT) - induced proliferation of PASMCs from the four cell groups by cell counting and BrdU incorporation. After 72 hours stimulation with the pulmonary vascular mitogen serotonin, a proliferative response was observed only in female PAH patient PASMCs (Figure 3.18 and Figure 3.19). This was inhibited by the 5-HT_{1B} selective antagonist SB224289 suggesting the proliferative response to serotonin was mediated by the 5-HT_{1B} receptor. In addition, the selective 5-HT_{1B} agonist, CP94253, again induced proliferation only in female patient PASMCs and this was inhibited by SB224289.
Figure 3-18 Serotonin (5-HT) -induced proliferation in human PASMCs from female PAH patients is mediated by the 5-HT1B receptor as determined by cell counts

Human PASMCs from female (A) and male (C) non-patients and female (B) and male (D) PAH patients were quiesced for 24 hours in 0.2% FBS. PASMCs were then pre-incubated with the selective 5-HT1B receptor antagonist SB224289 (300nM) for 1 hour prior to addition of 5-HT (1µM) or CP94253 (1µM) in the presence of 2.5% FBS. 10% FBS was utilised as a positive control. Cell counts were assessed 72 hours later by haemocytometer. n=3 human samples per group, in triplicate, *P<0.05, **P<0.01, ****P<0.0001, One-way ANOVA followed by Tukey’s post-hoc test. Data expressed as % of 2.5% control.
Figure 3-19 Serotonin (5-HT)-induced proliferation in human PASMCs from female PAH patients is mediated by the 5-HT$_{1B}$ receptor as determined by BrdU incorporation

Human PASMCs from female (A) and male (C) non-patients and female (B) and male (D) PAH patients were quiesced for 24 hours in 0.2% FBS. PASMCs were then pre-incubated with the selective 5-HT$_{1B}$ receptor antagonist SB224289 (300nM) for 1 hour prior to addition of 5-HT (1µM) or CP94253 (1µM) in the presence of 2.5% FBS. 10% FBS was utilised as a positive control. BrdU label was added 48 hours after the initial stimulation and BrdU incorporation was assessed 24 hours later. n=4-6 samples per group, in triplicate, *P<0.05, **P<0.01, ***P<0.001, One-way ANOVA followed by Tukey’s post-hoc test. Data expressed as % of 2.5% control.
3.2.9 Over-expression of miRNA-96 in vitro reduces 5-HT_{1B} expression and function

To validate the functional significance of the miRNA-96 and 5-HT_{1B} interaction we performed transient over-expression of miRNA-96 in vitro and analysed the subsequent effect on target 5-HT_{1B} mRNA and protein. Initially, a concentration gradient demonstrated the optimal pre-miRNA-96 concentration to obtain a significant increase in mature miRNA-96 expression and reduction in 5-HT_{1B} expression (Figure 3.20, Figure 3.21 and Figure 3.22). Therefore both pre-miRNA-96 and pre-miRNA-negative control were used at a concentration of 1nM for further expression and functional analysis experiments. Results again showed successful transfection of pre-miRNA-96 lead to significantly increased expression of mature miRNA-96 levels within PASMCs with no effect from pre-miRNA-negative control transfection (Figure 3.23). The increase in miRNA-96 expression correlated with a significant reduction in 5-HT_{1B} protein level however had no significant effect on 5-HT_{1B} mRNA level (Figure 3.24 and Figure 3.25). This suggests that over-expression of miRNA-96 has the ability to bind 5-HT_{1B} mRNA and prevent the translation of the transcript into protein but does not target the transcript for degradation. To examine the functional consequence we performed serotonin-induced proliferation in PASMCs from female PAH patients. Serotonin-induced proliferation was abolished in PASMCs transfected with pre-miRNA-96 whereas transfection with pre-miRNA-negative control had no effect on proliferative response to serotonin (Figure 3.26). Moreover, the effect of pre-miRNA-96 was comparable to the effect of the 5-HT_{1B} antagonist SB224289. These results suggest that serotonin-induced proliferation can be altered by modulating the expression of the 5-HT_{1B} receptor via regulation of miRNA-96.
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Figure 3-20 The expression of mature miRNA-96 is dependent on the transfected pre-miRNA-96 concentration

Human PASMCs were transfected with either pre-miRNA-96 or pre-miRNA negative control at a range of concentrations. The expression of mature miRNA-96 was assessed 48 hours post-transfection by Taqman quantitative Real Time-PCR. Results were normalised to the small nuclear RNA, RNU48. n=3 per group in triplicate, *p<0.05, ***p<0.001, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the RQ ± RQ\textsubscript{max}/RQ\textsubscript{min}. RQ= relative quantification, NTC= non-transfected control.
Figure 3-21 The expression of 5-HT\textsubscript{1B} mRNA is dependent on the transfected pre-miRNA-96 concentration

Human PASMCs were transfected with either pre-miRNA-96 or pre-miRNA negative control at a range of concentrations. The expression of 5-HT\textsubscript{1B} mRNA was assessed 48 hours post-transfection by Taqman quantitative Real Time-PCR. Results were normalised to the GAPDH. n=3 per group in triplicate, *p<0.05, **p<0.01, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the $RQ \pm \frac{RQ_{\text{max}}}{RQ_{\text{min}}}$. $RQ=$ relative quantification, NTC= non-transfected control.
Figure 3-22 The expression of 5-HT$_{1B}$ protein is dependent on the transfected pre-miRNA-96 concentration

Human PASMCs were transfected with either pre-miRNA-96 or pre-miRNA negative control at a range of concentrations. The expression of 5-HT$_{1B}$ protein was assessed 72 hours post-transfection by western blotting and analysed by densitometric analysis (A). Representative immunoblot (B). α-tubulin was used as the internal loading control. n=3 per group in duplicate, *p<0.05. One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the mean ± SEM. NTC= non-transfected control.
Figure 3-23 Transfection with 1nM pre-miRNA-96 increases the mature miRNA-96 expression in human PASMCs

Human PASMCs were transfected with either pre-miRNA-96 or pre-miRNA negative control at a concentration of 1nM. The expression of mature miRNA-96 was assessed 48 hours post-transfection by Taqman quantitative Real Time-PCR. Results were normalised to the small nuclear RNA, RNU48. n=3 per group in triplicate, ****p<0.0001, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the RQ ± RQ\text{max}/RQ\text{min}. RQ= relative quantification.
Figure 3-24 Transfection with 1nM pre-miRNA-96 does not affect 5-HT_{1B} mRNA expression in human PASMCs

Human PASMCs were transfected with either pre-miRNA-96 or pre-miRNA negative control at a concentration of 1nM. The expression of 5-HT_{1B} mRNA was assessed 48 hours post-transfection by Taqman quantitative Real Time-PCR. Results were normalised to GAPDH. n=3 per group in triplicate. Data was expressed as the RQ ± RQ^{max}/RQ^{min}. RQ= relative quantification.
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Figure 3-25 Transfection with 1nM pre-miRNA-96 decreases 5-HT$_{1B}$ protein expression in human PASMCs

Human PASMCs were transfected with either pre-miRNA-96 or pre-miRNA negative control at a concentration of 1nM. The expression of 5-HT$_{1B}$ protein was assessed 72 hours post-transfection by western blotting and analysed by densitometric analysis (A). Representative immunoblot (B). α-tubulin was used as the internal loading control. n=3 per group in triplicate, ***p<0.001, ****p<0.0001, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the mean ± SEM. NTC= non-transfection control.
Human PASMCs from female PAH patients were quiesced for 24 hours in 0.2% FBS. PASMCs were then transfected with 1nM pre-miRNA-96 for 6 hours or pre-incubated with the selective 5-HT\textsubscript{1B} receptor antagonist SB224289 (300nM) for 1 hour prior to addition of 5-HT (1\textmu M) in the presence of 2.5% FBS. 10% FBS was utilised as a positive control. Cell counts were assessed 72 hours later by haemocytometer. n=3 samples per group, in triplicate, *P<0.05, **P<0.01, ****P<0.0001, One-way ANOVA followed by Tukey’s post-hoc test. Data expressed as % of 2.5% control.
3.2.10 Estrogen affects miRNA-96 and 5-HT₁B expression

To understand why the miRNA-96/5-HT₁B axis was only prominent in female PAH PASMCs we investigated a role for estrogen in miRNA-96 regulation. Previous studies have demonstrated a link between estrogen and the serotonin system. More specifically, PASMCs stimulated with 17β-estradiol showed an increase in the 5-HT₁B receptor, SERT and TPH1 protein expression (White et al., 2011a). We therefore wished to examine the effect of 17β-estradiol on miRNA-96 expression. First we found that PASMCs stimulated with 17β-estradiol had a significantly decreased expression of miRNA-96 compared to un-stimulated cells (Figure 3.27). Secondly, we analysed lung tissue from mice with reduced circulating estrogen levels. Here we found that female mice dosed with anastrozole (inhibitor of estrogen synthesising enzyme aromatase) had significantly increased levels of miRNA-96 compared to female mice dosed with vehicle (Figure 3.28). The increase in miRNA-96 corresponded with a significant decrease in 5-HT₁B mRNA. Interestingly this observation was only noted in lung tissue from female mice as dosing with anastrozole had no effect on miRNA-96 or 5-HT₁B mRNA expression in lung tissue from male mice. These results indicate that estrogen may modulate the 5-HT₁B receptor via regulation of miRNA-96 in females.
Figure 3-27 17β-estradiol (E2) decreases the expression of miRNA-96

Human PASMCs were quiesced for 24 hours in 0.2% FBS. PASMCs were then stimulated with E2 (1nM) in the presence of 2.5% FBS. The expression of mature miRNA-96 was assessed 48 hours post-stimulation by Taqman quantitative Real Time-PCR. Results were normalised to the small nuclear RNA, RNU48. n=6 per group in triplicate, *P<0.05, unpaired t-test. Data was expressed as the \( RQ \pm \frac{RQ_{\text{max}}}{RQ_{\text{min}}} \).
Figure 3-28 Inhibiting estrogen synthesis with the aromatase inhibitor anastrozole, increases miRNA-96 expression and decreases 5-HT1B mRNA expression in female mice

Mice were dosed with anastrozole (3mg/kg) or vehicle (1% carboxymethylcellulose) via subcutaneous injection for 14 days to reduce endogenous estrogen levels (Mair et al., 2014). Whole lung homogenates were assessed for miRNA-96 expression in female (A) and male (C) tissue and 5-HT1B mRNA expression in female (B) and male (D) tissue by Taqman quantitative Real Time-PCR. Results for miRNA-96 expression were normalised to the small nuclear RNA, RNU48 and to GAPDH for 5-HT1B mRNA expression. n=5 per group in triplicate, *P<0.05, **P<0.01, un-paired t-test. Data was expressed as the RQ \pm \text{RQ}_{\text{max}}/\text{RQ}_{\text{min}}.
3.3 Discussion

Mutations within the BMPR-II gene are associated with HPAH however they occur with reduced disease penetrance. This suggests second hit factors are required for disease onset. Contributions from the serotonin system have been reported to have involvement in PAH development (MacLean et al., 1996b; Long et al., 2006; MacLean et al., 2009; Maclean et al., 2010). Furthermore, genetic modifications underpinned by alterations in miRNA expression are also thought to be key in PAH pathogenesis (Caruso et al., 2010; Bienertova-Vasku et al., 2015; Zhou et al., 2015). PAH occurs four-fold more frequently in women than in men; however, explanation towards the underlying reasons are limited (Ling et al., 2012). Emerging literature highlights evidence in support of a causative role for estrogens in female susceptibility to PAH development (Rajkumar et al., 2010; White et al., 2012; Mair et al., 2014). On the contrary estrogen has also been shown to improve right ventricular ejection fraction and cardiac output in the SU-HX rat model (Liu et al., 2014; Frump et al., 2015). This chapter summarises data which implicates a sex specific genetic change in the 5-HT1B receptor expression and function in both BMPR2\textsuperscript{R899X+/−} mouse model and human clinical PAH samples is underpinned by dysregulation in miRNA-96 which is potentially under the regulatory control of the female sex hormone estrogen.

It has recently been highlighted that female PASMCs exhibit reduced expression of lung BMPR-II, SMAD, Id1 and Id3 expression compared to male PASMCs which could predispose females to even less expression in the event of BMPR-II haploinsufficiency (Mair et al., 2015) or predispose females to an increased susceptibility of a second hit. This is thought to contribute to enhanced female susceptibility in PAH development related to BMPR-II deficiency. The BMPR2\textsuperscript{R899X+/−} mouse model generates a mild PH phenotype however sex was not taken into account when data analysed (Long et al., 2011; Long et al., 2015). Here we analysed remodelling of the small distal pulmonary arteries by measuring the muscularisation and proliferation of PASMCs in pulmonary arteries. Data revealed distinct sex differences in remodelling of BMPR2\textsuperscript{R899X+/−} mice. Female BMPR2\textsuperscript{R899X+/−} mice had significantly increased percentage of muscularisation and proliferation compared to WT mice. This difference was not observed in the male cohort. This could be explained by the above mentioned idea that females are predisposed to a reduction in BMPR-II pathway which in turn can lead to unopposed p38MAPK/ERK signalling and increased proliferation. Additionally, other contributing factors could be facilitating an augmented proliferative phenotype in female BMPR2\textsuperscript{R899X+/−} mice. For example, it has been reported
that exogenous administration of serotonin can uncover a PH phenotype in a similar BMPR2+/− mouse model (Long et al., 2006) therefore perhaps mediators of the serotonin system are enhanced in female BMPR2R899X+/− mice and not males, and contribute to enhanced proliferation. Furthermore, sex differences between female BMPR2R899X+/− mice and male BMPR2R899X+/− mice could be attributed to differences in sex hormone pathways. For example, in other mouse models which exhibit female susceptibility in remodelling of pulmonary arteries, anomalous estrogen metabolism was found to be associated with disease susceptibility (White et al., 2011a; White et al., 2011b). Therefore, it would be interesting to ascertain if alterations within the estrogen pathway are established within the female BMPR2R899X+/− and not males which could potentially play a role in the sex differences in remodelling observed within this study.

Another explanation for the sex differences observed in remodelling between female and male BMPR2R899X+/− mice could be due to differences within miRNA expression as a consequence of BMPR-II mutation. The data above mentioned presents the novel finding that sex does influence miRNA expression in PASMCs from the BMPR2R899X+/− mouse model of PH. Sexual dimorphism in miRNA expression has been previously observed in other models of sex bias diseases e.g. SLE (Dai et al., 2013; Ohta et al., 2013). Here, dysregulated miRNA signatures were found in female mice after disease onset and not in age-matched male mice (Dai et al., 2013). This is the first study to report sexual dimorphism in miRNA expression regarding PAH. As the model under investigation involves dysregulation of the BMPR-II gene, an aspect mirrored in human disease, it suggests that our results could potentially help us understand the sex bias in human PAH. As the sex differences were selective and not apparent for every miRNA under examination we can suggest that sex was not affecting the biogenesis of the miRNAs studied. However, the main female sex hormone 17β-estradiol has been reported to influence the expression of the biogenic DICER gene via an ERα binding site in its promoter region (Bhat-Nakshatri et al., 2009). 17β-estradiol was found to up-regulate DICER gene expression and subsequently miRNA expression depending on the cell type involved. Therefore, as 17β-estradiol levels are higher in females compared to males (Dighe et al., 2005) it could potentially affect the biogenesis of miRNAs. Without further examining the components of the miRNA biogenesis pathway e.g. expression of DICER or DROSHA we cannot be certain that they are not influenced by sex within this study. It is known that the BMPR-II signalling pathway can influence regulation of certain miRNAs via their biogenesis. For example, SMAD proteins interact with DROSHA to increase the
biogenic processing of pri-miRNA-21 into mature miRNA-21 (Davis et al., 2008). With regards to BMPR-II mutations downstream SMAD expression is reduced and therefore interaction with DROSHA could also be expected to be reduced thus reducing the biogenic processing of miRNAs. This could possibly explain dysregulation of miRNA expression when occurring in both female and male BMPR2\textsuperscript{R899X+/-} PASMCs e.g. miRNA-145 and miRNA-206. However, when the dysregulation is selective for one sex i.e. occurring in female BMPR2\textsuperscript{R899X+/-} PASMCs and not male BMPR2\textsuperscript{R899X+/-} PASMCs then it would suggest other factors independent of the BMPR-II pathway are playing a regulatory role. Likewise, for miRNAs that are oppositely dysregulated e.g. miRNA-155 is down-regulated in female BMPR2\textsuperscript{R899X+/-} PASMCs but up-regulated in male BMPR2\textsuperscript{R899X+/-} PASMCs compared to WT, this would also suggest secondary contributing factors are controlling miRNA expression. Ultimately, understanding these differences is imperative to improving PAH therapies. Consistent differences in miRNA expression profile could be one potential reason why women and men respond differently to current PAH treatment (Gabler et al., 2012) and could signify that women and men with PAH need to be treated depending on their sex.

For further examination in this study we focused on miRNA-96, a novel miRNA implicated in the context of PAH. Here we show miRNA-96 expression is significantly down-regulated in PASMCs from female BMPR2\textsuperscript{R899X+/-} mice only and not males. This sex specific difference observed was consistent with results from clinical PAH samples which also showed only PASMCs from female PAH patients and not males had decreased expression of miRNA-96. This translational finding suggests a decreased expression of miRNA-96 has a conserved role in PAH pathogenesis. Furthermore, as the female PAH patient samples were from a mixed PAH population and not solely from those harbouring a BMPR-II mutation we can suggest that down-regulation of miRNA-96 is not due specifically to dysregulation of BMPR-II expression/signalling but from a wider contributing factor in PAH pathogenesis. However, as BMPR-II is thought to also be dysfunctional in some reports of IPAH (Machado et al., 2006a), without knowing if BMPR-II expression and signalling is decreased in our patient cohort we cannot further elaborate on the relationship with BMPR-II and miRNA-96. More recently it was reported that BMP-4 stimulation down-regulated the expression of miRNA-96 (Kim et al., 2014). As BMP-4 stimulation increases proliferation of distal PASMCs and leads to pulmonary arterial remodelling (Yang et al., 2005), we can suggest that there is a pathologically link...
between decreased miRNA-96 expression and aberrant proliferation with regards to distal PASMCs.

As this was the first study implicating miRNA-96 in PAH we sought to investigate a functional role and a possible target in PAH pathobiology. In silico bioinformatic analysis suggested the 5-HT\textsubscript{1B} receptor as a putative target of miRNA-96. In addition, a polymorphism in the 3’UTR of the 5-HT\textsubscript{1B} receptor mRNA disrupts binding of miRNA-96 (Jensen et al., 2009). We validated the 5-HT\textsubscript{1B} receptor was a target of miRNA-96 through luciferase reporter assay and confirmed this with site-directed-mutagenesis. A reduction in miRNA-96 within PASMCs from female BMPR\textsubscript{II}\textsuperscript{R899X+/-} mice corresponded with an increase in target 5-HT\textsubscript{1B} receptor. This demonstrates an interesting interplay between the serotonin system and BMPR-II in a BMPR-II model of disease. This interaction has been proposed before when a PH phenotype was unmasked in BMPR\textsubscript{II}\textsuperscript{+/-} mice after exposure to heightened levels of serotonin (Long et al., 2006). Expression analysis of female PAH patient samples also showed a concomitant increase in 5-HT\textsubscript{1B} receptor expression in PASMCs with decreased miRNA-96 expression compared with female non-PAH patient samples. Increased expression of 5-HT\textsubscript{1B} receptor has been previously documented in remodelled pulmonary arteries from PAH patients as well as a number of animal models of PH (Heeley R.P., 1998; Launay et al., 2002; Morecroft et al., 2005), however, differences between sexes have not previously been distinguished. As the most abundant serotonin receptor within the pulmonary vasculature mediating both PASMC contraction and proliferation, the 5-HT\textsubscript{1B} receptor is an attractive therapeutic target (Morecroft et al., 1999). With regards to PAH the 5-HT\textsubscript{1B} receptor expression or function has not previously been targeted using epigenetic modulation. Here, we also demonstrated direct over-expression of miRNA-96 (via transfection of pre-miRNA-96) in human PASMCs could decrease expression of target 5-HT\textsubscript{1B} receptor protein in \textit{in vitro} cell culture. This acute over-expression of the miRNA-96 had no effect on 5-HT\textsubscript{1B} receptor mRNA suggesting that after 48 hours the miRNA-96 is binding to the 3’UTR of 5-HT\textsubscript{1B} receptor mRNA and silencing the translation to protein without actual degradation of the mRNA.

Serotonin is a well-documented mitogen within the pulmonary circulation (Eddahibi et al., 1999; Eddahibi et al., 2000; MacLean et al., 2000). Thus far there is no knowledge regarding its sex differences in mitogenic response. We demonstrated that serotonin-induced proliferation of human PASMCs from female patients was mediated by the 5-HT\textsubscript{1B} receptor as the effect was replicated by a 5-HT\textsubscript{1B} receptor agonist and abolished by a
5-HT\textsubscript{1B} receptor selective antagonist. These results are consistent with previous studies showing the importance of the 5-HT\textsubscript{1B} receptor mediating human PASMC proliferation (Lawrie \textit{et al.}, 2005; Morecroft \textit{et al.}, 2010; Liu \textit{et al.}, 2013). Interestingly, proliferation to serotonin in human PASMCs was only observed in female patient PASMCs. This correlates with low miRNA-96 and high 5-HT\textsubscript{1B} receptor expression in this patient cohort. No serotonin induced proliferation was demonstrated in any other cell line. This is despite there being low expression levels of the 5-HT\textsubscript{1B} receptor in male PAH patient and female and male non-patient PASMCs. The 5-HT\textsubscript{1B} receptor is coupled to the Gi protein and responses can be regulated by synergistic influences where a threshold for activation is required (Dickenson \textit{et al.}, 1996; Dickenson \textit{et al.}, 1998). Indeed, activity and/or expression of the 5-HT\textsubscript{1B} receptor can be increased synergistically by Gq-linked receptor stimulation (Sweeney \textit{et al.}, 1995; MacLean, 1999; MacLean \textit{et al.}, 2001; Lawrie \textit{et al.}, 2005), estrogen (White \textit{et al.}, 2011a), co-activation of the serotonin transporter (Lawrie \textit{et al.}, 2005; Morecroft \textit{et al.}, 2005; Morecroft \textit{et al.}, 2010) and pERK (Liu \textit{et al.}, 2004; Mair \textit{et al.}, 2008). Estrogen synthesis is increased in female human PASMCs (Mair \textit{et al.}, 2014) providing a stimulus for over-expression of 5-HT\textsubscript{1B} and SERT (White \textit{et al.}, 2011a) which is over-expressed in patient PASMCs. In addition, pERK2 expression is elevated in female human PASMCs and further enhanced in PASMCs from PAH patients (Yang \textit{et al.}, 2005; Mair \textit{et al.}, 2008). In addition, there is increased expression of Gq-linked receptors (e.g. PDGF, endothelin-1) in PASMCs from patients with PAH (Davie \textit{et al.}, 2002). Hence female PASMC from PAH patients are influenced by a unique combination of synergins (SERT, Gq-stimulation, estrogen) that can facilitate 5-HT\textsubscript{1B} receptor-mediated responses.

We further show miRNA-96 is expressed more in the lung compared to the liver, spleen and brain. Specifically, within the lung miRNA-96 localises to the smooth muscle cell layer of the pulmonary artery indicating that miRNA-96 may potentially play a role in the PASMCs i.e. the proliferative processes of PASMCs within PAH development. This was confirmed by the ability of over-expression of miRNA-96 to prevent serotonin-induced proliferation of PASMCs from female PAH patients. Thus within the female PAH patient PASMCs, down-regulation of miRNA-96 may lead to over-expression of 5-HT\textsubscript{1B} receptor which subsequently increased the capacity of serotonin to induce proliferation. This is the first report that miRNA-96 expression influences distal human PASMCs proliferation and that manipulation of miRNA-96 can influence proliferation through targeting the 5-HT\textsubscript{1B} receptor.
Little is currently known about the role miRNA-96 plays in vascular physiology and pathology. Aberrant expression of miRNA-96 has been observed in cancer biology where it has been described as being either oncogenic (Haflidadottir et al., 2013) or anti-oncogenic (Yu et al., 2010). MiRNA-96 has previously been associated with breast cancer (Lin et al., 2010) a disease strongly influenced by estrogen and suggests that there could be an association between estrogen and miRNA-96 expression. Indeed estrogen can alter miRNA expression through three mechanisms, firstly through estrogen-response-elements (ERE) in the promoter element of the pri-miRNA gene (Di Leva et al., 2010), secondly through transcription of estrogen related genes e.g. c-MYC which can in turn interact with transcription of primary-miRNA gene (Castellano et al., 2009) and thirdly through interaction with miRNA biogenesis as there is an ERα binding site in the promoter region of the Dicer gene (Bhat-Nakshatri et al., 2009). We have previously demonstrated that estrogen increases the protein expression of the 5-HT1B receptor in human PASMCs and also estrogen-induced proliferation of human PASMCs can be inhibited by a 5-HT1B selective antagonist (White et al., 2011a). It was therefore of interest to investigate if estrogen could potentially influence 5-HT1B receptor expression through modulating epigenetic control. Stimulation of human PASMCs with estrogen decreased the expression of miRNA-96 suggesting that estrogen could influence 5-HT1B receptor expression via regulation of miRNA-96. This is consistent with bioinformatic analysis showing the primary-miRNA-96 gene harbours an ERE in the promoter region (Matys et al., 2003). As estrogen is also a potent mitogen within PASMCs and mediates its proliferative effect through the 5-HT1B receptor, it would be interesting to see if over-expression of miRNA-96 (which would reduce 5-HT1B receptor expression) could abolish estrogen induced proliferation of PASMCs. A potential role for estrogen in regulation of miRNA-96 expression could help explain the sexual dimorphism of miRNA-96 expression within both PASMCs from BMPR2R899X+/- mice and human patient samples.

Previous studies have shown that exogenously administered estrogen can protect against PH in male mice (Lahm et al., 2012). We recently demonstrated expression of aromatase (the estrogen synthesising enzyme) in human, rat and mouse pulmonary artery smooth muscle which was greater in females (Mair et al., 2014). In addition, we showed that inhibition of endogenous aromatase by anastrozole can prevent and reverse PH in the hypoxic mouse and rat SU-HX model but only in females (Mair et al., 2014). Also anastrozole treatment restored a reduced expression of BMPR-II in lung samples from hypoxic female mice but not males (Mair et al., 2014). Anastrozole decreases endogenous
circulating estrogen. This suggests that endogenous estrogen plays a role in the development of PH in female mice and rats, but not males, and that the combination of circulating estrogen and local endogenous synthesis of estrogen in pulmonary arteries drives a PH phenotype in females. Here, we found lungs from mice treated with anastrozole demonstrated a significantly higher expression of miRNA-96 compared to control lung. This increase in miRNA-96 was associated with a decrease in 5-HT\(_{1B}\) receptor expression and was only observed in lungs from female mice not males. Collectively, our results suggest that endogenous estrogen regulates miRNA-96 expression only in female lung. However, we must also consider the input from male sex hormones. Inhibiting aromatase with anastrozole would favour the conversion of androstenedione to testosterone. Testosterone’s metabolite 5α-dihydrotestosterone (DHT) is known to regulate miRNA expression in prostate adenocarcinoma (Waltering et al., 2011). It would be of interest to assess the effect of testosterone and its metabolites on miRNA-96 expression to greater understand the hormonal control of miRNA-96 and reasons underlying its differential expression between sexes. Although it is also worthy to note that unpublished data has shown castration (thus removal of male sex hormones) has no effect on the development of PH in male mice (Audrey Wright, unpublished). Altogether this supports the hypothesis that sex plays an important role in the regulation of miRNAs.

In summary, our data indicates that estrogen and BMPR-II deficiency can decrease miRNA-96 expression in PASMCs causing an increase in 5-HT\(_{1B}\) receptor expression which may influence the pathobiology of PAH in females. Furthermore, increasing the expression of miRNA-96 has the ability to prevent proliferation which is a hallmark of PAH disease and makes enhancing miRNA-96 levels an attractive target for PAH therapy. Overall this data strengthens the presence of sex differences within PAH disease and a stratified approach of 5-HT\(_{1B}\) receptor or miR-96 targeted therapies aimed at female patients may prove beneficial.
Chapter Four

4 The Effect of Restoring miRNA-96 Levels *In Vivo*
4.1 Introduction

The 5-HT\textsubscript{1B} receptor and the wider serotonin system have well documented associations with PAH development. A general hyperactivity of serotonin synthesis and signalling leads to pulmonary artery vasoconstriction and remodelling, two major hallmarks of PAH (Herve et al., 1995; Morecroft et al., 1999; Eddahibi et al., 2006). The majority of receptor led serotonin signalling in the human pulmonary circulation is mediated via the 5-HT\textsubscript{1B} receptor making it a desirable target for pulmonary artery specificity (Morecroft et al., 1999). The 5-HT\textsubscript{1B} receptor is a GPCR which is coupled with the G\textsubscript{i/o} protein and upon receptor activation will inhibit adenylate cyclase and reduce the production of cAMP. Increased expression of 5-HT\textsubscript{1B} receptor has been documented in remodelled pulmonary arteries of PAH patient lungs and also within a variety of animal models including the hypoxic model (Heeley R.P., 1998; Launay et al., 2002). In Chapter 3 we showed an increase in 5-HT\textsubscript{1B} receptor expression and proliferative function specific to female PAH patient PASMCs. Experimentally the 5-HT\textsubscript{1B} receptor has successfully been therapeutically challenged by classical pharmacological antagonism in the hypoxic rat model (Keegan et al., 2001). However, classical pharmacological antagonism does not resolve the underlying over-expression of the 5-HT\textsubscript{1B} receptor documented within PAH and therefore an upstream regulator of this serotonin receptor may prove more beneficial.

In recent years the advent of miRNA based therapy has grown considerable interest. MiRNAs are non-coding RNAs which can direct expression of target genes in a post-transcriptional manner, some of which can be similarly dysregulated in disease. The idea of manipulating a possible subset of pathologically linked genes through one single miRNA provided promise of a very powerful therapeutic tool. Influencing miRNA levels in vivo can be achieved by either miRNA mimic or antagonir. A dynamic expression profile of miRNAs has previously been associated with PAH development (Caruso et al., 2010). Results from in vivo studies have revealed inhibition of miRNA-17 via an antagonir had the ability to reverse both a hypoxic and monocrotaline PH phenotype in rats (Pullamsetti et al., 2012). This was thought to be partly via up-regulation of the pro-apoptotic miRNA-17 target p21. Furthermore, delivery of a miRNA-204 mimic can reverse monocrotaline induced PH in rats via reducing the expression of the proliferative target nuclear factor of activated T cells (NFAT) (Courboulin et al., 2011). Thus the imbalance of proliferative and apoptotic genes (and their downstream effect) involved in PAH pathogenesis can be modulated in vivo by miRNA regulation.
As PAH is a sex bias disease (Ling et al., 2012) and sexual dimorphism in miRNAs is known to exist (Marco et al., 2013; Wu et al., 2013), we hypothesised in Chapter 3 that sex differences in miRNA expression could underlie female susceptibility in PAH development. In Chapter 3 we found that miRNA-96 was differentially expressed between sexes. The down-regulation of miRNA-96 in females contributed to an enhanced proliferative phenotype only observed in females via targeting the 5-HT$_{1B}$ receptor. We confirmed restoration of miRNA-96 levels in PASMCs in vitro inhibited proliferation, however, it remained unclear if restoration of miRNA-96 could prevent and/or reverse the onset of PAH development.

Here, we examine the effect of intravenous in vivo delivery of miRNA-96 mimic on the ability to prevent a PH phenotype in female WT mice and also reverse a mild PH phenotype in female BMPR2$^{R899X+/-}$ mice. Our data shows successful delivery of miRNA-96 mimic to the pulmonary arteries by intravenous tail vein injection. Additionally, we highlight this is able to restore deficiencies of miRNA-96 in the lungs of both hypoxic and BMPR2$^{R899X+/-}$ mice. Most importantly we demonstrate intravenous in vivo delivery of miRNA-96 mimic can prevent the induction of a hypoxic PH phenotype and also reverse an established disease phenotype in BMPR2$^{R899X+/-}$ mice. Furthermore, we show data confirming the therapeutic effect is mediated through targeted reduction of the 5-HT$_{1B}$ receptor in both of these models.

Aims of this chapter:

1. To examine if intravenous tail vein injection successfully delivered miRNA-96 mimic to the pulmonary arteries.

2. To evaluate if miRNA-96 mimic can prevent and reverse a PH phenotype in female mice.

3. To ascertain if miRNA-96 mimic can effectively regulate the 5-HT$_{1B}$ receptor in vivo.
4.2 Results

4.2.1 Intravenous delivery of miRNA-96 mimic increases miRNA-96 expression in the pulmonary artery

One of the major hurdles in PAH therapy is successful delivery of the drug to the pulmonary arteries of the lung. To ensure intravenous tail vein injection of miRNA-96 mimic successfully reached the pulmonary artery and more specifically the smooth muscle cell layer we performed Taqman q-RT-PCR and *in situ* hybridisation. Our results indicate that dosing with 1.5mg/kg (2nmoles) miRNA-96 mimic on day 0 and day 7 as per Figure 2.3 is substantial to obtain an increase in miRNA-96 expression within the pulmonary arteries (Figure 4.1). Furthermore, miRNA-96 expression was localised within smooth muscle cells of the small distal pulmonary arteries as shown by the staining for α-smooth muscle actin in mice dosed with miRNA-96 mimic (Figure 4.2).

![Figure 4-1 Intravenous dosing with a miRNA-96 mimic increases the miRNA-96 expression in pulmonary arteries](image)

Mice were dosed with a miRNA-96 mimic (1.5mg/kg) or negative control (1.5mg/kg) via intravenous tail vein injection on day 0 and day 7. On day 14 pulmonary artery homogenates were assessed for miRNA-96 expression by Taqman quantitative Real Time-PCR. Results for miRNA-96 expression were normalised to the small nuclear RNA, U6. n=5 per group in triplicate, *P<0.05, un-paired t-test. Data was expressed as the RQ ± RQ\text{max}/RQ\text{min}.
Figure 4-2 MiRNA-96 expression is localised to smooth muscle cell layer after intravenous miRNA-96 mimic dosing

Representative in situ hybridisation and immunohistochemistry localisation of miRNA-96, von-williebrand factor (a marker for endothelial cells) and α-smooth muscle actin (a marker for smooth muscle cells) in 5μm frontal plane lung sections from mice dosed with a miRNA-96 mimic or negative control. Positive staining for miRNA-96 was visualised by a purple colour, von-williebrand and α-smooth muscle actin was visualised by the dark orange/brown colour. Scale bar = 50μm.
4.2.2 Intravenous delivery of miRNA-96 mimic prevents a PH phenotype in female hypoxic mice

To investigate the effect of a miRNA-96 mimic on the development of a PH phenotype we assessed right ventricular systolic pressure (RVSP), right ventricular hypertrophy (RVH) and remodelling in hypoxic female mice. Importantly our data demonstrates successful induction of a hypoxic PH phenotype as witnessed by the significant increase in all three measured parameters within the normoxic PBS dosed mice vs. hypoxic PBS dosed mice. Treatment with negative control mimic showed no effect on RVSP, RVH and remodelling of both normoxic and hypoxic mice. We observed that delivery of miRNA-96 mimic to the pulmonary arteries reduced RVSP in hypoxic mice as compared to both PBS and negative control mimic dosed mice (Figure 4.3). In hypoxia a miRNA-96 mimic also decreased the weight of the right ventricle reducing RVH compared to control mice (Figure 4.4). In addition, to ascertain if a miRNA-96 mimic affected remodelling of the small distal pulmonary arteries we assessed the percentage of pulmonary arteries with double elastic lamina within lung sections (Figure 4.5). Here our data indicates that treatment with miRNA-96 significantly reduced the number of remodelled pulmonary arteries in hypoxic mice compared to both PBS and negative control mimic dosed mice. As the number of remodelled pulmonary arteries in the hypoxic miRNA-96 mimic dosed group is not significantly different from the normoxic miRNA-96 mimic dosed group we can suggest that treatment with miRNA-96 mimic prevented the processes involved in hypoxia-induced remodelling. Administration of miRNA-96 mimic had no significant effect under normoxic conditions in all three assessed PH parameters. From this data we can suggest that miRNA-96 has a critical function in modulating hypoxia-induced PH in female mice. However, as all three parameters are not reduced to normoxic levels we can suggest that miRNA-96 may not solely be responsible for hypoxic induced PH and that other contributing factors are potentially playing a role.
Figure 4-3 Reduction in hypoxia-induced right ventricular systolic pressure by a miRNA-96 mimic

Female wild-type C57Bl/6 mice were exposed to hypoxia (10% oxygen) or normoxia for 14 days. On day 0 and day 7 mice were dosed with 1.5mg/kg miRNA-96 mimic, negative control or PBS via intravenous tail vein injection. Right ventricular systolic pressure (RVSP) was assessed by right heart catheterisation. n=10, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, One-way ANOVA followed by a Tukey’s post-hoc test. Data was expressed as the mean ± SEM.
Figure 4-4 Reduction in hypoxia-induced right ventricular hypertrophy by a miRNA-96 mimic

Female wild-type C57Bl/6 mice were exposed to hypoxia (10% oxygen) or normoxia for 14 days. On day 0 and day 7 mice were dosed with 1.5mg/kg miRNA-96 mimic, negative control or PBS via intravenous tail vein injection. Right ventricular hypertrophy (RVH) was assessed by measuring the right ventricle weight over the left ventricle plus septum (RV/LV+S). n=10, *p<0.05, **p<0.01, ****p<0.0001, One-way ANOVA followed by a Tukey’s post-hoc test. Data was expressed as the mean ± SEM.
Female wild-type C57Bl/6 mice were exposed to hypoxia (10% oxygen) or normoxia for 14 days. On day 0 and day 7 mice were dosed with 1.5mg/kg miRNA-96 mimic, negative control or PBS via intravenous tail vein injection. Remodelling of pulmonary arteries was assessed by counting the number of remodelled (double elastic lamina) and non-remodelled arteries (single elastic lamina) and expressing the percentage of remodelled arteries over the total number of arteries counted (A). Representative images are shown in the bottom panel (B). n=10, ***p<0.001, ****p<0.0001, One-way ANOVA followed by a Tukey’s post-hoc test. Data was expressed as the mean ± SEM. Scale bar =50µm.

Figure 4-5 Reduction in hypoxia-induced pulmonary artery remodelling by a miRNA-96 mimic
4.2.3 Intravenous delivery of miRNA-96 mimic had no effect on systemic parameters in female hypoxic mice

As the haemodynamics of mice administered a miRNA-96 mimic had not previously been explored, it was crucial to also examine the effects on both systemic arterial pressure (SAP) (Figure 4.6) and heart rate (HR) (Figure 4.7). Here our data highlighted that neither hypoxic conditions nor administration of PBS, negative control mimic or miRNA-96 mimic had any significant effect on both SAP and HR. This data importantly suggests that effects modulated by the miRNA-96 mimic are specific to the pulmonary circulation and therefore unwanted side effects mediated by the systemic circulation may be limited. In addition no significant differences were observed between body weights of all six experimental groups (Figure 4.8).

![Graph showing mean systemic arterial pressure (mSAP) in normoxic and hypoxic conditions for different groups](image)

**Figure 4-6 Administration of a miRNA-96 mimic or hypoxia had no effect on mean systemic arterial pressure in hypoxia-induced pulmonary hypertension**

Female wild-type C57Bl/6 mice were exposed to hypoxia (10% oxygen) or normoxia for 14 days. On day 0 and day 7 mice were dosed with 1.5mg/kg miRNA-96 mimic, negative control or PBS via intravenous tail vein injection. Mean systemic arterial pressure (mSAP) was assessed by catheterisation of the left common carotid artery. n=10, data was expressed as the mean ± SEM.
Figure 4-7 Administration of a miRNA-96 mimic or hypoxia had no effect on heart rate in hypoxia-induced pulmonary hypertension

Female wild-type C57Bl/6 mice were exposed to hypoxia (10% oxygen) or normoxia for 14 days. On day 0 and day 7 mice were dosed with 1.5mg/kg miRNA-96 mimic, negative control or PBS via intravenous tail vein injection. Heart rate was assessed by measuring the number of heart beats per minute (bpm). n=10, data was expressed as the mean ± SEM.
Figure 4-8 Administration of a miRNA-96 mimic or hypoxia had no effect on body weight in hypoxia-induced pulmonary hypertension

Female wild-type C57Bl/6 mice were exposed to hypoxia (10% oxygen) or normoxia for 14 days. On day 0 and day 7 mice were dosed with 1.5mg/kg miRNA-96 mimic, negative control or PBS via intravenous tail vein injection. Body weight was assessed before in vivo haemodynamics were performed. n=10, data was expressed as the mean ± SEM.
4.2.4 Intravenous delivery of miRNA-96 mimic restored the miRNA-96 deficiency in lung of female hypoxic mice

In chapter 3 we observed a miRNA-96 deficiency in both experimental animal model and clinical PAH samples from females. To assess if the deficiency was also evident in the hypoxic model we examined miRNA-96 expression in lung samples from both normoxic and hypoxic female mice. Here our data shows that hypoxia decreases the expression of miRNA-96 in the lung compared to normoxia (Figure 4.9). In addition, intravenous administration of miRNA-96 mimic increases the expression of miRNA-96 in both normoxic and hypoxic conditions and restores the observed hypoxic deficiency.

To assess the global tissue distribution of miRNA-96 after intravenous tail vein injection of miRNA-96 mimic we examined miRNA-96 expression levels in liver, kidney and spleen by Taqman qRT-PCR. In mice dosed with the miRNA-96 mimic there was no significant up-regulation of miRNA-96 expression compared to those dosed with the negative control mimic in any of the tissues analysed (Figure 4.10). Interestingly our data shows a deficiency in miRNA-96 expression at baseline in hypoxic spleens vs. normoxic spleens. These results strengthen the limited systemic side effects from intravenous miRNA-96 mimic administration.
Figure 4-9 Hypoxia and administration of a miRNA-96 mimic alters the expression of miRNA-96 in the lung

Female wild-type C57Bl/6 mice were exposed to hypoxia (10% oxygen) or normoxia for 14 days. On day 0 and day 7 mice were dosed with 1.5mg/kg miRNA-96 mimic or negative control via intravenous tail vein injection. The expression of miRNA-96 was assessed in whole lung homogenates by Taqman quantitative Real Time-PCR. Results were normalised to the small nuclear RNA, U6. n=6 in triplicate, *p<0.05, ***p<0.001, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the RQ ± RQ^max/RQ^min. RQ= relative quantification.
Figure 4-10 Hypoxia and administration of a miRNA-96 mimic alters the expression of miRNA-96 in the spleen but not kidney or liver

Female wild-type C57Bl/6 mice were exposed to hypoxia (10% oxygen) or normoxia for 14 days. On day 0 and day 7 mice were dosed with 1.5mg/kg miRNA-96 mimic or negative control via intravenous tail vein injection. The expression of miRNA-96 was assessed in whole liver (A), kidney (B) and spleen (C) homogenates by Taqman quantitative Real Time-PCR. Results were normalised to the small nuclear RNA, U6. n=6 in triplicate, *p<0.05, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the RQ ± RQ_{max}/RQ_{min}. RQ= relative quantification.
4.2.5 Intravenous delivery of miRNA-96 mimic decreased target 5-HT$_{1B}$ protein in lungs of female hypoxic mice

In Chapter 3 we confirmed by in vitro experiments that the 5-HT$_{1B}$ receptor was a true target of miRNA-96 and miRNA-96 may be controlling proliferation of PASMCs via modulation of the 5-HT$_{1B}$ receptor expression. To examine whether or not this regulation could be translated in vivo, we assessed the expression of the 5-HT$_{1B}$ receptor via Taqman and western blot in mice administered with the miRNA-96 mimic. Our results highlight a concomitant decrease in miRNA-96 expression with an increase in 5-HT$_{1B}$ protein expression in lungs from hypoxic control mice (Figure 4.12). Likewise, in lungs from hypoxic mice dosed with a miRNA-96 mimic there is an associated significant decrease in 5-HT$_{1B}$ protein expression compared to control mice. These results indicate that within an in vivo setting miRNA-96 has the ability to regulate the expression of the target 5-HT$_{1B}$ receptor at protein level. The 5-HT$_{1B}$ mRNA expression was not affected by either hypoxic or drug treatment (Figure 4.11).
Figure 4-11 Hypoxia and administration of a miRNA-96 mimic does not affect the expression of 5-HT$_{1B}$ mRNA in the lung

Female wild-type C57Bl/6 mice were exposed to hypoxia (10% oxygen) or normoxia for 14 days. On day 0 and day 7 mice were dosed with 1.5mg/kg miRNA-96 mimic or negative control via intravenous tail vein injection. The expression of 5-HT$_{1B}$ mRNA was assessed in whole lung homogenates by Taqman quantitative Real Time-PCR. Results were normalised to the GAPDH. n=6 in triplicate, data was expressed as the RQ $\pm$ RQ$^{\text{max}}$/RQ$^{\text{min}}$. RQ= relative quantification.
Figure 4-12 Hypoxia and administration of a miRNA-96 mimic alters the expression of 5-HT1B protein in the lung

Female wild-type C57Bl/6 mice were exposed to hypoxia (10% oxygen) or normoxia for 14 days. On day 0 and day 7 mice were dosed with 1.5mg/kg miRNA-96 mimic or negative control via intravenous tail vein injection. The expression of 5-HT1B protein was assessed by western blotting and analysed by densitometric analysis (A). Representative immunoblot (B). α-tubulin was used as the internal loading control. n=6 per group in duplicate, *p<0.05, ***p<0.001, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the mean ± SEM.
4.2.6 Intravenous delivery of miRNA-96 mimic increases components of the BMPR-II pathway in lungs of female hypoxic mice

As the BMPR-II pathway is integral to HPAH and some cases of IPAH we wished to investigate the effects of a miRNA-96 mimic on the components of the BMPR-II system within the lung. For this we examined the mRNA expression of BMPR-II, SMAD1, ID1 and ID3 by Taqman analysis (Figure 4.13). We found that hypoxia only significantly reduced BMPR-II expression but this significance was lost in those mice dosed with a miRNA-96 mimic. Interestingly we also demonstrated that in hypoxic mice dosed with miRNA-96 mimic there was a significant increase in both SMAD1 and ID1 expression compared to both normoxic mice dosed with miRNA-96 mimic and hypoxic mice dosed with negative control. There was no change observed in ID3 expression across any of the experimental groups. These results suggest that miRNA-96 mimic could also be possibly exerting beneficial therapeutic effects through increasing components of the BMPR-II pathway.
Figure 4-13 Hypoxia and administration of a miRNA-96 mimic alters the expression of the BMPR-II signalling pathway in the lung

Female wild-type C57Bl/6 mice were exposed to hypoxia (10% oxygen) or normoxia for 14 days. On day 0 and day 7 mice were dosed with 1.5mg/kg miRNA-96 mimic or negative control via intravenous tail vein injection. The expression of BMPR-II (A), SMAD1 (B), ID1 (C) and ID3 (D) mRNA were assessed in whole lung homogenates by Taqman quantitative Real Time-PCR. Results were normalised to the GAPDH. n=6 in triplicate, *p<0.05, ***p<0.001, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the RQ ± RQ\text{max}/RQ\text{min}. RQ= relative quantification.
4.2.7 Intravenous delivery of miRNA-96 mimic reverses a PH phenotype in female BMPR2R899X+/- mice

Having demonstrated the effectiveness of miRNA-96 mimic therapy at preventing a PH phenotype we wanted to investigate its potential at reversing a PH phenotype as this is more clinically relevant. For this we utilised the BMPR2R899X+/- mouse model of PH. At 6 months of age these mice exhibit an established mild phenotype of PH as observed by increased RVSP, RVH and remodelling compared to WT littermates (Figure 4.14, Figure 4.15 and Figure 4.16). Treatment with miRNA-96 mimic reversed the increases in both RVSP and remodelling. The RVH was not significantly different in BMPR2R899X+/- miRNA-96 mimic dosed mice compared to negative control mimic dosed mice. However, the RVH of the BMPR2R899X+/- miRNA-96 mimic dosed mice was also not significantly different from baseline WT level. This result could be due to the mild phenotype observed not providing a large enough therapeutic window or suggests that perhaps the BMPR-II mutation may have independent effects on the heart not associated with increased RVSP that are not targeted by miRNA-96 treatment. Overall, our data further indicates the fundamental role miRNA-96 plays in PH development.

We once again demonstrated no effects on systemic parameters including mSAP, HR and body weight with miRNA-96 mimic treatment (Figure 4.17, Figure 4.18 and Figure 4.19). These results reiterate the potential pulmonary circulation selectiveness of miRNA-96 mimic therapy.
Figure 4-14 Reduction in right ventricular systolic pressure by a miRNA-96 mimic in the BMPR2\textsuperscript{R899X+/e} model

Female wild-type and BMPR2\textsuperscript{R899X+/e} mice were dosed with 1.5mg/kg miRNA-96 mimic or negative control on day 0 and day 7 mice by intravenous tail vein injection. On day 14 right ventricular systolic pressure (RVSP) was assessed by right heart catheterisation. n=5-8, *p<0.05. One-way ANOVA followed by a Tukey’s post-hoc test. Data was expressed as the mean ± SEM.
Figure 4-15 Right ventricular hypertrophy is increased in the BMPR2<sup>R899X<sup>+/−</sup> model but not affected by miRNA-96 mimic

Female wild-type and BMPR2<sup>R899X<sup>+/−</sup> mice were dosed with 1.5mg/kg miRNA-96 mimic or negative control on day 0 and day 7 mice by intravenous tail vein injection. On day 14 right ventricular hypertrophy (RVH) was assessed by measuring the right ventricle weight over the left ventricle plus septum (RV/LV+S). n=5-8, *p<0.05, One-way ANOVA followed by a Tukey’s post-hoc test. Data was expressed as the mean ± SEM.
Female wild-type and BMPR2<sup>R899X+/−</sup> mice were dosed with 1.5mg/kg miRNA-96 mimic or negative control on day 0 and day 7 mice by intravenous tail vein injection. On day 14 remodelling of pulmonary arteries was assessed by counting the number of remodelled (double elastic lamina) and non-remodelled arteries (single elastic lamina) and expressing the percentage of remodelled arteries over the total number of arteries counted (A). Representative images are shown in the bottom panel (B). n=5-8, ****p<0.0001, One-way ANOVA followed by a Tukey’s post-hoc test. Data was expressed as the mean ± SEM. Scale bar =50µm.
Figure 4-17 Administration of a miRNA-96 mimic or BMPR2<sup>R899X+/-</sup> mutation had no effect on mean systemic arterial pressure

Female wild-type and BMPR2<sup>R899X+/-</sup> mice were dosed with 1.5mg/kg miRNA-96 mimic or negative control on day 0 and day 7 mice by intravenous tail vein injection. On day 14 mean systemic arterial pressure (mSAP) was assessed by catheterisation of the left common carotid artery. n=5-8, data was expressed as the mean ± SEM.
Female wild-type and BMPR2^{R899X+/-} mice were dosed with 1.5mg/kg miRNA-96 mimic or negative control on day 0 and day 7 by intravenous tail vein injection. Heart rate was assessed by measuring the number of heart beats per minute (bpm). n=5-8, data was expressed as the mean ± SEM.
Figure 4-19 Administration of a miRNA-96 mimic or BMPR2^{R899X+/−} mutation had no effect on body weight

Female wild-type and BMPR2^{R899X+/−} mice were dosed with 1.5mg/kg miRNA-96 mimic or negative control on day 0 and day 7 by intravenous tail vein injection. Body weight was assessed before in vivo haemodynamics were performed. n=5-8, data was expressed as the mean ± SEM.
4.2.8 Intravenous delivery of miRNA-96 mimic restored the miRNA-96 deficiency in lungs of female BMPR2<sup>R899X+/-</sup> mice

Thus far we have demonstrated a reduction of miRNA-96 expression in PASMCs from female BMPR2<sup>R899X+/-</sup> mice, female PAH patient samples and female hypoxic lungs. Here, we confirm a reduction of miRNA-96 expression in female BMPR2<sup>R899X+/-</sup> lungs at baseline. Administration of a miRNA-96 mimic increases the miRNA-96 expression in BMPR2<sup>R899X+/-</sup> lungs and restores the deficiency (Figure 4.20).

![Figure 4-20](image_url)

**Figure 4-20** BMPR2<sup>R899X+/-</sup> mutation and administration of a miRNA-96 mimic alters the expression of miRNA-96 in the lung

Female wild-type and BMPR2<sup>R899X+/-</sup> mice were dosed with 1.5mg/kg miRNA-96 mimic or negative control on day 0 and day 7 by intravenous tail vein injection. On day 14 the expression of miRNA-96 was assessed in whole lung homogenates by Taqman quantitative Real Time-PCR. Results were normalised to the small nuclear RNA, U6. n=5-8 in triplicate, *p<0.05, ***p<0.001. One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the RQ ± RQ<sub>max</sub>/RQ<sub>min</sub>. RQ= relative quantification.
4.2.9 Intravenous delivery of miRNA-96 mimic reverses the increase in 5-HT\textsubscript{1B} receptor protein in lungs of female BMPR\textsubscript{2}^{R899X+/-} mice

We previously demonstrated a decrease in miRNA-96 expression in female BMPR\textsubscript{2}^{R899X+/-} PASMCs was associated with an increase in target 5-HT\textsubscript{1B} receptor expression. Here our results show this is also true for 5-HT\textsubscript{1B} receptor protein expression in whole lung from female BMPR\textsubscript{2}^{R899X+/-} mice. Similar to the hypoxic model we show that intravenous delivery of miRNA-96 mimic can reduce the expression of 5-HT\textsubscript{1B} protein expression within the lung compared to control mice restoring it to baseline level (Figure 4.22). This result strengthens the successful ability of a miRNA-96 mimic to control target 5-HT\textsubscript{1B} receptor protein expression \textit{in vivo}. The 5-HT\textsubscript{1B} mRNA expression was not affected by either genotype or drug treatment (Figure 4.21).
Figure 4-21 BMPR2^{R899X+/-} mutation and administration of a miRNA-96 mimic does not affect the expression of 5-HT_{1B} mRNA in the lung

Female wild-type and BMPR2^{R899X+/-} mice were dosed with 1.5mg/kg miRNA-96 mimic or negative control on day 0 and day 7 by intravenous tail vein injection. On day 14 the expression of 5-HT_{1B} mRNA was assessed in whole lung homogenates by Taqman quantitative Real Time-PCR. Results were normalised to the GAPDH. n=5-8 in triplicate, data was expressed as the RQ \pm RQ^{max}/RQ^{min}. RQ= relative quantification.
Female wild-type and BMPR2<sup>R899X+/-</sup> mice were dosed with 1.5mg/kg miRNA-96 mimic or negative control on day 0 and day 7 by intravenous tail vein injection. On day 14 the expression of 5-HT<sub>1B</sub> protein was assessed by western blotting and analysed by densitometric analysis (A). Representative immunoblot (B). α-tubulin was used as the internal loading control. n=5-6 per group in duplicate, *p<0.05, **p<0.01, One-way ANOVA followed by Tukey’s post-hoc test. Data was expressed as the mean ± SEM.
4.3 Discussion

According to the REVEAL registry it is recognised that 80% of PAH patients are female (Badesch et al., 2010). This has led researchers to believe that female sex may predispose to the development of PAH. However, sex differences in PAH are highly understudied. In Chapter 3 we explored sex differences in miRNA expression as a possible factor in female susceptibility and identified a potential role for miRNA-96 in the pathogenesis of female PAH. Through in vitro investigations we highlighted a deficiency in miRNA-96 led to an increase in target 5-HT$_{1B}$ receptor and ultimately increased proliferation of female PASMCs. Aberrant proliferation of PASMCs is a major hallmark of PAH development as it is an initiating process in the remodelling of pulmonary arteries (Zhang et al., 2003). A role for miRNA-96 in disease development had not been investigated in vivo. This chapter summarises data which implicates re-establishing miRNA-96 expression in models of PH which are deficient in miRNA-96 has the ability to both prevent and reverse PH disease parameters including remodelling of small distal pulmonary arteries by potentially reducing disease associated increases in 5-HT$_{1B}$ receptor expression.

Recent studies have demonstrated that dysregulation of miRNA-96 is associated with certain forms of cancer (Yu et al., 2010). For example a decrease in miRNA-96 expression is highlighted in pancreatic cancer (Feng et al., 2014). As cancer is a disease also coupled with aberrant proliferation, perhaps miRNA-96 deficiency may underlie processes which contribute to anomalous cellular proliferation. Thought to serve as a tumour suppressor in pancreatic cancer, up-regulation of miRNA-96 represses pancreatic cell proliferation, migration and invasion in vitro (Feng et al., 2014). In addition, the growth of pancreatic tumours is significantly decreased upon introduction of miRNA-96 in an in vivo mouse model. This result was due to repression of the up-regulated pro-proliferative oncogene HERG1. Thus therapeutic success was achieved by decreasing a pro-proliferative gene via enhancing miRNA-96 expression.

In Chapter 3 we showed that augmenting levels of miRNA-96 could decrease 5-HT$_{1B}$ receptor expression and prevent serotonin induced proliferation of PASMCs. To investigate if restoration of miRNA-96 expression in vivo can protect against and reverse a PH phenotype via the 5-HT$_{1B}$ receptor, we performed two separate in vivo studies to examine the effects of a miRNA-96 mimic. We utilised the hypoxic mouse model and BMPR2$^{R899X/+}$ mouse model to investigate the prevention and reversal of disease respectively. We first confirmed that our chosen route of administration, intravenous tail
In vivo injection, successfully delivered miRNA-96 mimic to the pulmonary arteries of the lung. This was indicated by the increase in miRNA-96 expression in the pulmonary arteries. We also show that the increase in miRNA-96 expression via the miRNA-96 mimic was located within the smooth muscle cell layer of the distal pulmonary arteries. This is a key strength of this study as targeting the small distal pulmonary arteries is a major hurdle in PAH therapeutics. Furthermore, our previous data demonstrated a deficiency in miRNA-96 within distal PASMCs and therefore augmentation of expression here would be necessary to restore the imbalance. As our present research has focused on PASMCs we do not know the role, if any, miRNA-96 plays in PAECs. However, interestingly our in situ hybridisation results do not indicate detection of miRNA-96 expression within PAEC. Therefore we can suggest that within this study miRNA-96 is not playing a role within PAEC.

The hypoxic mouse model of PH produces a robust disease phenotype including decreased BMPR-II signalling (Long et al., 2009; Mair et al., 2015). Deficiencies in the BMPR-II signalling pathway play a key involvement in ~70% of HPAH and ~40% IPAH patients (Machado et al., 2006a). Deficiencies in the BMPR-II pathway can occur due to mutations with the BMPR-II gene (Atkinson et al., 2002; Yang et al., 2005). The BMPR2^R899X+/− mouse is a model with a knock-in BMPR-II gene mutation which develops mild PH at 6 months of age (Long et al., 2011; Long et al., 2015). Here, we report that within both the hypoxic and BMPR2^R899X+/− mouse models there is a significantly reduced expression of miRNA-96 and increased expression of 5-HT_{1B} receptor protein expression within lung tissue of control dosed mice. Results from both of our in vivo studies indicate that intravenous administration of miRNA-96 mimic can both prevent and reverse increases in PH characteristics including RVSP, RVH and pulmonary arterial remodelling. This was associated with an increase in lung miRNA-96 expression and decrease in 5-HT_{1B} receptor expression. This substantiates our hypothesis that PH is associated with increased 5-HT_{1B} receptor mediated remodelling/proliferation under the control of miRNA-96. The miRNA-96 mimic had no effect on mean systemic arterial pressure or heart rate in either model suggesting that this therapeutic strategy could be pulmonary selective.

For investigations into the in vivo effects of a miRNA-96 mimic on PH we focused on a dose of 1.5mg/kg on day 0 and day 7. This was based on a previous study which showed at this dose a miRNA mimic delivered using the MaxSuppressor in vivo RNALancerII kit accumulated mostly within lung tissue and lung tissue had the highest expression increase
compared to non-treated mice (Trang et al., 2011). Within the in vivo data presented here, the selected dose of miRNA-96 mimic increased the miRNA-96 between ~threefold and fivefold within the whole lung. This seems a small increase in miRNA-96 expression however this dose still demonstrated a therapeutic effect and it is known that the extent of change within miRNA expression does not correlate with its effective outcome (Calin et al., 2004; He et al., 2005). It would be of interest to increase the administered dose to potentially observe a greater therapeutic outcome. However, as with all exogenously administered drugs, increasing the dose could potentially lead to unwanted off target side effects. Perhaps only the need for restoring levels and re-balancing the deficiency is sufficient for therapeutic success rather than excessive increases in expression. Similarly, altering the route of administration may increase delivery to smooth muscle cells of small distal pulmonary arteries. For example, more direct local routes of administration i.e. intratracheal or intranasal have demonstrated successful delivery of miRNA agents to pulmonary arteries (Courboulin et al., 2011; Kim et al., 2013). These routes of administration are also perhaps more clinically relevant. We examined the effects of a miRNA-96 mimic in both the hypoxic and BMPR2^{R899X+/-} mouse models. Even though these models produce a PH phenotype they fail to recapitulate aspects of human PAH such as plexiform lesions. The SU-HX model of PH is thought to best mirror the formation of plexiform lesions similar to human disease thus investigating the therapeutic effect of a miRNA-96 mimic in vivo within the SU-HX model is imperative.

The therapeutic effects observed with a miRNA-96 mimic could be extrapolated from both an increase in miRNA-96 expression and decrease in 5-HT_{1B} receptor expression. The 5-HT_{1B} receptor has a well-defined role in PAH with involvement in mediating both proliferation and contraction of PASMCs. Excessive 5-HT_{1B} receptor expression is documented in remodelled pulmonary arteries of PAH patient lungs (Launay et al., 2002), within a variety of animal models (Heeley R.P., 1998; Rondelet et al., 2003; Morecroft et al., 2005) and we have also previously shown in Chapter 3 a sex-specific increase in 5-HT_{1B} receptor expression in PASMCs from female PAH patients. Decreasing the expression of the 5-HT_{1B} receptor would greatly reduce its involvement in the above mentioned functions. Previous in vivo analysis has demonstrated inhibition of the 5-HT_{1B} receptor by receptor antagonism is able to reduce the development of hypoxia-induced pulmonary artery remodelling, contraction and overall PAH phenotype (Keegan et al., 2001). However, these experiments have not shown whether the therapeutic effect matched with a decrease in 5-HT_{1B} receptor expression or signalling. Tackling the source of the
increase in 5-HT$_{1B}$ expression (via miRNA-96) rather than inhibiting the 5-HT$_{1B}$ activity (via a pharmacological antagonist) could perhaps prove more beneficial.

By definition, miRNAs have the ability to direct the expression of more than one target gene, with many miRNAs regulating up to 1000 genes. Therefore the beneficial effects from increasing miRNA-96 will not solely be through regulation of the 5-HT$_{1B}$ receptor but potentially through a plethora of other targets. This concept raises the concern of many other possible “on-target” and “off-target” effects mediated by miRNA-96. Within this present study we began to explore these possible effects first by examining the distribution of the miRNA-96 mimic. We found no significant changes in miRNA-96 expression in liver, kidney or spleen within our dosed mice. However, without further global tissue analysis we cannot comment on the complete miRNA-96 mimic distribution which could potentially generate negative systemic side effects. We only explored the 5-HT$_{1B}$ receptor as a target of miRNA-96 although there are other putative and published targets. The therapeutic effect observed from the miRNA-96 mimic could perhaps be from modulation of other miRNA-96 targets which could also be assisting disease prevention and regression. Further investigation into other miRNA-96 targets and their association with PAH is warranted.

It has previously been demonstrated that BMPR-II expression is reduced in the hypoxic mouse, the monocrotaline rat and the SU-HX rat models of PH (Long et al., 2009; Mair et al., 2014). We confirmed that BMPR-II mRNA expression was reduced in the lungs of the hypoxic mice. This reduction was lost in mice dosed with a miRNA-96 mimic suggesting that increasing miRNA-96 expression was augmenting BMPR-II expression. We also showed that via increasing miRNA-96 levels we could increase the expression of SMAD1 and ID1 mRNA within whole lung. The BMPR-II pathway has been shown to be integral in PAH biology with a general reduction in the pathway associated with the development of PAH (Teichert-Kuliszewska et al., 2006). Experimental therapies restoring the BMPR-II signalling pathway represents a novel therapeutic strategy. Evidence from mice deficient in BMPR-II and the SU-HX rat model highlights rescuing the BMPR-II pathway with FK506 prevents a PH phenotype (Spiekerkoetter et al., 2013). A miRNA-96 mimic is potentially a novel therapeutic way to increase a deficient BMPR-II pathway. This preliminary data warrants further examination of the relationship between miRNA-96 and BMPR-II signalling. We could assume that miRNA-96 does not increases the BMPR-II pathway by directly targeting the above mentioned genes as an increase in miRNA-96 would typically
reduce any putative target. It suggests that possibly BMPR-II, SMAD1 and ID1 are indirect targets of miRNA-96 with another intermediate gene/miRNA mediator existing between them. This data reiterates the necessity to investigate further targets of miRNA-96 which may or may not be direct targets of miRNA-96.

In summary, the data presented proposes restoration of deficiencies in miRNA-96 expression in the lungs of both hypoxic and BMPR2^{R899X+/−} mice by intravenous in vivo delivery of miRNA-96 mimic can prevent the induction of a hypoxic PH phenotype and also reverse an established disease phenotype in BMPR2^{R899X+/−} mice. Furthermore, increasing the expression of miRNA-96 in vivo has the ability to reduce increases in the target 5-HT_{1B} receptor in both of these models. Overall, this data further implicates the importance of miRNA-96 in PAH biology and reinforces the possibility of miRNA-96 based therapies for PAH.
5.1 **General Discussion**

PAH is currently an incurable vasculopathy which affects the arteries of the pulmonary circulation. In the 1950’s Dresdale documented an increase in frequency of female PAH patients (Dresdale et al., 1951). This recognised female susceptibility to PAH remains today, as recent advanced epidemiological studies highlight 70-80% of PAH patients are female (Badesch et al., 2010; Ling et al., 2012). This is in contrast to other PAH demographics which have changed over time i.e. the age of diagnosis now ranges from 50-65 years old compared to 36 years old and patients are now diagnosed with more severe PAH (Hoep et al., 2014). However, the reasons underlying the imbalanced female to male ratio regarding disease prevalence remain relatively obscure. Current PAH therapies do not incorporate this sex bias. This is in despite of recent studies having shown sex differences in treatment response (Gabler et al., 2012; Shapiro et al., 2012). It is important to further understand the reasons owing to this to improve clinical outcome.

One suggestion towards explaining the female susceptibility in PAH is that the female sex hormone estrogen plays a causative role. In support of this, eliminating circulating estrogen levels via ovariectomy can abolish a PH phenotype in female susceptible experimental models including SERT+ mice (White et al., 2011a), MTS1+ mice (Dempse et al., 2011) and dexfenfluramine mice (Dempse et al., 2013). In addition, reduction of local estrogen within the pulmonary arteries via the inhibition of the estrogen synthesising enzyme aromatase can also prevent and reverse a PH phenotype in the hypoxic mouse and SU-HX rat model respectively (Mair et al., 2014). Furthermore, the estrogen receptor ERα is upregulated in PAH and is thought to be linked to pathological signalling pathways (Rajkumar et al., 2010; Mair et al., 2014; Wright et al., 2015). Indeed inhibition of ERα prevents the development of a hypoxic phenotype in female mice (Mair et al., 2014). There is also substantial evidence to suggest the metabolites of estrogen play an important role in PAH development. The formation of 16α-OHE1 by the estrogen metabolising enzyme CYP1B1 can lead to aberrant proliferation of PASMCs and induction of a PH phenotype (White et al., 2012). Therapeutic success by inhibition of CYP1B1 has been proven in both hypoxic and SU-HX models of PH. As of yet there are no approved estrogen based therapies for PAH. However, the aforementioned research supports the fundamental importance of the estrogen pathway in PAH pathogenesis.

Estrogen can exert control over a number of genes including TPH1, SERT and 5-HT1B in PASMCs which are known mediators of PAH (White et al., 2011a). In addition, estrogen...
can also facilitate the regulation of miRNAs (Bhat-Nakshatri et al., 2009). In circumstances when estrogen or estrogen signalling is dysregulated then miRNAs which are under the control of estrogen may also become dysregulated and contribute to disease processes (Morgan et al., 2011). The estrogen pathway is known to be affected in diseases such as breast cancer and SLE both of which have a higher female prevalence (Ohta et al., 2013). In an experimental model of SLE which mirrors the female susceptibility there was recognition of sexual dimorphism of miRNA expression after the disease onset (Dai et al., 2013). Thus female specific changes in miRNAs were associated with a female susceptible disease. Regarding this evidence, the aim of this thesis was to investigate the potential of sexual dimorphism of miRNAs in PAH that could account for the sex bias in disease and provide a new miRNA candidate for a novel PAH therapy.

In Chapter 3 the sex differences of 20 miRNAs were investigated in PASMCs from BMPR2<sup>R899X<sup>+/-</sup> mice by qRT-PCR. Mutations in BMPR-II cause deficiencies within the BMPR-II signalling pathway and play a key involvement in ~70% of HPAH and ~40% of IPAH patients (Atkinson et al., 2002; Yang et al., 2005; Machado et al., 2006a). It has recently been documented that female control PASMCs are pre-disposed to reduced expression of BMPR-II and downstream signalling components compared to male PASMCs (Mair et al., 2015). This could contribute to an enhanced consequence of further BMPR-II reduction brought about by mutation in female PASMCs. Thus even in WT PASMCs differential BMPR-II levels could be affecting miRNA expression. This is the first study to report sexual dimorphism of miRNAs within the context of PAH. Some of the miRNAs studied have been previously implicated in PAH, however, the differential expression between sexes was not known e.g. miRNA-21 (White et al., 2014), miRNA-424, miRNA-503 (Kim et al., 2013). These novel findings could underpin the sex bias observed in PAH susceptibility. We focused the subsequent research on miRNA-96 as this was a novel miRNA in relation with PAH development.

The BMPR2<sup>R899X<sup>+/-</sup> model of PH produces a mild phenotype in vivo (Long et al., 2011; Long et al., 2015) and does not completely recapitulate all characteristics of human PAH therefore we also investigated the expression pattern of miRNA-96 in human PASMCs from PAH patients and non-patients. Similar down-regulation of miRNA-96 in mouse female disease PAMCs was confirmed in human PASMCs from distal pulmonary arteries. Conservation of miRNA expression pattern is not always found between experimental and human samples. For example, miRNA-451 has been shown to be up-regulated in the
monocrotaline rat model of PH but unchanged in human PAH samples (Caruso et al., 2010). The conserved expression pattern of miRNA-96 suggests that dysregulation of miRNA-96 plays a vital role in female PAH pathogenesis. As both the mouse BMPR2R899X+/− PASMCs and human PASMCs are representative systems of pre-existing/end-stage PAH, we do not know if the miRNA-96 dysregulation observed is one of which occurs at the initial stage of PAH pathology and persists throughout disease or one which occurs as a consequence of disease. To begin to understand this it would be necessary to observe if PAH characteristics could be induced by incurring a decrease in miRNA-96. This could be done in vitro by anti-miRNA-96 treatment to PASMCs or in vivo by inducing miRNA-96 knockdown.

The list of miRNAs examined was by no means extensive and a complete unbiased microarray analysis of miRNAs should be performed to assess sexual dimorphism of other miRNAs in relation to PAH. This would ultimately produce more novel miRNAs with differential expression pattern dependent on sex which could help elucidate the underlying genetic changes contributing to the sex bias in PAH. Further to this, examination of miRNA expression within PASMCs provides specific insight into the changes within this one cell type. It is known miRNA biology greatly depends on the cell type under investigation (He et al., 2012). In the pulmonary vasculature it is both PASMCs and PAECs which contribute to PAH development (Zhang et al., 2003; Teichert-Kuliszewska et al., 2006). Therefore, further analysis of sexual dimorphism of miRNAs within PAECs would significantly add to our findings.

In Chapter 3 our results revealed the 5-HT1B receptor was a direct target of miRNA-96. In addition, a reduction in miRNA-96 expression was associated with an increase in 5-HT1B receptor expression in both female BMPR2R899X+/− PASMCs and human PASMCs from female PAH patients. The 5-HT1B receptor has previously been pathologically associated with PAH. Within the pulmonary vasculature the 5-HT1B receptor is the most abundantly expressed serotonin receptor involved in the aberrant proliferation and contraction of PASMCs (Morecroft et al., 1999). An increased expression of 5-HT1B receptor has been shown in remodelled pulmonary arteries from PAH patients (Launay et al., 2002); however, our results highlighting its specific up-regulation within female disease, is a novel finding. Furthermore, the finding of serotonin-induced proliferation mediated by the 5-HT1B receptor specifically in female PASMCs was also an original observation. This suggests that the estrogen system may be influencing the 5-HT1B expression here.
The estrogen system has previously been shown to interplay with the serotonin system (White et al., 2011a). White et al. showed that stimulation of PASMCs with estrogen can up-regulate the expression of the 5-HT$_{1B}$ receptor. In addition, estrogen-induced proliferation of PASMCs was also found to be mediated by the 5-HT$_{1B}$ receptor. However, the mechanism for such regulation was not investigated. Here, within this thesis we suggest that the influence estrogen was having on the expression of 5-HT$_{1B}$ receptor expression was through epigenetic control via miRNA-96. We show stimulation of PASMCs with estrogen can down-regulate miRNA-96 expression. We further show that an inhibitor of the estrogen synthesising enzyme aromatase can trigger an increase in miRNA-96 and a decrease in 5-HT$_{1B}$ receptor expression. In addition, we show by augmenting the miRNA-96 expression both in vitro and in vivo can decrease directly the expression of the 5-HT$_{1B}$ receptor. With this regulation axis in mind we can propose that a mechanism for the original miRNA-96 dysregulation in both the female BMPR2$^{R899X+/-}$ and human PASMCs originates from estrogen. Dysregulated estrogen levels could lead to the reduction in miRNA-96 and subsequent increase in 5-HT$_{1B}$ receptor. Investigating the estrogen levels and estrogen signalling pathway within the female BMPR2$^{R899X+/-}$ mice and female human PASMCs utilised would provide insight into whether dysregulation of the estrogen system was occurring. Previous evidence using similar female PASMC samples has shown that the expression of aromatase is unchanged between female PAH patient and non-patients, however, changes in aromatase activity or estrogen levels are unknown (Mair et al., 2014).

Within the vasculature the role of miRNA-96 is relatively elusive. However, a role for miRNA-96 has been described in the cancer field. Aberrant expression of miRNA-96 is thought to contribute to both oncogenic and anti-oncogenic properties perhaps depending on which cell type under investigation (Yu et al., 2010; Haflidadottir et al., 2013; Feng et al., 2014). As anomalous proliferation is a major hallmark of PASMCs within PAH we investigated the role of miRNA-96 with regards to both PASMC proliferation and pulmonary artery remodelling. We found that by restoring miRNA-96 deficiencies by premiRNA-96 and miRNA-96 mimics in vitro and in vivo, respectively, we could inhibit serotonin-induced proliferation of PASMCs and inhibit the remodelling of small distal pulmonary arteries within the lung. More importantly our data showed that within the BMPR2$^{R899X+/-}$ mice with an established mild PH phenotype, the miRNA-96 mimic could reverse the increased percentage of remodelled pulmonary arteries. Regression of PAH disease characteristics especially remodelled pulmonary arteries is therapeutically
attractive. Current PAH therapies focus on alleviating the intense vasoconstriction of the pulmonary arteries and therefore a treatment which incorporates regression of remodelled pulmonary arteries may prove additionally beneficial. Research into the ability of miRNA-96 to regulate regeneration of pulmonary arteries is warranted. In addition, we have shown that by restoring miRNA-96 expression in the lung we can improve pulmonary vascular haemodynamics and reduce hypertrophy of the right ventricle in female mice. Even though miRNA-96 dysregulation was only observed in females, it would be of interest to examine whether miRNA-96 mimic has a therapeutic effect in male mice to begin to understand if a miRNA-96 based therapy would be specific to female patients.

Overall this thesis has introduced the novel finding that sexual dimorphism of miRNA expression does exist within PAH and this could contribute to sex differences in PAH prevalence. Furthermore, this research implicates a novel miRNA dysregulated in PAH pathogenesis, miRNA-96. Through miRNA-96 we have linked two highly associated contributing factors of PAH, the estrogen and serotonin system. Under the proposed control of estrogen, miRNA-96 targets the 5-HT\textsubscript{1B} receptor to exert control of PASMC proliferation, a major hallmark of PAH pathogenesis (Figure 5.1). Even though further research into the female specific miRNA-96/5-HT\textsubscript{1B} axis is warranted this thesis provides extensive knowledge into a novel miRNA-96 based therapeutic for PAH.

MiRNA based research and implications in disease has grown exponentially over the past two decades, however, the reality of miRNA based therapies not only in PAH but in general is still questionable. Out of the hundreds of miRNAs found to be implicated in disease pathologies only two targets have progressed to the clinical trials stage (miR-122, miR-34) (He et al., 2007; Liu et al., 2011; Hu et al., 2013; Janssen et al., 2013). However, considering miRNAs were only discovered in humans ~15 years ago, the rapid progression to clinical development is remarkable and promising. With regards to miRNA based therapeutics, two main challenges are faced. Firstly, successful delivery to target cell type and secondly appropriate modulation of gene target(s). From a pharmacokinetic view, miRNA drug molecules are negatively charged due to their phosphate backbone. Negatively charged molecules do not cross the cellular membrane well thus increasing the lipophilicity of miRNA drug molecules is crucial to increase their therapeutic capacity. This typically involves encapsulating the miRNA molecule into a lipid formulation. With regards to the miRNA-96 mimic utilised in vivo within this thesis, we incorporated the miRNA-96 mimic into a neutral lipid compartment using the MaxSuppressor In Vivo
RNA-LANCER II technique (Trang et al., 2011). With this method we demonstrated successful accumulation of miRNA-96 within PASMCs from mice dosed with miRNA-96 mimic. Other similar miRNA carriers have also proven successful with delivery to the lung but the magnitude of the delivery does depend on the carrier tested and does not always favour the lung over other tissues (Wu et al., 2011; Bala et al., 2015). Optimising the best carrier formulation for miRNA based drugs will be vital for successful delivery to the pulmonary arteries. However, before the miRNA drug molecule can enter the desired cell type it first must reach the intended cell type. For such tissue/cell specific delivery the miRNA drug molecule must be sufficiently “tagged”. For example, the LNA- antisense oligonucleotide against miRNA-122 has a phosphorothiate modification which leads to its preferential congregation within the liver where its target gene is located (Janssen et al., 2013). Finding an appropriate modification suited to PASMC accumulation could greatly improve miRNA selective delivery and therapeutic effectiveness in PAH. Similarly adeno-associated virus (AAV) - assisted delivery of miRNAs could allow tissue specificity due to their natural tropism for different tissues and ability to utilise tissue specific promoters (Robson et al., 2003). We did not find any significant increases in miRNA-96 expression within either liver, kidney or spleen tissue after in vivo miRNA-96 mimic delivery, however, full global tissue analysis was not completed and further tissue analysis would be required to confirm tissue specificity. One tissue in particular requiring examination would be the brain. As miRNA-96 targets the 5-HT1B receptor which is highly expressed within the brain, any target modulation within the brain by a miRNA-96 mimic could potentially trigger unwanted side effects. It will be crucial to see if a miRNA-96 mimic has the capacity to cross the blood brain barrier and function within brain tissue to clarify any potential side effects.

One key miRNA may modulate multiple disease associated signalling pathways. Therefore within a pathological setting, a single miRNA could be the common denominator involved in several complex dysregulated gene networks. Therapeutically, even minor alterations in the expression of a single miRNA could accumulate to have a profound synergistic effect through modifying numerous gene targets. However, this could prove undesirable when a miRNA target is not involved/dysregulated within the disease or the gene targeted is altered in the opposite direction to the primary gene target. Identification and investigation of further miRNA-96 targets would inform us of other potential signalling pathways which could be synergistically facilitating the therapeutic success of miRNA-96 mimic observed within the in vivo studies of this thesis. With regards to the miRNA-96 mimic utilised in
this thesis, achieving the correct level of miRNA-96 restoration is crucial. In theory, replenishing the deficient PASMCs with miRNA-96 should also restore normal function of the miRNA and therefore not have any unwanted side effects. This is greatly dependent on the correct dose of miRNA-96 administered, as too much could offset the balance in the opposite direction. Any potential unwanted effects are likely to arise when the mimic is introduced into cells where it is not normally endogenously expressed. Further examination of global miRNA-96 expression and function after *in vivo* miRNA-96 mimic administration would need to be investigated before commenting on the potential side effects in these present studies. Knowledge of long-term effects of miRNA based therapies is limited due to the lack of longitudinal experimental studies. To better understand the tolerability of a miRNA-96 mimic a toxicity report should be performed to provide insight into cytokine levels as well as liver and kidney function tests. Examining the half-life of a miRNA-96 mimic would assist further *in vivo* studies where dose and route of administration are being investigated.

Another therapeutic alternative to restore miRNA-96 expression within PAH could be to find a small molecule drug which could endogenously up-regulate miRNA-96. For example, we showed a link between estrogen levels and endogenous miRNA-96 expression. Administration of anastrozole, which is an inhibitor of the estrogen synthesising enzyme aromatase, decreases estrogen levels (Mair et al., 2014). Within this thesis we further show that this is associated with an up-regulation in endogenous miRNA-96 expression and a decrease in target 5-HT$_{1B}$ receptor expression. Therefore there is potential to increase miRNA-96 expression via administration of anastrozole. Anastrozole has already proven to be a successful therapy in experimental models of PH (Mair et al., 2014). Perhaps combining anastrozole with miRNA-96 mimic could augment the therapeutic effect. Combining small molecules with miRNA mimics has been documented previously in cancer research. Here it was highlighted that using a combination of the tyrosine kinase inhibitor, Erlotinib, with the miRNA-34 mimic, MRX34, created a synergistic therapeutic effect (Zhao et al., 2014). Furthermore, this study found that Erlotinib resistant cancer cell lines became sensitive to drug treatment when MRX34 was used in combination (Zhao et al., 2014). This type of adjunctive therapy has not been examined in a PAH setting, however, could play an important role in stratification of PAH therapies.
Figure 5-1 Proposed mechanism of miRNA-96 dysregulation in PAH

A schematic overview of key findings from this thesis. We show that miRNA-96 targets the 5-HT$_{1B}$ receptor which exerts control of proliferation in female PAH PASMCs. In addition we show that BMPR-II mutations in females, hypoxia and estrogen can all down-regulate miRNA-96. This leads to a concomitant increase in 5-HT$_{1B}$ receptor expression which contributes to an increase in PASMC proliferation in females and ultimately a PAH phenotype.
5.2 Future Perspective

This investigation not only demonstrates the importance of sex in miRNA regulation with regards to PAH but also that restoring miRNA-96 expression represents a novel therapeutic target for female PAH. Going forward, a complete microarray analysis of sexually dimorphic miRNAs should be performed in female and male PAH patient vs. non-PAH patient samples. This would further our understanding of miRNAs which are dysregulated in females only, males only and also those which are dysregulated in both. The differences observed within this current study suggest that potential miRNAs for therapeutic exploitation would be individual to sex and therefore their use in PAH therapy would have to be stratified accordingly.

With regards to miRNA-96, we propose that successful miRNA-96 mimic treatment may be specific to the female PAH patient. However, without further investigation into the effect of a miRNA-96 mimic within male mice, we cannot say that this therapy would be sex-specific. Following on from this, identifying a network of further miRNA-96 target genes is imperative. This would clarify other potential miRNA-96 mediated pathways possibly moderating the therapeutic effect of a miRNA-96 mimic. Fine tuning the specific delivery and dose of a miRNA-96 mimic for targeting PASMCs would also be clinically relevant. For example, inhalational administration may allow for more targeted/direct delivery to the pulmonary circulation which could potentially minimise unwanted side effects. Additionally, an inhalational based miRNA therapy would have better patient compliance than intravenous administration. For the advancement of a miRNA-96 mimic therapy, longitudinal experimental studies focusing on the tolerability and potential toxicity of this miRNA are greatly advised. Standard toxicology studies, including those encompassing effects on all vital organs and effects of repeated administration of the drug candidate, should be performed. For miRNA based therapies the cause of toxicity can be classed into hybridisation-dependent and hybridisation-independent events, thus both should be taken into consideration (Cheng et al.). Hybridisation-dependent toxicology events include those which could occur due to exaggerated pharmacology i.e. when the miRNA-mimic accumulates to an extent where too much target repression occurs. Hybridisation-independent toxicology events include those in relation to the chemistry of the miRNA mimic. For example, as miRNA based therapies involve introduction of RNA into the body, this could produce an innate immune response and trigger inflammatory signalling.
Studies have shown miRNAs can be easily identified within accessible patient samples e.g. blood/plasma. Therefore examination of miRNA-96 as a feasible biomarker for (female) PAH is also warranted.

This thesis strongly emphasises the promise of miRNA based treatments within PAH however provides novel data which envisions that patient sex should be incorporated as a variable factor. The sex differences of treatment response have recently been highlighted within the PAH field and our data further emphasises the use of stratified medicine with regards to sex in PAH.


Cheng K, Mahato RI *Advanced delivery and therapeutic applications of RNAi*. edn.


