Exploring pulmonary hypertension through microRNA-155 manipulation: signalling pathways, remodelling and haemodynamics

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Abstract

Background and Aims: Improving survival in pulmonary arterial hypertension depends on developing novel therapies which target pulmonary vascular remodelling. MicroRNAs (miRNAs) are post-transcriptional modulators of gene expression, many of which have been shown to influence signalling pathways and cell processes which control pulmonary vascular remodelling. However, miRNAs have complex and often contradictory actions on these mechanisms, and better understanding of these complexities is needed. MicroRNA-155 has been linked to factors which drive pulmonary hypertension (PH) including TGF-β/BMP signalling, inflammation, vasoactive mediators and cellular response to hypoxia. Through manipulation of miR-155 levels, this thesis aims to establish if the pathobiology of PH can be better understood by examining links to some of these factors or others such as p38 MAPK signalling, and to evaluate the in vitro and in vivo effects of downregulating miR-155, with regards to pulmonary vascular remodelling.

Methods and results: Downregulation of miR-155 either via a knockout (KO) mouse model, or pharmacological antagonism in vitro, was studied in the hypoxic model of PH. In vitro, downregulation of miR-155 prevented the proliferative and migratory response of pulmonary artery fibroblasts (PAFs) to hypoxia. This was seen both in PAFs from the KO mice, and in rat PAFs treated with antimiR-155. Reduced hypoxic phosphorylation of p38 MAPK was also seen in both models. Hypoxic activation of Smad5, a member of the TGF-β/BMP family, was greater in KO mouse PAFs than in wild type (WT) controls. In vivo, hypoxic KO mice had a lower right ventricular systolic pressure than WT controls. Right ventricular hypertrophy did not develop in the KO mice exposed to hypoxia, and hypoxia-driven vascular remodelling was prevented in the KO mice.

Conclusions: Downregulation of miR-155 conveys a protective effect against PH. Remodelling mechanisms are attenuated in vitro, and this translates to an in vivo effect. p38 MAPK has been shown by others to control the hypoxic proliferation of PAFs, so the reduced hypoxic phosphorylation of p38 MAPK in this study may partly explain these results. Manipulation of miR-155 in PH should be studied further to fully elucidate the mechanisms behind these findings.

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McGlinchey N, Bradley C, McSharry C, Peacock AJ, Welsh DJ. MicroRNA-155 controls pulmonary artery fibroblast cell signalling and proliferation in a knockout mouse model: A role in pulmonary arterial hypertension. Eur Resp J 2014; 44: Suppl 58, P2359

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McGlinchey N, Samillan V, Kurowska-Stolarska M, McSharry C, Nilsen M, MacLean M, Peacock AJ, Welsh DJ. Elucidating signalling pathways in pulmonary hypertension using a microRNA knockout mouse model – In vitro and in vivo data. Eur Resp J 2015; 46: Suppl 59, PA592

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Author's Declaration

I declare that this thesis has been written by myself and is the result of my own work, except where explicit reference is made to the contribution of others. It has not been submitted for any other higher degree at the University of Glasgow or any other institution.

Neil McGlinchey
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Definitions/Abbreviations

ALK-1	activin receptor-like kinase 1
ANOVA	analysis of variance
BMP	bone morphogenetic protein
BMPR2	BMP type 2 receptor
cAMP	cyclic adenine monophosphate
cGMP	cyclic guanine monophosphate
CH	chronic hypoxia
CHD	congenital heart disease
CHPAF	chronic hypoxic pulmonary artery fibroblast
COPD	chronic obstructive pulmonary disease
CO_2	carbon dioxide
CPM	counts per million
CTD	connective tissue disease
CTEPH	chronic thromboembolic pulmonary hypertension
DGCR8	DiGeorge syndrome critical region 8
DLBCL	diffuse large B-cell lymphoma
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus

EC	endothelial cell
ECL	enhanced chemiluminescence
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
ERK	extracellular regulated kinase
ERS	European Respiratory Society
ESC	European Society of Cardiology
ET-1	endothelin-1
ET_A	endothelin receptor type A
ET_B	endothelin receptor type B
FCS	fetal calf serum
HCl	hydrochloric acid
HHT	hereditary haemorrhagic telangiectasia
HIF	hypoxia inducible factor
HIV	human immunodeficiency virus
HPAH	heritable pulmonary arterial hypertension
HPV	hypoxic pulmonary vasoconstriction
HRP	horseradish peroxidase
IL-6	interleukin-6
ILD	interstitial lung disease
IPAH	idiopathic pulmonary arterial hypertension
IV	intravenous
JNK	c-Jun N-terminal kinase

KCNK3	potassium channel subfamily K member 3
KO	knockout
K_V	voltage gated potassium channel
LV	left ventricle
LV+S	left ventricle + septum
MAPK	mitogen-activated protein kinase
MCT	monocrotaline
MPAF	mouse pulmonary artery fibroblast
mPAP	mean pulmonary artery pressure
miR/miRNA	microRNA
mRNA	messenger ribonucleic acid
NaOH	sodium hydroxide
NFAT	nuclear factor of activated T-cells
NIH	National Institutes of Health
NO	nitric oxide
NOS	nitric oxide synthase
nt	nucleotide
O_2	oxygen
PAEC	pulmonary artery endothelial cell
PAF	pulmonary artery fibroblast
PAH	pulmonary arterial hypertension
PAP	pulmonary artery pressure
PASMC	pulmonary artery smooth muscle cell

PAWP	pulmonary artery wedge pressure
PBS	phosphate buffered saline
PBS/T	phosphate buffered saline/Tween
PDE	phosphodiesterase
PH	pulmonary hypertension
PPAR-γ	peroxisome proliferator-activated receptor gamma
PVR	pulmonary vascular resistance
RHC	right heart catheterisation
RIPA	radioimmunoprecipitation assay (buffer)
RISC	RNA-induced silencing complex
RPAF	rat pulmonary artery fibroblast
RV	right ventricle
RVH	right ventricular hypertrophy
RVSP	right ventricular systolic pressure
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SERT	serotonin transporter
SFM	serum-free media
sGC	soluble guanylate cyclase
SLE	systemic lupus erythematosus
SMC	smooth muscle cell
SPVU	Scottish Pulmonary Vascular Unit

STAT-3	signal transducer and activator of transcription 3
TAK-1	transforming growth factor-β activated kinase 1
TCA	trichloroacetic acid
TGF-β	transforming growth factor-β
TNF-α	tumour necrosis factor-α
TPH	tryptophan hydroxylase
UTR	untranslated region
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
VQ	ventilation/perfusion
WHO	World Health Organisation
WT	wild type

Chapter 1

Introduction

1.1 Pulmonary Hypertension

1.1.1 General Introduction

The focus of this thesis is a condition called pulmonary hypertension (PH). Rather than representing a single disease entity, this is a widely-encompassing term applied to a collection of diseases with the unifying feature of raised pulmonary artery pressure (PAP). This results in increased pulmonary vascular resistance (PVR) and subsequent right ventricular (RV) failure and premature death. Clinically, patients present with worsening breathlessness, peripheral oedema, chest pain and exertional syncope.

Much progress has been made in recent years with regards understanding pulmonary vascular biology and, in particular, with the development of disease-targeted treatments for pulmonary arterial hypertension (PAH). However there remains an unmet clinical need as demonstrated by an ongoing unacceptably high mortality rate. This chapter will briefly outline the definition, classification and treatment of PH, as well as explore the current understanding of the pathogenesis of the condition.

1.1.2 Definition of pulmonary hypertension

PH is defined as the presence at right heart catheterisation (RHC) of a persistent resting mean PAP of greater than 25mmHg [1]. However, this is simply a haemodynamic measurement rather than a diagnosis and gives no insight into the underlying aetiology of the condition. Further measurements made at RHC help to characterise the nature of PH. The presence of an elevated pulmonary artery wedge pressure (PAWP), i.e. >15mmHg, indicates post-capillary PH, which is due to left heart disease. PH with a normal PAWP is termed pre-capillary PH and describes all other classes of PH. This is outlined in table 1-1 [2]. PVR is also calculated from measurements taken at RHC. An elevation of PVR to greater than 3 Wood units is necessary for the diagnosis of PAH, and is a feature of other causes of PH which may also respond to disease-targeted therapy.

Definition	Characteristics	Clinical Group
Pulmonary hypertension (PH)	mPAP ≥ 25mmHg	All
Pre-capillary PH	mPAP ≥ 25mmHg PAWP ≤ 15mmHg	1. Pulmonary arterial hypertension 3. PH due to lung disease and/or hypoxia 4. Chronic thromboembolic pulmonary hypertension 5. PH with unclear multifactorial mechanisms
Post-capillary PH	mPAP ≥ 25mmHg PAWP > 15mmHg	2. PH due to left heart disease

Table 1-1 Haemodynamic definitions of pulmonary hypertension
Adapted from "2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension" by Galie, N. et al. Eur Heart J, 2016. **37**(1): p. 67-119. mPAP, mean pulmonary artery pressure; PAWP, pulmonary artery wedge pressure.

1.1.3 Classification and diagnosis of pulmonary hypertension

Following an outbreak of PH due to the appetite suppressant Aminorex in the 1970s, a forum of international experts was called by the World Health Organisation. Here, a simple classification and haemodynamic definition of PH was agreed upon. These forums have continued at regular intervals, with further refinements of the classification system based on haemodynamic and pathological characteristics, as well as management strategies. The most recent forum in Nice, France in 2013 produced a comprehensive overview of the clinical and pathophysiological facets of PH [3].

The clinical classification of PH, as agreed at the Nice symposium, is outlined in table 1-2 [4]. This divides PH into five discrete groups, of which groups 1, 4 and 5 (PAH, chronic thromboembolic PH, and miscellaneous, respectively) are the groups which are currently shown to derive benefit from specific PH-targeted therapies. These groups are the broad focus of this thesis, and in particular PAH.

Updated Classification of Pulmonary Hypertension
1. Pulmonary arterial hypertension 1.1 Idiopathic PAH 1.2 Heritable PAH 1.2.1 BMPR2 1.2.2 ALK-1, ENG, SMAD9, CAV1, KCNK3 1.2.3 Unknown 1.3 Drug and toxin induced 1.4 Associated with 1.4.1 Connective tissue disease 1.4.2 HIV infection 1.4.3 Portal hypertension 1.4.4 Congenital heart diseases 1.4.5 Schistosomiasis 1' Pulmonary veno-occlusive disease and/or pulmonary capillary haemangiomatosis 1'' Persistent pulmonary hypertension of the newborn (PPHN)
2. Pulmonary hypertension due to left heart disease 2.1 Left ventricular systolic dysfunction 2.2 Left ventricular diastolic dysfunction 2.3 Valvular disease 2.4 Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies
3. Pulmonary hypertension due to lung diseases and/or hypoxia 3.1 Chronic obstructive pulmonary disease 3.2 Interstitial lung disease 3.3 Other pulmonary diseases with mixed restrictive and obstructive pattern 3.4 Sleep-disordered breathing 3.5 Alveolar hypoventilation disorders 3.6 Chronic exposure to high altitude 3.7 Developmental lung disease
4. Chronic thromboembolic pulmonary hypertension (CTEPH)
5. Pulmonary hypertension with unclear multifactorial mechanisms 5.1 Haematologic disorders: chronic haemolytic anaemia, myeloproliferative disorders, splenectomy 5.2 Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis 5.3 Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders 5.4 Others: tumoural obstruction, fibrosing mediastinitis, chronic renal failure, segmental PH

Table 1-2 Clinical classification of pulmonary hypertension
Adapted from "Updated clinical classification of pulmonary hypertension" by Simonneau, G. et al. J Am Coll Cardiol, 2013. **62**(25 Suppl): p. D34-41.

In order to determine the exact aetiology of pulmonary hypertension, and therefore assess the appropriateness of disease-targeted therapy, patients should undergo thorough investigation by a specialist pulmonary vascular team. Unfortunately, a major hurdle to reaching this stage is the often subtle and non-specific nature of the symptoms reported by patients, which require a high index of clinical suspicion on the part of the physician undertaking the initial assessment. Often, the average time from symptom onset to diagnosis can be as long as 4 years [5]. To help improve this, an argument could be made that screening programmes are warranted for those at high risk, such as patients with connective tissue disease, congenital heart disease and HIV.

The evaluation of a patient with suspected pulmonary hypertension should aim to confirm the diagnosis of PH, as well as clarifying its aetiology and severity. As a screening tool, echocardiography adds weight to a clinical suspicion of PH, as well as evaluating the possibility of left heart disease. However, the gold standard test is right heart catheterisation, which confirms the haemodynamics that define PH. Further assessment should include computed tomography, pulmonary function testing, magnetic resonance imaging and ventilation/perfusion scintigraphy (VQ).

The process is laid out in the 2015 ESC/ERS guidelines [2] and summarised in figure 1-1.

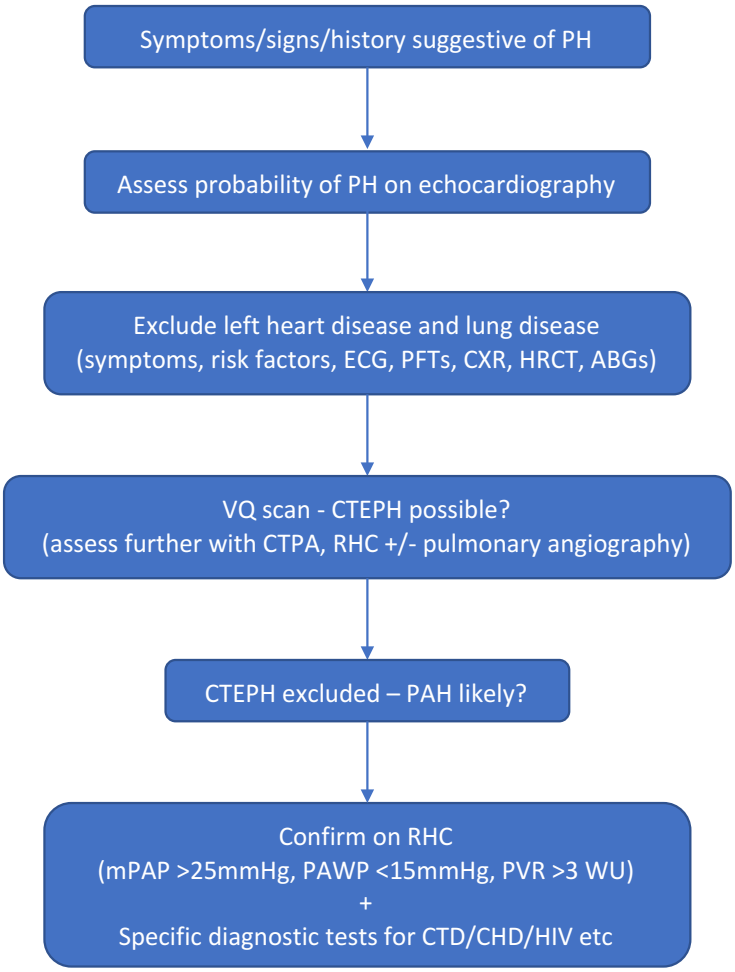


Figure 1-1 Simplified algorithm for the investigation of suspected pulmonary hypertension
Adapted from "2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension" by Galie, N. et al. Eur Heart J, 2016. **37**(1): p. 67-119. ECG, electrocardiograph; PFTs, pulmonary function tests; CXR, chest radiograph; HRCT, high resolution computed tomography; ABGs, arterial blood gases; CTPA, computed tomography pulmonary angiography; CTEPH, chronic thromboembolic PH; WU, Wood units; CTD, connective tissue disease; CHD, congenital heart disease; HIV, human immunodeficiency virus.

1.1.4 Epidemiology and prognosis of pulmonary arterial hypertension

PAH remains a rare disease. Recent indications are that incidence is in the range of 5-10 cases per million adults, with a prevalence of 15-60 per million [2]. Modern registries from several countries, including France, the United States and the UK, reflect these numbers [6-8]. These registries also indicate a female preponderance towards PAH, ranging from 65% to 80% of patients being female. Over time, there have been changes in the results collected by these registries [9]. The landmark NIH registry in the US, which collected data between 1981 and 1988, recorded the mean age at diagnosis as 36 years [10]. This has increased to

50-65 years in modern registries, reflecting the changing clinical profile of patients. Several possible explanations for this increasing age of patients at diagnosis have been suggested. These include increased physician and patient awareness and understanding of the disease; an increase in the frequency of non-invasive screening such as by echocardiography; and improvements in the clinical diagnosis of PAH, as it's possible that in the past patients with comorbidities such as lung disease or left heart disease may have been misclassified [11].

Importantly, and likely as a consequence of disease-targeted therapies, PAH patients are living longer. Median survival has increased from 2.8 years in the NIH registry [10] to 7 years in the REVEAL database [12], with survival at one year improved from 68% in the NIH registry to over 90% in the UK registry [8]. Despite these improvements, PAH remains a progressive disease. The consequences of the vascular remodelling that characterises the disease are an increase in pulmonary vascular resistance and increased work for the right ventricle, leading to RV hypertrophy. The RV ultimately dilates and fails, culminating in premature death [13]. Current treatments can delay this, mostly by ameliorating the pulmonary vasoconstriction that accompanies the process. However, treatments which prevent or reverse remodelling are desperately needed.

1.1.5 Current treatments for pulmonary arterial hypertension

Over the last 20 years, the introduction of disease-targeted drugs has revolutionised PAH treatment. These primarily act as pulmonary vasodilators through smooth muscle relaxation and hence reduction of right ventricular overload. Their effect on pulmonary vascular remodelling, the key underlying pathological process, is minimal. Unfortunately, this means that the condition will still progress, albeit at a reduced rate, ultimately resulting in premature death.

The currently available targeted therapies exert their vasodilatory effect on the pulmonary circulation via three distinct pathways. These pathways, and the drug classes which act on them, are the nitric oxide pathway (phosphodiesterase-5 inhibitors and soluble guanylate cyclase stimulators); the endothelin pathway (endothelin receptor antagonists); and the prostacyclin pathway (prostanoids). Epoprostenol, a short-acting pulmonary vasodilator delivered as a continuous IV

infusion, falls into this last group. It was the first agent used to directly treat PAH and was shown to improve exercise capacity and survival [14]. Whilst multiple clinical trials have shown the functional and haemodynamic improvement with other PAH-specific drugs since epoprostenol, the only other drug to show a clear survival benefit is the endothelin receptor antagonist macitentan [15].

Response to treatment should be regularly assessed by improvement in symptoms and WHO functional class, as well as exercise capacity, typically using the six-minute walk test. Combination therapy with drugs from two or more classes can be used when initial improvement is inadequate or, increasingly, from the point of diagnosis in those with severe disease. Supportive medications such as diuretics, oxygen, anticoagulants and digoxin also remain important in the general management of PAH patients. If response to treatment remains poor despite optimal combination therapy and supportive measures, then lung transplantation should be considered.

This section highlights the need for improved disease-specific treatments for PAH. Drugs which target the underlying pulmonary vascular remodelling process are the next step in advancing the management for these patients, and understanding the pathobiology of PAH is key to this.

1.2 Pathobiology of pulmonary hypertension

1.2.1 The normal pulmonary circulation

The pulmonary circulation is a low pressure, high flow circuit with thin-walled compliant vessels, and a relatively under-perfused vascular bed in normal conditions at rest. These features allow it to cope with significant changes in volume and flow, such as with exercise-induced increases in cardiac output, without a dramatic increase in pressure, which the thin-walled right ventricle cannot generate.

Between the main pulmonary artery and the capillary bed there are 15 divisions of the pulmonary arterial system [16]. Whilst there are distinct differences in

these vessels as the network progresses from proximal to distal, the vessel wall largely has the same structure. There are three distinct cellular layers in the wall of a normal pulmonary artery. Working from the outside of the vessel in, the first of these is the adventitia. This layer consists of fibroblasts and extracellular matrix, along with the vasa vasorum which are blood vessels arising from the systemic bronchial circulation. The middle layer, or media, largely comprises of smooth muscle cells. The intima is the innermost layer and is made up of endothelial cells. Separating these layers are external and internal elastic laminae, respectively.

The proximal vessels in the pulmonary arterial tree (divisions 15-13) have a medial smooth muscle layer that is generally thinner than that found in the medium size vessels (divisions 12-4). In contrast, the smaller vessels and arterioles, especially those with a diameter less than 100μm, usually lack a smooth muscle layer [17]. These smaller vessels are contiguous with the pulmonary capillary network which forms the interface with the alveolar network. It is here where the main function of the pulmonary circulation occurs, namely gas exchange.

1.2.2 The pulmonary circulation in pulmonary hypertension

The pathobiology of pulmonary hypertension is complex and multifaceted. The key pathological processes are vasoconstriction, pulmonary vascular remodelling, loss of smaller distal pulmonary vessels (vascular pruning) and vascular thrombosis in situ [18]. These processes occur to a varying extent in all forms of pulmonary hypertension, depending on the exact aetiology. For example, in chronic thromboembolic pulmonary hypertension (CTEPH), extensive thrombosis, both in proximal and distal vessels, is a core component of the condition's pathogenesis. In contrast, thrombosis in PAH tends to be present inconsistently and in smaller arterioles [19].

Vasoconstriction undoubtedly plays a part in the development of PH. This may be driven by factors such as hypoxia or intracellular calcium levels. Clinically, vasoconstriction is thought to be a principal feature in a distinct subset (<10%) of idiopathic PAH patients who demonstrate a vasoreactive phenotype. This is assessed at right heart catheterisation by way of acute pulmonary vasodilator

testing, for example with inhaled nitric oxide. Patients in this subset show a significant response to vasoreactivity testing, often with near-normalisation of PAP and PVR, and benefit from calcium channel blockers. [20].

Various vasoactive mediators have also been identified and the majority of current treatments target these pathways. However, the fact that these treatments largely do not improve survival suggests that vasoconstriction is not the main factor in the development of PAH in the majority of cases. There is often significant overlap between vasoconstriction and vascular remodelling, with many molecular mechanisms and signalling pathways involved in both processes. These are explored later in this chapter.

Overall, it is now clear that pulmonary vascular remodelling is the dominant pathological factor in severe pulmonary hypertension. Characterised by cellular hypertrophy, proliferation and migration, remodelling causes restriction of blood flow and hence an increase in pulmonary vascular resistance and increased RV afterload. After initial adaptation to these conditions through processes including RV hypertrophy, the right heart ultimately decompensates and fails, resulting in death.

The exact triggers of these processes are unknown, but it is a widely held consensus that PAH is caused by a combination of environmental, inflammatory and genetic factors which interact in a “multiple-hit” mechanism [21].

1.2.3 Pulmonary vascular remodelling: key cellular and signalling mechanisms

In health, the structure of the pulmonary arterial wall is kept constant by a balance between apoptosis and proliferation of adventitial fibroblasts, smooth muscle cells in the medial layer, and endothelial cells. In PAH, this balance becomes disrupted leading to adventitial and intimal thickening and muscularisation of smaller distal vessels which were previously non-muscularised. These processes are the main drivers of pulmonary vascular remodelling, a hallmark feature of PAH [18]. The result of this is luminal narrowing, and this in turn leads to elevated pulmonary vascular resistance.

Pulmonary vascular remodelling is a complex process and our understanding of it remains incomplete. All layers and cell types of the vessel wall are involved, and a range of molecular signalling pathways are involved. These will now be individually considered.

1.2.3.1 Pulmonary artery fibroblasts

Pulmonary artery fibroblasts (PAFs) are the most abundant cellular component of the adventitia. They play an important role in providing mechanical support to the vessel wall by producing extracellular matrix (ECM). However, evidence has emerged over recent years to show that their role is much more active than just forming a supporting framework. They are important for both the initiation and propagation of pulmonary vascular remodelling. In hypoxic animal models of PH, there is evidence of early adventitial remodelling and increased fibroblast proliferation [22]. There is also evidence from animal models that the most significant proliferative response to hypoxia of all vessel wall cell types is that of the adventitial fibroblast [23].

Activation of fibroblasts by numerous stimuli leads to their differentiation into myofibroblasts. Amongst these simulants are growth factors such as TGF-β, which stimulates the expression of α-smooth muscle actin and collagen production [24, 25] facilitating the transformation towards a myofibroblast phenotype. Inflammatory cytokines including IL-6 also promotes this transition [26], as does hypoxia [27, 28]. These myofibroblasts demonstrate contractile functions and have the ability to migrate through the vessel wall, contributing to the distal muscularisation of previously non-muscularised vessels [29-31].

Fibroblasts are the principle regulators of the adventitial extracellular matrix. The production of ECM increases substantially in response to injurious stimuli leading to an accumulation of proteins including collagen, elastin, osteopontin and fibronectin. This results in vascular remodelling, with vessel stiffening and decreased compliance [32, 33]. Activated fibroblasts have also been shown to release growth factors and reactive oxygen species that have paracrine effects on

nearby smooth muscle cells and endothelial cells, further stimulating proliferation and propagating vascular remodelling [34].

In summary, PAFs show an early proliferative response to hypoxia; their transformation into myofibroblasts causes vessel muscularisation; they cause an increase in ECM deposition; and they stimulate the proliferation of other vessel wall cell types. These features make PAFs key players in pulmonary vascular remodelling.

1.2.3.2 Pulmonary artery smooth muscle cells

The medial compartment of the pulmonary vascular wall responds to hypoxia, inflammatory cytokines and other stimuli to cause further structural change of the vessel. Pulmonary artery smooth muscle cells (PASMCs) proliferate, hypertrophy, migrate and produce matrix proteins in response to these stimuli, causing medial hypertrophy and the muscularisation of small arteries/arterioles [35]. In remodelling, imbalance between proliferation and apoptosis of PASMCs results in medial thickening. This is supported by evidence from human PAH, with increased resistance of PASMCs to apoptotic stimuli such as bone morphogenetic protein (BMP)-2 and -7, in comparison to normal PASMCs [36].

In PASMCs, cytosolic Ca^{2+} levels are important determinants of cellular proliferation, migration and contraction. Elevated Ca^{2+} levels result in the activation of various transcription factors, such as nuclear factor of activated T-cells (NFAT), which promotes PASMC proliferation [37, 38]. Contraction of smooth muscle is an important component of pulmonary vasoconstriction. This is also driven by elevated Ca^{2+} levels, ultimately initiating the interaction of myosin filaments with actin filaments in a process known as crossbridge cycling.

The influx of Ca^{2+} into PASMCs can be controlled by voltage-gated calcium channels and non-selective cation channels such as transient receptor potential channels (TRPC). In patients with PAH, there is increased expression of TRPC proteins, which suggest one way in which cellular calcium homeostasis may be upset in PAH [39]. The homeostasis of intracellular potassium also has a knock-on effect on calcium levels, as K^{+} ions control cell membrane depolarisation, which activates

voltage-gated calcium channels. In PAH, the down-regulation of voltage-sensitive potassium channels (Kv) in PASMCs results in an increase of intracellular K^+ ions and therefore depolarisation of the cell membrane [40].

1.2.3.3 Pulmonary artery endothelial cells

The intimal layer of pulmonary arteries is predominantly formed by pulmonary artery endothelial cells (PAECs). The function of the normal endothelium is to act as a semipermeable membrane and regulate vascular tone, cellular proliferation and coagulation. Given its constant contact with blood, the endothelium is the component of the vessel wall that is most readily exposed to damage from shear stress, toxins and inflammation, and therefore is primely positioned to play a key role in the early stages of remodelling. Increased PAEC proliferation along with increased collagen deposition and inward migration of myofibroblasts contribute to neointimal thickening and hence pulmonary vascular remodelling [41].

Another important feature of vessel remodelling is the formation of plexiform lesions, which in part are due to disorganised proliferation and apoptosis of PAECs [42]. Most commonly seen in IPAH and less commonly in other forms of PAH, these lesions contain PAECs that are monoclonal [43]. This implies a semi-malignant process, a theory which is further supported by the decreased expression in plexiform lesions of peroxisome proliferator-activated receptor-γ (PPAR-γ), which has anti-proliferative and pro-apoptotic functions and is speculated to function as a tumour suppressor gene [44]. PPAR-γ has also previously been shown to convey a protective effect in experimental PH [45].

Endothelial dysfunction further propagates the pathogenesis of PH through dysregulation of various vasoactive mediators. These include endothelin-1 and thromboxane, which are pro-proliferative and induce vasoconstriction, and nitric oxide and prostacyclin, which are vasodilators and anti-proliferative.

1.2.3.4 Endothelin-1

Endothelin-1 (ET-1) is primarily a potent vasoconstrictor and pro-proliferative peptide which, in the lungs, is principally released by endothelial cells [46].

Elevated levels of ET-1 have been found in both patients with PAH and animal models [47, 48] and endothelin receptor antagonism in an animal model of chronic hypoxic PH demonstrated a protective effect [49]. This benefit has successfully been translated to humans, and endothelin receptor antagonists (ETRAs) are now an important treatment modality for patients, leading to improvements in quality of life and exercise tolerance.

ET-1 exerts its effects via the G-protein coupled receptors ET_A and ET_B. These receptors vary not only in their distribution within the pulmonary vasculature, but also the effects they mediate. The detrimental effects of ET-1, such as vasoconstriction and cell proliferation are primarily as a result of its action on ET_A receptors, which are found on both PASMCs and PAFs [50, 51].

The role of ET_B receptors however is more complex. They are principally found on endothelial cells, but to a lesser extent on PASMCs and PAFs. ET-1 acts on endothelial cells via the ET_B receptor to stimulate the release of the vasodilators nitric oxide and prostacyclin [51, 52]. ET_B receptors also contribute to the clearance of ET-1, likely due to the internalisation of the ET-1/ET_B receptor complex intracellularly after binding [53]. Conversely, the binding of ET-1 on ET_B receptors located on smooth muscle cells appears to result in pulmonary vasoconstriction [54]. It is also becoming clear that the roles of the ET_A and ET_B receptors is different in disease, compared with healthy conditions. For example, the vasoconstrictive role of ET_B receptors may be more pronounced in PH [55]. This may be as a result of the upregulation of ET_B receptors, which has been identified in the distal pulmonary vessels of PAH patients [50]. It is this complexity of the role of the ET_B receptor which has led to debate about whether ETRAs which are selective for the ET_A receptor, or those which target both ET_A and ET_B, confer the greater therapeutic benefit.

1.2.3.5 Nitric oxide

In the pulmonary circulation, nitric oxide (NO) is derived primarily from endothelial cells through the actions of endothelial nitric oxide synthase (eNOS) on L-Arginine [56]. It exerts its effects on adjacent PASMCs in a paracrine fashion via activation of soluble guanylate cyclase (sGC) and a subsequent increase of the

secondary messenger cyclic guanosine monophosphate (cGMP). Ultimately this results in relaxation of smooth muscle cells and vasodilation. NO also has an inhibitory effect on the proliferation and migration of PASMCs. In the pulmonary circulation, the action of phosphodiesterase type 5 (PDE5) depletes cGMP, terminating the effect of NO.

The importance of the NO system to the pulmonary vasculature has been demonstrated in both humans and animals. Genetic knockout of eNOS in mice results in reduced cGMP and causes PH. This is partially ameliorated by restoration of the gene through adenoviral transfection [57] [58]. The administration of specific NOS antagonists to healthy humans results in an elevated pulmonary vascular resistance [59], whilst the pulmonary vessels from the explanted lungs of patients with IPAH show reduced eNOS levels [60]. The use of inhaled NO as a part of the diagnostic process to assess pulmonary vasoreactivity in humans has already been discussed, and in intensive care units NO can be used for therapeutic benefit.

The clinical translation of these findings has led to the manipulation of the NO pathway as a treatment for PH. This is most well established with the PDE5 inhibitors including sildenafil and tadalafil. However, stimulators of sGC are now used in clinical practice. Riociguat, the first drug in this family, is effective in both PAH [61] and CTEPH [62].

1.2.3.6 Prostacyclin and Thromboxane A_2

Prostacyclin (PGI_2) is a metabolite of arachidonic acid, produced by PAECs through the action of cyclooxygenase and subsequently prostacyclin synthase. It acts via the G-coupled IP receptor, causing an increase in cyclic adenosine monophosphate (cAMP), the consequences of which are vasodilation, decreased platelet aggregation and inhibited proliferation of smooth muscle cells [63]. In animal models, administration of prostanoids has led to reversal of markers of vascular remodelling [64] [65]. Thromboxane A_2 is another metabolite of arachidonic acid with effects that are antagonistic to prostacyclin, inducing vasoconstriction and platelet aggregation. Imbalance between these two compounds contributes to endothelial dysfunction and, in human PAH, PAECs have been shown to have decreased levels of prostacyclin synthase and increased levels of thromboxane A_2

[66]. These observations led to the development of prostacyclin analogues for the treatment of PAH, with intravenous epoprostenol still considered the gold standard therapy [2].

1.2.4 Pulmonary vascular remodelling: other important factors

1.2.4.1 TGF-β/BMP signalling

The transforming growth factor beta (TGF-β) superfamily is a complex signal transduction pathway, components of which play an important role as regulators of cardiovascular development and disease, including PAH [67]. The family is composed of more than 60 members, including the TGF-β ligands themselves, activins and bone morphogenetic proteins (BMPs) [68]. All the ligands in this superfamily exert their effect through a similar signalling cascade. This involves the activation of type I and type II receptors, followed sequentially by receptor-specific cytoplasmic transcription factors, known as Smads, which then translocate to the nucleus and modulate gene function.

Among the type I receptors are TGF-βR1, activin receptor-like kinase 1 (ALK-1, also known as ACVRL-1), BMPR1a and BMPR1b. Type II receptors include TGF-βR1 and BMPR2. Both receptor types are trans-membrane serine/threonine kinases, which exist in their resting state in pairs i.e. homodimers. When the specific ligand binds, a heterotetrameric receptor complex forms. This comprises of two homodimers, one each of type I and type II receptors. The type II receptors are constitutively active so, when the ligand binds, these then phosphorylate the inactive type I receptors. This results in activation of the cytoplasmic Smads.

Three Smad sub-groups exit: the receptor-regulated Smads (R-Smads including Smad1, Smad2, Smad3, Smad5 and Smad8); the common mediator Smad (Co-Smad i.e. Smad4) and inhibitory Smads (I-Smads, including Smad6 and Smad7) [68-70]. Once the type I receptor has been activated, exactly which R-Smads it recruits and phosphorylates depends on which ligand has bound to which combination of receptors. It should be noted that the same ligand can induce differing signalling pathways and hence responses, depending on exactly which receptors comprise the activated complex.

It is recognised that TGF-βR1 recruits Smad2/3 through the action of a TGF-β ligand. However, TGF-β ligands can also act via ALK-1 to activate Smad1/5/8. The BMP ligands, acting through the BMPR1a or BMPR1b, will also recruit Smad1/5/8. Once the R-Smads have been phosphorylated they dissociate from the receptor complex and translocate to the nucleus, facilitated by their interaction with the co-Smad, Smad4. One of the main targets of TGF-β/BMP signalling are the inhibitor of differentiation (Id) proteins. Once transcription factors bind to these proteins they are prevented from binding to DNA, preventing cellular differentiation and promoting proliferation.

The TGF-β/BMP signalling pathway is illustrated in figure 1-2.

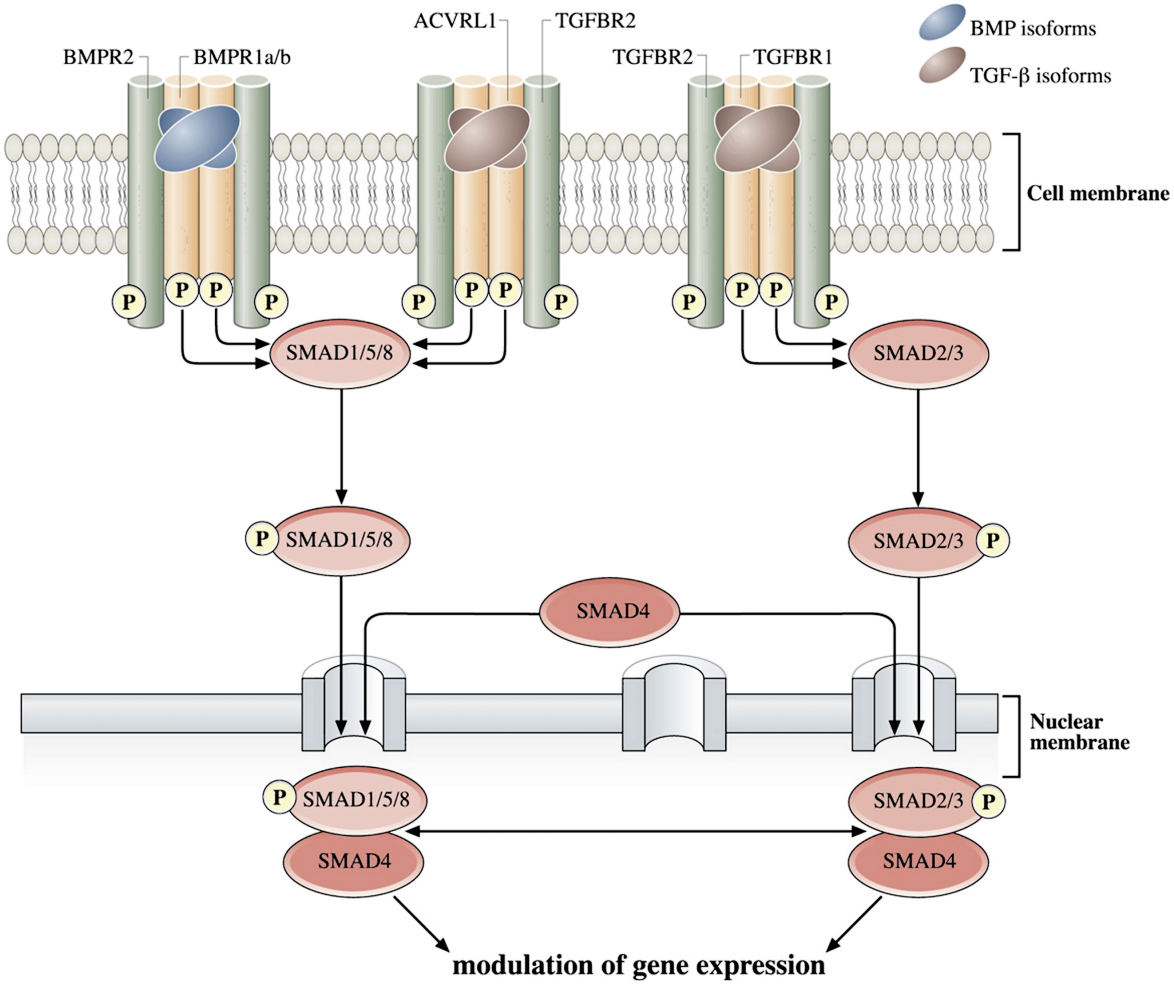


Figure 1-2 The TGF-β/BMP signalling pathway
Ligands from the TGF-β superfamily bind to the heterotetrameric receptor complex in the cell membrane, resulting in the recruitment of R-Smads. Facilitated by Smad4, R-Smads then translocate to the nucleus where they regulate gene expression. Ⓟ indicates phosphorylation. Replicated with permission from "Transforming Growth Factor β/Bone Morphogenic Protein Signaling in Pulmonary Arterial Hypertension: Remodeling Revisited" by Eickelberg, O. and Morty, R. E. *Trends in Cardiovascular Medicine*, 2007, 17(8): p263-9.

TGF-β/BMP signalling has been shown to regulate PASMC and PAEC proliferation, differentiation and migration. As explained above, the processes are complex and depend on which combination of receptors are activated. Activation of TGF-βR1 by TGF-β inhibits PAEC proliferation and migration via the phosphorylation of Smad2/3. However, when TGF-β activates ALK-1, PAEC proliferation and migration is promoted via the phosphorylation of Smad1/5/8 [71, 72].

BMPs have also been shown to signal in a Smad-independent manner. When Smad signalling is supressed, such as through a mutation in the BMPR2 receptor, BMP ligands can signal via mitogen-activated protein kinase (MAPK) pathways. Specifically, this is through activation of TGF-β activated kinase 1 (TAK1), a member of the MAPKKK family. Ordinarily TAK1 is bound to BMPR2, but this relationship is reduced in the presence of a BMPR2 mutation, freeing TAK1 and making it accessible for BMP signalling. TAK1 activation by BMPs leads to the phosphorylation of p38 MAPK which, in PASMCs with a BMPR mutation, results in increased cellular proliferation. This suggests an important interaction between the BMP and MAPK signalling pathways [73, 74].

1.2.4.2 Genetic factors

Reports of families with PH, which date back to at least 1954, indicate a clear underlying genetic susceptibility to the disease [75, 76]. The most common genetic mutations in PAH are those within the gene encoding the bone morphogenetic protein receptor 2 (BMPR2), a key component of the TGF-β/BMP signalling family. Over 300 such mutations have been identified to date, and these are found in approximately 75% of cases of heritable PAH (HPAH) and 25% of apparently idiopathic PAH cases [77]. BMPR2 mutations are inherited in an autosomal dominant fashion, however there is incomplete penetrance of approximately 20% [78], meaning that up to 80% of carrier will not be affected, and lending some weight to the “multiple-hit” hypothesis of causation of PAH. The development of HPAH, like IPAH, has a marked female predominance and exhibits genetic anticipation, suggesting that younger females are at increased risk of developing HPAH.

Animal models have been developed in an effort to emulate PH caused by BMPR2 mutations. A knockout (KO) mouse model that is heterozygous for BMPR2 displays decreased signalling through Smad1/5/8 in PASMCs [79]. Phenotypically, it exhibits only mild PH with distal vascular remodelling but without RV hypertrophy. A second stimulus, such as hypoxia, is necessary for significant PH to develop.

Alternative pathogenic mutations arising from the TGFβ/BMP family are also linked to the development of HPAH. These are especially prominent in individuals affected by hereditary haemorrhagic telangiectasia (HHT), with the most well documented mutations affecting the activin receptor-like kinase-1 (ALK-1) gene and the endoglin gene [80, 81].
Recently, a novel mutation in the gene encoding potassium channel subfamily K member 3 (KCNK3) was identified as responsible for PAH in members of the same family in which a previously known PAH-causing mutation could not be identified [82]. This mutation results in loss of function of the potassium channel, although this could be remedied by pharmacological manipulation.

1.2.4.3 The role of p38 MAPK

Mitogen-activated protein kinases (MAPKs) constitute a pivotal signalling pathway that allows cells to process a diverse variety of external stimuli and orchestrate an appropriate response. They regulate an array of cell functions including differentiation, proliferation and apoptosis [83]. Once an external stimulus activates a cell membrane receptor complex, a signalling cascade is triggered by sequential phosphorylation. This cascade consists of at least three core components (MAP3K, MAPKK and MAPK) and ultimately exerts its effect through phosphorylation of cytosolic substrate proteins or by activating nuclear transcription factors [84].

Among the distinct subgroups of MAPKs are the extracellular signal-regulated kinases (ERK) family; the c-Jun N-terminal kinases (JNK) family and the p38 MAPK family. The p38 family (of which there are 4 isomers - α, β, γ, δ) is of particular interest as it has been shown to be activated by hypoxia and specific inflammatory cytokines and growth factors that are important in PH [85].

Our laboratory has an ongoing core interest in the interactions between hypoxia, p38 MAPK and pulmonary artery fibroblasts, and the contribution of these interactions to the pathogenesis of PH. Among the notable findings, from our group and others, which link these three factors are:

- Exposure to acute hypoxia results in increased cellular proliferation of normal rat, human and bovine PAFs [86, 87].
- PAFs from chronic hypoxic animals (CHPAFs) which are cultured in normoxia are constitutively hyperproliferative compared to normal PAFs, suggesting a phenotypic switch [88-90].
- p38 MAPK plays an essential role in the proliferation of PAFs in acute hypoxia, and is constitutively upregulated in CHPAFs. Inhibition of p38MAPK can prevent or reverse the hyperproliferative effect in both acute and chronic hypoxia [89-91]
- The effects of acute hypoxia on fibroblasts and the p38 MAPK pathway are specific to the pulmonary circulation, with no effect seen in systemic fibroblasts [92].
- Hypoxia-induced upregulation of p38 MAPK causes endothelial dysfunction and decreased nitric oxide, which can be reversed by inhibition of p38 MAPK [93].
- p38 MAPK is important in BMPR2 mutations. PASMCs with BMPR2 mutations have ineffective Smad signalling, and p38 MAPK signalling becomes active [74].
- Only the α and γ isoforms of p38 MAPK are phosphorylated in hypoxia-exposed PAFs [94].
- Antagonism of the α isoform of p38 MAPK with a novel clinically available drug reverses PH in the chronic hypoxic and monocrotaline animal models. Given the presence of elevated levels of phosphorylated p38 MAPK and p38 MAPKα in pulmonary vessels from IPAH patients, this novel drug has potential as a therapeutic agent [95].

When considered together, these observations provide cohesive evidence that that p38 MAPK is an important signalling mechanism in the development of pulmonary vascular remodelling. It also provides a route through which further exploration of the pathobiology of PH can be explored.

1.2.4.4 Inflammation and pulmonary hypertension

Evidence for the role of inflammatory processes in the initiation and propagation of PH and pulmonary vascular remodelling is growing continually. A clear connection between inflammatory conditions/autoimmunity and PAH exists in the form of connective tissue disease associated PAH. Conditions such as systemic sclerosis and systemic lupus erythematosus (SLE) are established causes of PAH and as well as responding to PAH-targeted therapy, there is a role for immunosuppression in SLE-PAH [96, 97]. An early link between inflammation and plexiform lesion was identified [42], and more recently, increased levels of inflammatory cells, such as macrophages, mast cells, T cells and B cells, in the adventitia of IPAH patients lends further evidence [98].

Cytokines such as IL-6 are produced by a number of immune cells. Elevated levels of cytokines, including IL-6, are found in the circulation of IPAH, familial PAH and systemic sclerosis patients and have been noted to correlate with survival [99, 100]. Serum IL-6 levels also correlate to the severity of PH in patients with chronic obstructive pulmonary disease (COPD) [101]. It is also implicated in animal models of PH, with evidence of increased migration of PASMCs, suggesting a contribution to pulmonary vascular remodelling [102, 103]. Overall, the role of inflammation in PAH clearly warrants further investigation.

1.2.4.5 Hypoxia: vasoconstriction and remodelling

Hypoxia is the condition of insufficient oxygen supply to maintain normal function of cells, tissues or organisms. It is one of the most common aetiologies of pulmonary hypertension. How the systemic and pulmonary circulations respond to acute hypoxia differs. In the systemic circulation, hypoxia results in vasodilation and an increase of oxygen supply to tissues. In the pulmonary circulation however, it results in hypoxic pulmonary vasoconstriction (HPV). This can be considered an adaptive response, to shunt blood away from inadequately ventilated areas of the lung and hence improve ventilation-perfusion (VQ) mismatch. However, it is also one of the key pathological processes in PH.

Several possible mechanisms are postulated to be possible for HPV. Increased intracellular calcium concentrations in PASMCs is partly caused by influx through voltage gated calcium channels and non-specific cationic channels [39]. This promotes cellular contraction and vasoconstriction. Simultaneously, hypoxia results in altered activity of potassium channels, increasing membrane depolarisation resulting in further cell contraction [40]. The paracrine function of ET-1 released from neighbouring PAECs, along with reduced eNOS activity, can further promote the vasoconstriction, and this his has been discussed earlier in this chapter.

Whilst HPV is a typical response to acute hypoxia, the pulmonary circulation undergoes remodelling when hypoxia is chronic [104]. The effect of chronic hypoxia on individual cell types in the pulmonary vessels wall has been considered previously. Many of the effects of chronic hypoxia are mediated through hypoxia inducible factor 1 (HIF-1), a ubiquitous regulator of the cellular response to hypoxia. HIF-1 is a heterodimeric protein composed of individual α and β components. Under normal oxygen concentrations HIF-1α is degraded, however this degradation is reduced in hypoxia with stabilisation of HIF-1α [105]. HIF-1α subsequently translocates to the nucleus, regulating the transcription of various genes that are influential in PH such as ET-1, calcium channels and VEGF [106]. The importance of HIF-1 is further highlighted by the finding that transgenic mice heterozygous for HIF-1α, whilst phenotypically normal under baseline conditions, are protected against hypoxia-induced PH [107, 108]. Hypoxia is undoubtedly an important factor in PH, both due to acute HPV and chronic hypoxic remodelling.

1.2.4.6 Serotonin

Interest in the “serotonin hypothesis of pulmonary hypertension” arose with the outbreak of anorexigen-associated PAH. These agents, such as Aminorex, are substrates for the serotonin transporter (SERT), causing increased extracellular serotonin [109]. The conversion of L-trytophan to 5-hydroxy-L-tyrptophan through the action of tryptophan hydroxylase (TPH) is an early step in the synthesis of serotonin (5-HT) [110]. Increased expression of pulmonary arterial endothelial TPH-1 has been observed in PAH patients and serotonin derived from the endothelium can act on neighbouring PASMCs in a paracrine fashion [111]. In vitro,

serotonin activates serotonin receptors on human PASMCs to induce cellular proliferation and migration [112]. The incubation of rat PAFs with serotonin stimulates cell proliferation and migration, as well as causing increased expression of α-SMA, a marker of cellular differentiation into myofibroblasts [113].

The complex interaction between serotonin, gender and female sex hormones has been implicated in the pathogenesis of PAH. The overexpression of SERT in male mice has no effect on indicators of PH such as RV systolic pressure or pulmonary vascular remodelling. Female mice overexpressing SERT, however, do develop PH which becomes even more marked following exposure to chronic hypoxia [114]. Ovariectomy leads to attenuation of PH in the SERT+ mice, however the PH phenotype is re-established by administration of 17β oestradiol to both the hypoxic and normoxic SERT+ve mice. The increased prevalence of PAH in females may in part be explained by the serotonin hypothesis.

1.3 Animal models of pulmonary hypertension

Understanding the pathobiology of PAH is vital to the development of new therapies for the disease. Animal models are important tools in elucidating the exact molecular mechanisms involved, and although no perfect model exists which perfectly replicates human disease, there are a number of well-established and newer models. The chronic hypoxic and monocrotaline models are the most extensively studied, however they lack the complex plexiform arteriopathy seen in severe human disease. Modifications of these classic models often incorporates a second “hit”, therefore theoretically more closely reflecting the pathogenesis and histological findings seen in PAH. By developing models which more closely replicate the human disease, research should lead to the discovery of more potential treatments which are not just successful in preclinical trials, but which also translate to benefits in humans.

1.3.1 Chronic hypoxia model

Humans and animals that reside at high altitude develop elevated PA pressures and RV hypertrophy [115, 116]. In addition, chronic hypoxia leads to the PH

associated with various conditions including COPD, interstitial lung disease and sleep apnoea (Group 3 in the PH classification). Acute and chronic hypoxia influence the development of PH as outlined above, and the relative ease with which hypoxic conditions can be reproduced makes it an appealing model to study. Rodents exposed to hypobaric hypoxia are most commonly studied in PAH. This technique maintains the oxygen fraction in the air, but reduces atmospheric pressure, replicating conditions at high altitude. Rats exposed to hypobaric hypoxia for 2 weeks develop elevated PA pressures, RV hypertrophy, muscularised and thickened distal vessels, and vascular pruning relatively quickly [117]. However, the response varies across species and strains. The hypoxic mouse model for example develops haemodynamic changes but remodelling is much less marked [118, 119]. Plexiform lesions are not seen in this model, and removal of the hypoxic stimulus causes disease reversal, suggesting it is a less robust model.

1.3.2 Monocrotaline rat model

Monocrotaline (MCT) is a pyrrolizidine alkaloid, derived from the seeds of the *Crotalaria spectabilis* plant. It is most commonly utilised in rats, and is administered as a single subcutaneous or intraperitoneal injection in a pro-drug form, which is then metabolised to its active form by cytochrome P450 CYP3A in the liver [120]. Pulmonary hypertension and pulmonary vascular remodelling typically develop over 1 to 2 weeks and progressively worsen [121]. Although not fully understood, monocrotaline is thought to produce its effect through direct toxicity of its metabolites on the lung [122]. There is activation of proliferative and anti-apoptotic factors and along with dysregulated NO signalling [123, 124], arising from damage to the endothelial cell membrane and loss of essential membrane proteins including caveolin-1 [125]. Once again, plexiform lesions are not seen [126]. Death frequently occurs after treatment with monocrotaline, however this may be due to hepatic damage or other toxic effects rather than PH [127].

1.3.3 SU5416/Hypoxia model

This model was developed in an attempt to more realistically recapitulate human PAH, and reinforces the “multiple-hit” hypothesis in disease development. It

involves the administration of the VEGF receptor-2 inhibitor SU5416 ("Sugen") to rats or mice, which are then exposed to chronic hypoxia. Vascular endothelial growth factor (VEGF) is involved in the regulation of endothelial cell function and maintenance [128], and administration of the VEGFR-2 inhibitor combined with chronic hypoxia produces a severe form of PH, with extensive remodelling and, importantly, the development of plexiform lesions [129, 130]. This model, and others which more closely resemble the complex remodelling processes seen in humans, offer promising new tools for elucidating the mechanisms behind PAH.

1.4 MicroRNAs

1.4.1 Introduction

MicroRNAs (miRNAs, miRs) are small, single strands of non-coding RNA, consisting of 22-25 nucleotide bases, which act as post-transcriptional regulators of gene expression. They bind to the 3' untranslated region (UTR) of complementary target mRNA, resulting in translational repression or causing degradation of the mRNA [131, 132]. The first miRNA, named lin-4, was identified in 1993 in the nematode *Caenorhabditis elegans* [133, 134]. It was the discovery of a second miRNA in 2000, and the realisation that it was conserved across many species, that triggered the subsequent exponential growth in miRNA research [135, 136]. According to version 21 of the online database miRBase (released June 2014), microRNAs have been recognised in over 200 species so far, with more than 2500 mature miRNAs identified in humans, and many more predicted. MicroRNA-mRNA interactions are complex. A single miRNA is capable of regulating hundreds of genes and conversely, a single gene can be regulated by multiple miRNAs [137]. As a result of this, miRNAs can help with understanding the disease aetiology and signalling pathways, even if they are not utilised directly as therapeutic agents.

1.4.2 MicroRNA biogenesis

The biogenesis of miRNAs involves multiple steps, which are illustrated in figure 1-3. The process begins in the nucleus with the transcription of the miRNA gene by RNA polymerase II, creating the primary miRNA (pri-miRNA) [138]. This primary

transcript can be over 1000 bases long and will often include the sequences for multiple mature miRNAs (polycistronic) [131, 139]. Cleavage of the pri-miRNA then occurs to form the pre-miRNA, a stem loop molecule ~70 nucleotides long. This step is controlled by the action of Drosha, a nuclear RNase III enzyme, which forms a complex with the DiGeorge syndrome critical region gene 8 (DGCR8), a double-stranded RNA binding protein [140, 141]. After processing by Drosha and DGCR8, the pre-miRNA binds to Exportin 5, a nucleo-cytoplasmic transport factor, which facilitates its export to the cytoplasm.

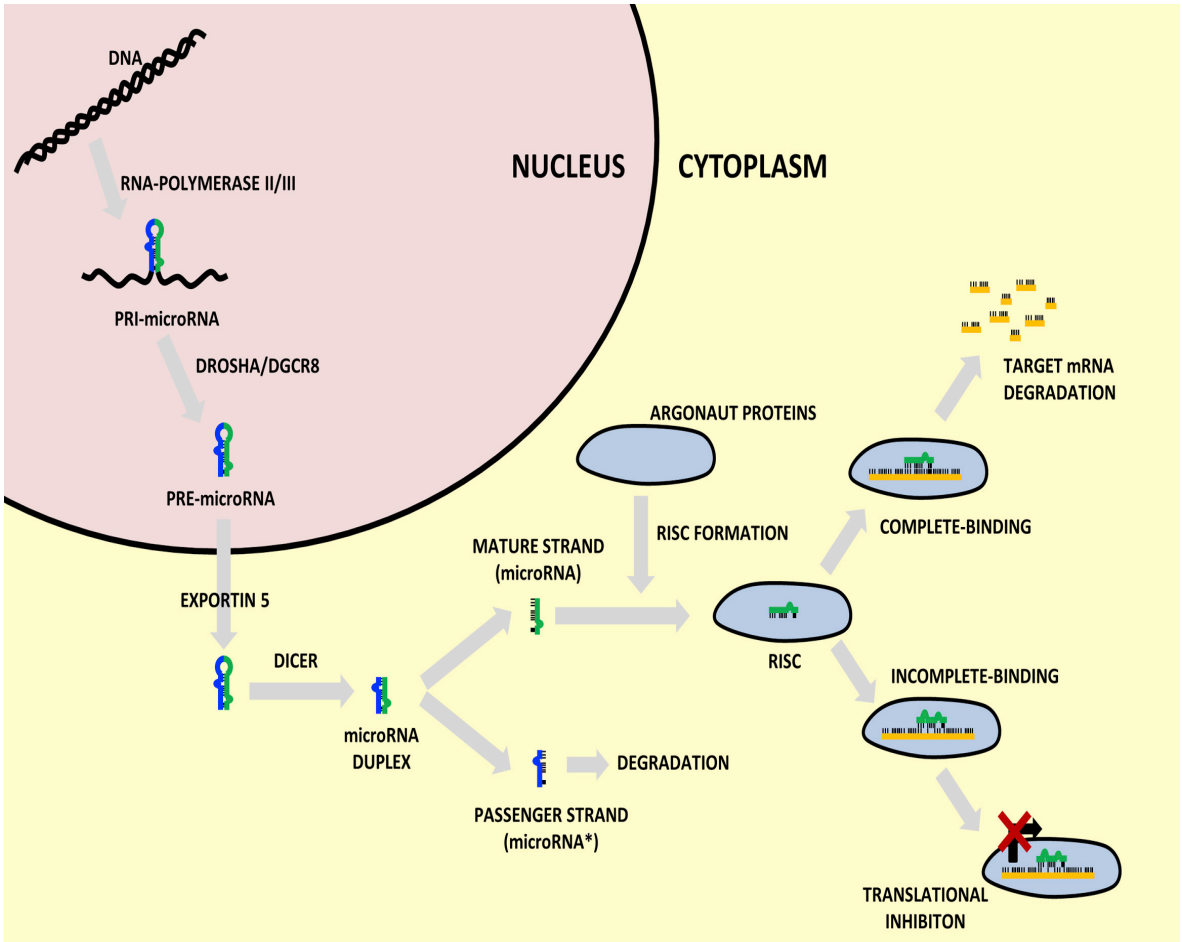


Figure 1-3 The biogenesis of miRNA

The biogenesis of microRNAs begins in the nucleus with the translation of the pri-miRNA. A series of cleavage steps by various enzymes in both the nucleus and the cytoplasm before the mature miRNA is integrated into the RISC which aids binding with, and subsequent repression or degradation of, the target mRNA. Replicated with permission from [142] “MicroRNAs in pulmonary arterial hypertension: pathogenesis, diagnosis and treatment.” Journal of the American Society of Hypertension, 2015 9(3): p221-34.

In the cytoplasm, the pre-miRNA undergoes a further cleavage step by the cytoplasmic RNase III endonuclease enzyme termed Dicer. This creates a temporary double-stranded miRNA duplex. Through the action of helicases, the duplex is separated into the guide strand (the mature miRNA) and the passenger strand, which is usually degraded. The mature miRNA then binds to the Argonaut protein (Ago), a component of the RNA-induced silencing complex (RISC).

1.4.3 MicroRNA mechanisms of action and regulation

Once loaded into the RISC, the mature miRNA then binds to the target mRNA. The manner in which this happens is thought to influence the mechanism by which the miRNA regulates gene expression. The binding process involves the so-called “seed sequence” of the miRNA, found on the 5’ end of the miRNA and comprising of nucleotides 2-8. Broadly speaking, if there is complete complementarity between the miRNA “seed sequence” and the 3’ UTR of the target mRNA (based on Watson-Crick pairing), then mRNA degradation results. Partial complementarity, however, usually leads to repressed translation of the mRNA. In various animals species, most miRNA-mRNA binding tends to be imperfect, resulting in features such as nucleotide bulging [143]. This suggests that mRNA repression is the primary mode of action in animal species.

Although likely less important, alternative mechanisms of binding have been identified. These mechanisms help to improve overall recognition of target mRNA by miRNA, and include 3’-end pairing, where there is complementarity between the 3’ ends of both the miRNA and its target [144]. Binding between the 5’ ends has also been reported (which may actually lead to enhanced translation) [145], and to “centred sites” where the midsection of the miRNA binds [146].

Many miRNAs, along with their targets, are widely conserved across species, whereas some are specific to a small number of animal or plant species. Even if a miRNA is widely conserved, it may not be expressed to the same extent in different species, and this may also vary within a specific species, based on its stage of development [147]. Furthermore, these regulators of mRNA expression are themselves subject to regulation through mechnisms such as methylation, RNA editing and the action of single nucleotide polymorphisms (SNPs) [148].

1.4.4 MicroRNAs as therapeutic targets

As miRNAs are so intricately involved in the regulation of gene expression, they clearly have the potential to modify signalling pathways and hence influence disease processes. This makes techniques which manipulate miRNA levels attractive as prospective therapies. Manipulation of miRNA levels can be achieved by two broad strategies. Expression of miRNAs can be enhanced by a miR-mimic which substitutes for a downregulated miRNA. Levels can also be suppressed by an antimiR, an oligonucleotide with a sequence complementary to the miRNA being targeted, permitting binding and inactivation.

One advantage of using miRNAs as treatments could be the ability to target multiple genes within a network, potentially enhancing the therapeutic effect. Their relatively small size is attractive with regards ease of delivery into tissues. However, manipulation of miRNA levels with miR-mimics or antimiRs also poses challenges. Targeting the specific tissue where the miRNA imbalance exists can prove difficult. This therefore creates issues with off-target effects. This could be amplified by the ability of a single microRNA to have many targets. Toxicity may also arise as a consequence of an immune response to either the miR-mimic or antimiR, or the carrier agent used to get it into its target tissue. Despites the potential hurdles, miRNAs are being investigated as therapies in human clinical trials, such as with the miR-122 inhibitor Miravirsen in hepatitis C [149].

1.5 MicroRNAs in pulmonary hypertension

The development of the lungs, and maintenance of their homeostasis is dependent on miRNAs [150, 151], and microRNAs have been shown to be implicated in the pathogenesis of a wide range of pulmonary diseases, including lung cancer, COPD and pulmonary fibrosis [152, 153]. With regards pulmonary hypertension, many causative stimuli are able to modify the expression of miRNA in vascular cells. The dysregulation of miRNAs influences disease processes that are important in vascular remodelling, including cell proliferation, apoptosis and migration via a multitude of signalling pathways. What is also clear is that miRNA expression is

dynamic, with variable expression as the disease progresses [154]. Several of the more notable miRNA that have recently been studied in PAH will now be reviewed.

1.5.1 miR-21

Research conducted into miR-21 in PAH suggests it plays an important role, although some findings are apparently contradictory. Upregulation of miR-21 has been demonstrated in response to hypoxia in human PAECs [155] and human PASMCs [156]. In vitro, treatment of human PASMCs with antimir-21 results in reduced proliferation and migration in response to hypoxia [156]. Conversely, exogenous overexpression of miR-21 using a miR mimic enhances the proliferation of human PASMCs [157]. Upregulation of miR-21 has also been noted in lung tissue from various animal models including chronic hypoxic mice [157] as well as IL-6 overexpressing mice and the SU5416/hypoxia mouse model [155]. Increased mir-21 staining has been demonstrated by in situ hybridisation in the distal pulmonary vessels from PAH patients [155]. In vivo, downregulation of miR-21 has been shown to both prevent and reverse the development of PH in mice exposed to chronic hypoxia [157]. Taken together, these results suggest that miR-21 plays a contributory role in the development of PH. However, evidence that contradicts these findings has been seen in miR-21 knockout mice exposed to SU5416/hypoxia. These mice develop a pronounced PH phenotype compared to their wild type controls, suggesting that miR-21 is protective [155]. There is also contradictory evidence in the monocrotaline rat model, with Caruso et al finding downregulation of miR-21 [154], but Parikh and colleagues demonstrating upregulation [155]. It is clear that the role of miR-21 in the development of PH is complex. Some of these contradictory finding may be explained by the variety of cells, species and PH models examined, and overall it may not be possible to directly compare them.

1.5.2 miR-124

Downregulation of miR-124 has been observed in various animal models of PH, as well as in human IPAH samples. PAFs from calves with hypoxia-induced PH and PAFs from patients with IPAH show lower levels of miR-124, along with a proliferative and migratory phenotype [158]. Augmentation of miR-124 levels in bovine and human PAFs reduces PAF proliferation and migration, and

pharmacological reduction of miR-124 in control PAFs causes a proliferative phenotype [158]. Reduced expression of miR-124 has also been observed in human PASMCs exposed to hypoxia and in lung tissue from mice with chronic hypoxia-induced PH [159]. NFAT is an important transcription factor in the regulation of cell proliferation and apoptosis. miR-124 has been shown to have an inhibitory effect on the NFAT pathway by regulating NFAT phosphorylation and its nuclear translocation. Over expression of miR-124 inhibits PASMC proliferation and promote cellular differentiation possibly through repression of NFAT [159]. miR-124 therefore could be considered as anti-proliferative and pro-differentiation.

1.5.3 miR-145

miR-145 is organised in a bicistronic cluster with miR-143, with the pair transcribed from a common pri-miRNA. Both miRNAs are highly expressed in SMCs and fundamental in SMC physiology. In the systemic circulation miR-145 has been postulated to have a protective role, as carotid arteries with neointimal formation have reduced miR-145 levels, and miR-145 over-expression reduces neointimal formation in a balloon injury model [160]. However, a converse role for miR-145 has been identified in the pulmonary circulation. Upregulation of miR-145 in PASMCs, pulmonary arteries, and whole lung homogenate from PAH patients has been demonstrated [161]. This is corroborated by findings from lung and RV tissue from chronic hypoxic mice. In vivo data has shown a reduction in PH in hypoxic mice that under-express miR-145 through both transgenic and pharmacological methods [161]. Elevated miR-143/145 expression has also been identified in vascular concentric lesions in human PAH samples, suggesting a tendency towards vessel muscularisation.

1.5.4 miR-204

One of the first miRNAs to be associated with pulmonary vascular remodelling was miR-204. Downregulation of miR-204 in chronic hypoxic and monocrotaline rat models of PH has been proven [154] [162]. The treatment of monocrotaline rats with nebulised miR-204 mimics decreased pulmonary artery pressures on echo, and reduced RV wall thickness [162]. Decreased expression of miR-204 has also been seen in human PAH, correlating with disease severity as indicated by

pulmonary vascular resistance [162]. Human PAH-PASMCs also display greater proliferation and resistance to apoptosis, compared with control PASMCs. These findings were replicated in normal hPASMCs treated with a miR-204 antagomiR. The pro-proliferative/anti-apoptotic phenotype observed with miR-204 downregulation appears to be regulated via the STAT3-NFAT pathway, with increased STAT3 and NFATc2 expression identified in PAH-PASMCs and PAH lung tissue. ET-1 and other factors important in the pathogenesis of PAH activate STAT3, a transcription factor which binds to miR-204 causing its downregulation. This results in an increase in miR-204 target gene SHP2, and subsequently a further increase in STAT3, creating a positive feedback loop and a more severe PH phenotype. STAT3 directly induces NFATc2 expression, which is necessary for ongoing PAH-PASMC proliferation and resistance to apoptosis [162].

1.5.5 miR-150 and miR-26a – potential biomarkers in PAH

The first miRNA to be suggested as an independent predictor of survival in PAH is miR-150. A small cohort study of PAH patients identified 58 miRNAs with plasma levels that were altered in PAH. Of these, miR-150 was most downregulated, and it was subsequently shown to correlate with survival. Levels of miR-150 were also found to be decreased in the lungs of monocrotaline rats [163]. A further potential candidate as a biomarker for PAH is miR-26a [164]. Reduced plasma levels of miR-26a have been shown in both monocrotaline rats and PAH patients compared with controls. Expression of miR-26a in lung and RV tissue from monocrotaline rats has also been demonstrated as substantially lower, correlating with haemodynamic parameters and RV hypertrophy. A positive correlation between miR-26a levels and the six-minute walk distance has also been demonstrated.

1.6 MicroRNA-155 and links to pulmonary hypertension

1.6.1 Introduction to miR-155

MicroRNA-155 is a ‘typical multifunctional miRNA’ with roles in numerous biological processes and diseases including inflammation, immunity,

haematopoiesis, solid cancers and viral infection [165]. The precursor for miR-155 is processed from the B-cell Integration Cluster (BIC) gene, which is located on chromosome 21. The BIC gene itself was first identified due to its role in avian viral-induced lymphomas [166]. Further links between BIC/miR-155 and haematological malignancies have been identified, including the role miR-155 plays in diffuse large B cell lymphoma (DLBCL) [167, 168]. The role of miR-155 in the activation of the Epstein-Barr virus and the subsequent role it plays in EBV-related malignancies has also been established [169]. The effect of miR-155 on TGF-β/BMP signalling pathway, and in particular Smad5, will now be explored, along with other potential links between miR-155 and pulmonary hypertension pathobiology.

1.6.2 miR-155 and TGF-β pathway

MicroRNA-155 has been linked to the TGF-β pathway through its roles in the development of DLBCL and in the pathobiology of the Epstein-Barr virus related malignancies. Overexpression of miR-155 is associated with aggressive forms of DLBCL [170, 171]. Using unbiased genome-wide screening tools, *Rai et al* [167] identified that miR-155 binds to the 3' UTR of the Smad5 gene, making Smad5 a direct target of miR-155. Ectopic overexpression of miR-155 in DLBCL cell lines resulted in downregulation of Smad5 and, conversely, inhibiting miR-155 with an antimiR led to elevated Smad5 levels. This confirms the inverse correlation between miR-155 and Smad5. DLBCL cells overexpressing miR-155 were also found to be resistant to the growth-inhibitory effects of TGF-β1 and BMP 2 and 4, and had a decreased response to TGF-β1-mediated cell cycle arrest. Yin et al postulated that the induction of miR-155 plays a role in EBV-mediated B-cell activation and oncogenesis, partly by its effect on Smad5. miR-155 has been shown to inhibit BMP-mediated EBV reactivation by targeting several members of the TGF-β/BMP signalling pathway including Smad5. miR-155 also plays a role in reversing BMP-mediated cell growth inhibition in lung epithelial cells [169]. Through these studies, not only have links between miR-155 and a PAH-relevant pathway been established, but miR-155 overexpression has been shown to convey resistance to regulation of cell growth.

1.6.3 miR-155 and Inflammation

Elevated levels of inflammatory cells and cytokines such as IL-6 have been identified in PAH patients, as previously discussed. Many links between miR-155 and inflammation and immunity have been established. Increased BIC/miR-155 expression is found in activated B and T lymphocytes, as well as activated macrophages and monocytes [165]. Elevated miR-155 levels have been identified in synovial fluid and tissue from patients with rheumatoid arthritis, a chronic inflammatory condition [172]. miR-155 is also upregulated in rheumatoid arthritis synovial macrophages and monocytes, and it promotes the production of pro-inflammatory cytokines including IL-6 and TNF-α [173].

1.6.4 miR-155 and HIF-1α

A role for miR-155 in the cellular response to hypoxia has been identified [174]. HIF-1α is recognised as a master regulator of the cellular response to hypoxia. Hypoxic upregulation of miR-155 has been demonstrated in colonic epithelial cells and, based on miRNA target prediction algorithms, HIF-1α is predicted to be a target of miR-155. This is confirmed by the observation that exogenous over expression of miR-155 decreased HIF-1α levels and activity in these cells, and downregulation of endogenous miR-155 with an antimiR restored HIF-1α levels and activity. A negative feedback loop between miR-155 and HIF-1α therefore appears to exist in this model.

1.6.5 miR-155 and eNOS

Nitric oxide produced by PAECs is a potent pulmonary vasodilator and in PAH the NO-sGC-cGMP pathway is dysregulated. This is the basis for the treatment of PAH with PDE5 inhibitors and stimulators of sGC, as discussed earlier. NO is generated through the action of endothelial NO synthase (eNOS). eNOS has been proven to be a direct target of miR-155, and miR-155 overexpression has been shown to decrease the production of NO from endothelial cells in the systemic circulation, through its effect on eNOS [175]. Furthermore, inhibition if miR-155 resulted in

increased eNOS expression and NO levels. Whether this effect translates to the pulmonary circulation has not been proven.

1.6.6 Summary

Many triggers, pathways and processes are involved in the pathogenesis of PAH and pulmonary vascular remodelling and, although it has not yet specifically been examined in the disease, miR-155 appears to influence some of these:

- The TGF-β/BMP pathway is clearly important in PAH, not least because of the pre-eminence of BMPR2 mutations as the major genetic cause of the disease. miR-155 directly controls Smad5, one of the signalling intermediaries from this pathway, with an inverse correlation demonstrated.
- Elevated levels of miR-155 are found in inflammatory conditions such as rheumatoid arthritis and it promotes the production of IL-6, which is upregulated in PH, along with other cytokines.
- eNOS is downregulated by miR-155, reducing NO levels and increasing vascular tone.
- Hypoxia upregulates miR-155, which in turn downregulates HIF-1α, suggesting the presence of a negative feedback loop and therefore impacting on the cellular response to hypoxia.

If these findings were to directly translate to the pulmonary circulation and PAH, it would imply that miR-155 has a detrimental influence through its actions on Smad5, IL-6 and eNOS. However, miR-155 may suppress the HIF-1α-mediated effects of hypoxia, offering a protective effect against PAH.

1.7 Aims and hypothesis

There have been significant advances in the understanding of the pathobiology of PAH over the past three decades. This has resulted in the availability of disease specific treatments with subsequent improvements in quality of life, exercise

tolerance and survival. However, these treatments primarily target vasoconstriction in the pulmonary circulation, and not the pulmonary vascular remodelling that is a cardinal feature of PAH.

MicroRNAs have complex and, at times, contradictory actions. Although they have often been explored as therapeutic agents, it is clear that they are also important tools in the investigation of disease pathobiology. As outlined, microRNA-155 has a number of potential links to PH pathobiology.

The principal aim of this thesis is to explore how the manipulation of microRNA-155 can be used as a tool to understand the pathways and mechanisms which lead to pulmonary vascular remodelling in PH. Ultimately this should contribute to the development of treatments which more effectively target this remodelling, as these are needed to further improve survival in patients.

The hypothesis behind this work is that microRNA-155 is an important regulator of the signalling pathways and mechanisms behind pulmonary vascular remodelling. Manipulation of miR-155 levels will lead to increased understanding of the pathobiology of PH, and its downregulation may possibly convey a protective effect against vascular remodelling processes by restoring normal Smad5 signalling and reducing p38 MAPK activity.

The main basis for this work is a miR-155 knockout mouse model. The aims are:

1. To establish the *in vitro* effect of miR-155 knockout on mouse PAFs with regards remodelling processes, including cell migration and proliferation, and to establish whether p38 MAPK and Smad5 signalling may be involved in these remodelling processes
2. To validate these *in vitro* findings by the use of a second model, which will explore whether they are replicated across species and by using a different technique for miR-155 downregulation, namely pharmacological knockdown in rat PAFs
3. To assess the effects *in vivo* of miR-155 knockout on the development of PH in the chronic hypoxic mouse model, as evaluated by RVSP, RVH and vascular remodelling.

Chapter 2

Materials and Methods

2.1 General equipment, reagents & solutions

Plasticware for tissue culture was obtained from Greiner Labortechnick Ltd (Gloucestershire, UK) and Corning Life Sciences (New York, USA). All general chemicals were of analar grade. Details about the composition and source of specific reagents, solutions and antibodies are laid out in table 2-1.

Solution/Reagent/Antibody	Source/Composition
Serum Free Medium	Dulbecco's Modified Eagle's Medium (DMEM) 2mM L-glutamine Penicillin 100iu/ml Streptomycin 100μg/ml (Obtained from Sigma, UK or Invitrogen, UK)
Cell Culture Medium	As above, with either 10% or 20% Fetal Calf Serum (FCS) (Sigma, UK)
Phosphate buffered saline (PBS) solution	Dissolve 1 PBS tablet (Sigma, UK) in 200mls distilled water to obtain a 137mM NaCl, 2.7 mM KCl and 10mM Phosphate buffer solution (pH 7.4 at 25°C)
Trypsin Solution	100ml 0.5% Trypsin 10X (Fisher Scientific, UK) 400ml PBS solution
Cryopreservation Solution	1ml dimethylsulphoxide (DMSO) (Sigma, UK) 9ml 10% culture medium
[^{3}H]-Thymidine (1mCi/ml)	Amersham, UK
Ethanol	Sigma, UK
Trichloroacetic acid 5%	4.1g trichloroacetic acid powder (Sigma, UK) 500mls distilled water
Sodium Hydroxide 0.3M	6g sodium hydroxide (Sigma, UK) 500mls distilled water
Ecoscint scintillation fluid	National Diagnostics
Trypan Blue	Gibco, UK
Radioimmunoprecipitation assay buffer (RIPA)	ThermoFisher Scientific
NuPAGE SDS buffers/reagents	ThermoFisher Scientific
PBS/T wash buffer	2 litres PBS solution 4ml Tween-20 (ThermoFisher Scientific)
Non-fat milk powder	Marvel (Premier Foods, UK)
ECL solution reagents	ThermoFisher Scientific
Total p38 MAPK antibody	Cell signaling
Phosphorylated p38 MAPK antibody	Cell signaling
Phosphorylated Smad5 antibody	Abcam
Anti-rabbit IgG HRP-linked secondary antibody	Cell signaling
RNaseZap	ThermoFisher Scientific
AntimiR-155 (mmu-miR-155-5p)	mirVana, Life Technologies
Negative control antimiR #1	mirVana, Life Technologies
Lipofectamine 2000	ThermoFisher Scientific
Opti-MEM reduced serum medium	Life Technologies

Table 2-1 Details of specific reagents, antibodies and solutions used in vitro

2.2 In vitro methods

2.2.1 Primary cell culture

Adventitial fibroblasts used throughout these studies were obtained from primary culture by explant, using rat or mouse pulmonary arteries. A combination of freshly explanted cells, along with cells which had previously been stored in liquid nitrogen were used. This technique has been employed in the Scottish Pulmonary Vascular Unit laboratory for many years and is well established.

To prevent contamination of cell lines, procedures involving the preparation or use of culture medium, or the preparation of drugs and reagents were conducted under sterile conditions using a clean Microflow laminar flow hood (figure 2-1) that was liberally sprayed with 70% ethanol before each use. Sterlisation of non-sterile equipment and consumables was performed using a Prestige Medical Classic 200 autoclave. Non-sterile reagents were filtered through a 0.2µm pore filter.

Figure 2-1 Microflow laminar flow hood

Galaxy-R incubators (figure2-2) were supplied by Wolf Laboratories (York, UK).

Figure 2-2 Galaxy-R incubator

2.2.1.1 Adventitial fibroblast explant/culture

The main pulmonary artery was dissected free from a heart & lung tissue block using a dissecting microscope (Zeiss STEMI SV11). Excess adherent tissue was removed, and a section of the artery was opened longitudinally into a flat sheet. Fibroblasts were isolated using the Freshney technique [176] with some modifications [92]. This has previously been shown to result in a culture of pure fibroblasts [177]. Briefly, the intima and media layers of the vessel wall were removed by gentle abrasion with a sterile razor blade, leaving behind just the adventitia. This was subsequently dissected into 5mm^2 pieces that were transferred to a 25cm^3 culture flask, placed on the base at regular intervals. To the flask was added 2mls of 20% culture medium.

The flask was then incubated at 37°C with 21% O_2 and 5% CO_2. Cells subsequently grew out from the tissue segments within a few days, and once a monolayer of cells had formed the tissue fragments were aspirated away. Cells were then lifted from the base of the flask using trypsin (see section 2.2.1.2) and resuspended in culture medium in a fresh 25cm^3 flask. Cells were trypsinised again after a further seven days and subsequently grown in 75cm^3 culture flasks.

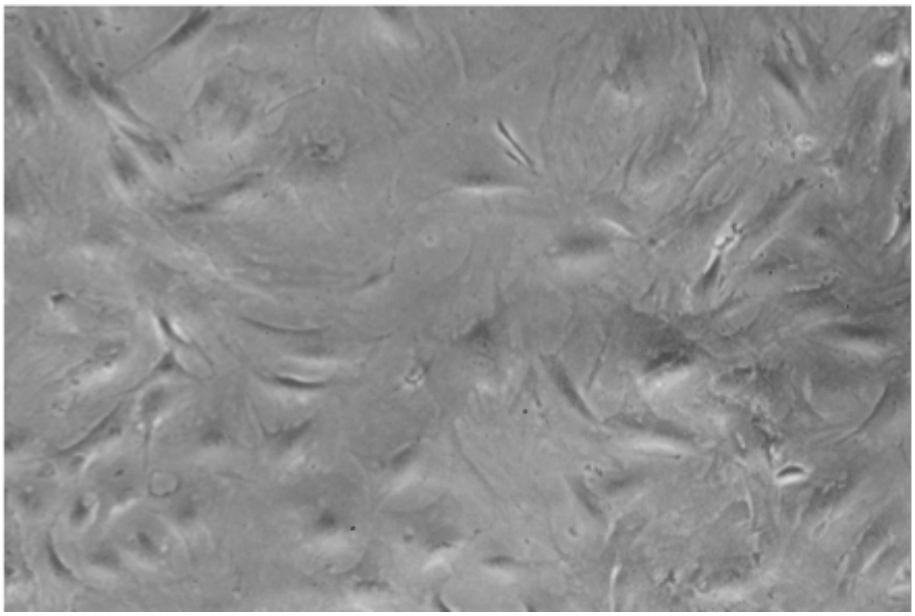


Figure 2-3 Typical appearances of rat PAFs

2.2.1.2 Routine cell maintenance

Cells were routinely grown in 75cm^3 culture flasks containing 10ml of 10% culture medium. The flasks were kept in a Galaxy R incubator set at 37°C. Cells were visually inspected using a light microscope (Olympus CK2), and had the culture medium replaced regularly, every 2 to 3 days. The cells were grown until almost fully confluent before passage.

To passage cells, the culture medium was aspirated and cells were washed twice with 2ml of trypsin solution for approximately 10 seconds per wash. After the second wash was removed, cells were incubated for 5 to 7 minutes until they were seen to be detaching from the flask surface. This was aided further by gentle agitation, before 10ml of 10% culture medium was added to inactivate the trypsin and to resuspend the cells. A fraction of this cell suspension was then aliquoted

into a fresh 75cm^3 culture flask containing suffient new 10% culture medium so that the total volume in the new flask was 10mls. The remainder of the cell suspension could be divided in 6, 12 or 24 well culture plates as required. All cells used for experiments were between passage 2 and 6.

2.2.1.3 Cell Storage

Fully confluent cells at an early passage were trypsinised and resuspended in 10ml of 10% culture medium. The suspension was then centrifuged for 10 minutes at 3000rpm. The resulting cell pellet was then resuspended in 1ml of cryopreservative solution and transferred to a cryotube. The cells were subsequently cooled at +4°C and -20°C for 1 hour each, before being placed in the -80°C freezer overnight. The cryotubes were then transferred to liquid nitrogen for longer term storage.

When required, cryotubes of frozen cells were rapidly thawed at 37°C in a water bath. The thawed contents of the cryotube were resuspended in 9mls of 10% culture medium and transferred to a cell culture flask. Once the cells had adhered to the flask surface, the culture medium was exchanged to minimise exposure of the defrosted cells to dimethylsulphoxide.

2.2.2 Acute hypoxia

Maintenance of cells in the Galaxy R incubator allows the control of oxygen concentration between 0% and 21%, through the supplementation of the atmosphere with nitrogen. When hypoxic conditions were required, a Pneuchange automatic gas cyclinder change-over unit (NTC, Woulton, Liverpool, UK), was used to supply fresh nitrogen from cylinders on demand, until the desired degree of hypoxia was achieved. The incubator is also connected to a CO_2 cylinder which supplements the chamber to maintain CO_2 levels at 5%.

An environment of 5% O_2 has previously been established by the laboratory to maintain a cell pO_2 of 35mmHg with a stable physiological pH. Experimental controls were maintained in a second incubator set at normal atmospheric oxygen levels (21%).

2.2.3 Assessment of cell proliferation

2.2.3.1 [^{3}H]-Thymidine proliferation assay

Use of this technique allows determination of cellular proliferation by measuring the incorporation of [^{3}H]-Thymidine into DNA during cell division.

PAFs were seeded at a density of 5x10^3 cells per well into 24-well plates. Each well contained 500µl of 10% culture medium, and the plates were incubated until cells reached 60% confluency. Culture medium was then replaced with 500µl serum-free medium for 24 hours to quiesce the cells. The appropriate reagents or conditions under investigation were then applied, and plates were incubated for a further 24 hours in hypoxia or normal atmospheric conditions, as required.

A dilute solution of [^{3}H]-Thymidine was prepared by adding 10µl of the stock solution (1mCi/ml) to 2.5ml of serum-free culture medium. For the final 4 hours of an experiment, 25µl of the dilute [^{3}H]-Thymidine solution was added to each well.

At the end of the experiment, the reaction was halted by washing each well twice with 500µl of ice-cold phosphate-buffered saline. Precipitation of proteins was achieved by washing 3 times with 5% trichloroacetic acid (TCA, 500µl/well/wash). Washing each well twice with 500µl of 100% ethanol resulted in lipid fractions being solubilised. The resultant material was then dissolved in 0.3M sodium hydroxide, 200µl per well, for 30mins. Each well's contents were then transferred to a 1ml eppendorf tube, and 1.2ml of Ecoscint scintillation solution was added. Each eppendorf was gently agitated to mix the contents and allowed to settle overnight in darkness. Radioactivity was then quantified using a Wallac 1409 scintillation counter, and expressed as either degradations or counts per minute (DPM or CPM, respectively).

2.2.3.2 Cell counting & trypan blue staining

Cells seeded into approriate well plates were used for experiments as desired. Once these were complete, the cells were treated briefly twice with 400µl of

trypsin solution before being resuspended in 1ml PBS solution. 100µl of the cell suspension was added to 100µl of 0.4% trypan blue solution, and 20µl of the resulting solution was transferred to a Neubauer Haemocytometer. Cells which stain blue are non-viable, and by counting the total cells and the non-viable cells in 4 of the squares marked out on the haemocytometer's grid (using a light microscope at low power), the percentage of viable cells could be calculated.

To ascertain the total number of cells in the initial solution, the following equation was used:

No. of cells in original solution = 2x [Total no. of cells in 4 squares/4] x10000

2.2.4 Assessment of cell migration

Cellular migration was assessed using a scratch wound assay. Six-well plates of fully confluent fibroblasts were serum starved for 24 hours before vertical scratches were drawn through the confluent cell monolayer using a p200 pipette tip. Cells were washed with serum-free medium to remove any debris before fresh serum-free medium was added to each well. A photograph of the scratch in the cell monolayer was then taken, using a digital SLR camera. Prior to use, the well plates had been lightly pre-scored on the underside to aid orientation when photographs were being taken.

The 6-well plates were incubated for a further 24 hours after experimental conditions were applied, before being re-photographed at the same location.
Using the photographs, quantitative assessment of migration was made by calculating the percentage closure over 24 hours. The width of each scratch was measured 4 times, at regular intervals along its length. Percentage closure of the scratch was calculated as:

% closure = [width at 24 hours / width at 0 hours] x100

2.2.5 Detection & analysis of proteins

2.2.5.1 Preparation of cell samples for protein analysis

Cell were grown to 90% confluency in 6-well plates, before being quiesced using serum-free medium for 24 hours. The conditions under investigation were then applied. Once the experiment was complete, culture plates were placed on ice and the culture medium removed. Each well was washed twice with 500µl of ice-cold PBS solution. Cell lysis was achieved by the addition to each well of 50µl of radioimmunoprecipitation assay (RIPA) buffer for 10 minutes. This was augmented by mechanical disruption, using a cell scraper. The resulting solution was aspirated and stored in a 1ml eppendorf tube at -80°C until needed.

2.2.5.2 SDS-PAGE gel electrophoresis

Protein samples were analysed by sodium dodecyl sulfate polyacramide gel electrophoresis (SDS-PAGE). An appropriate quantity of the cell lysate, prepared as above, was added to SDS sample buffer and reducing agent. The samples were then incubated in a 70°C water bath for 5 minutes to denature proteins and disrupt disulphide bonds.

The samples were then loaded into individual lanes of precast 10% gel cassettes (Life Technologies, UK), using loading tips. The first lane of each cassette was loaded with See-Blue prestained molecular weight marker, to allow protein sizes to be determined. The SDS-PAGE cassettes were then assembled into a XCell-II electrophoresis/blotting cube. The two compartments of the cube were subsequently filled with SDS Running Buffer and the gels were then subjected to electrical current. A voltage of 150V was applied for 100 minutes, whilst the cube was cooled by surounding its base with ice (figure 2-4).

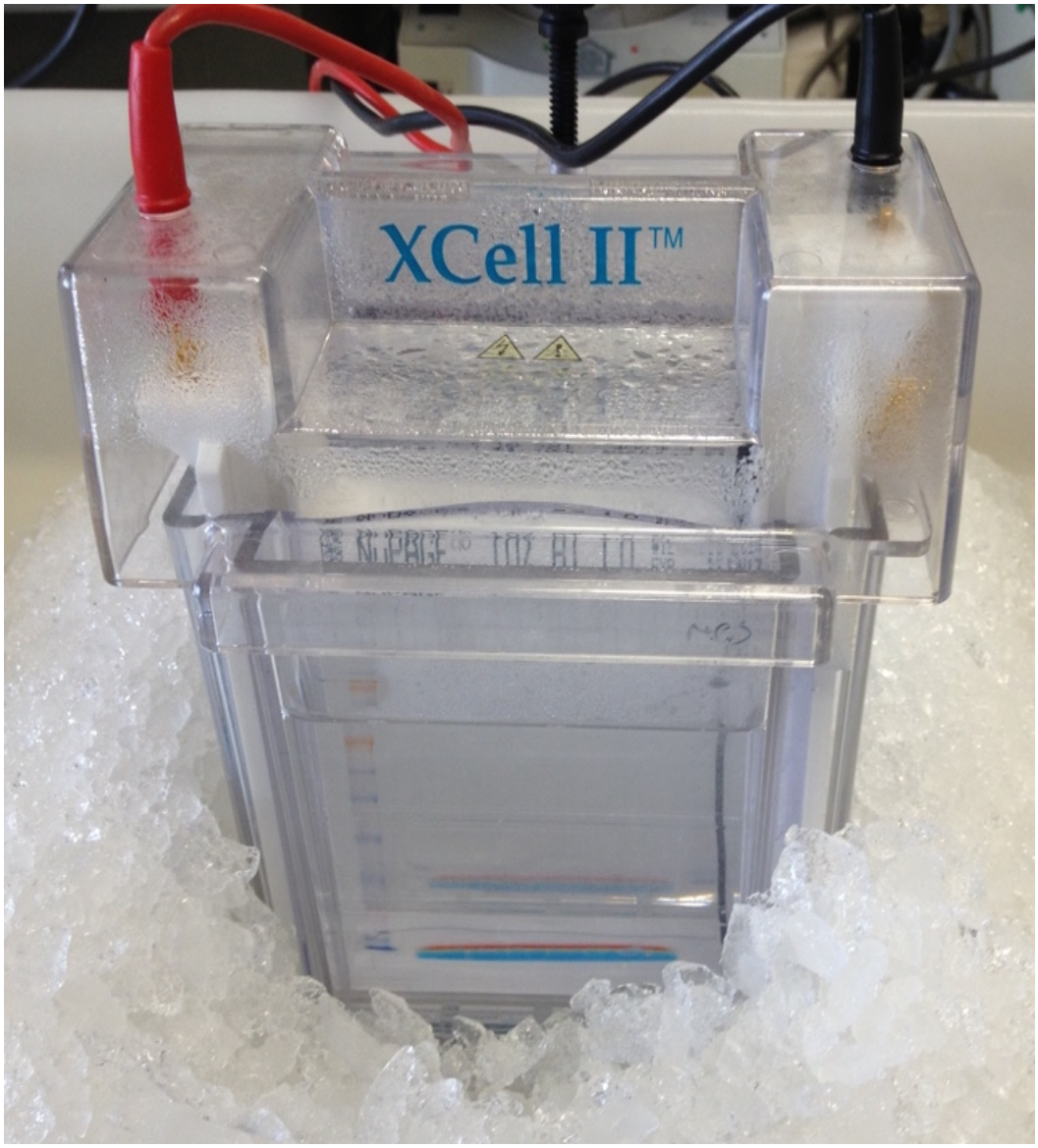


Figure 2-4 XCell II electrophoresis/blotting cube

2.2.5.3 Transfer to nitrocellulose membrane

Once electrophoresis was complete, the apparatus was disassembled and the gels carefully removed from their plastic cassettes. The gels were then layered into a transfer module, with a nitrocellulose membrane (which had been pre-activated with methanol) positioned between the gel and the cathode. The transfer module was then inserted into the blotting cube, and Transfer Buffer was added. A voltage of 30V was applied for 60mins, resulting in the transfer of negatively charged proteins onto the membrane.

2.2.5.4 Immunoblotting

The nitrocellulose blots were washed in PBS/T wash buffer and blocked for non-specific binding for 1 hour on a Gyro-Rocker platform in PBS/T supplemented with 10% non-fat milk (w/v), at room temperature. Blots were then incubated for 1 hour in PBS/T with 5% non-fat milk (w/v) containing the primary antibody at an appropriate dilution. The blots were rinsed and washed 4 times with PBS/T and then incubated for 60 minutes with the secondary antibody, usually anti-rabbit IgG conjugated to Horseradish peroxidase (HRP), in PBS/T with 5% non-fat milk at a concentraion of 1:2000. The blots were then washed as before.

2.2.5.5 Antibody detection

Following completion of washing procedures, proteins were detected using a method of enhanced chemilumenescence (ECL). Briefly, light emission from the action of HRP on its substrate within the ECL solution is detected on x-ray film as bands representing the proteins that have bound to the HRP-linked antibody. Blots were incubated in ECL solution for 30 seconds and then sandwiched between acetate film, ensuring all air bubbles were dispelled. Blots were then placed in a cassette with x-ray film for between 1 and 10 minutes, before the x-ray film was fed through a KODAK M35-M X-OMAT processor.

2.2.5.6 Re-probing membranes

If it was desirable for a nitrocellulose membrane to be probed for different proteins, primary and secondary antibodies were stripped from the membrane by incubating it in Stripping Buffer (Restore, ThermoFisher Scientific) for 30mins. The membranes were then rinsed with PBS/T, before the immunoblotting procedure was repeated with a different primary antibody.

2.2.5.7 Densitometry

Each protein band from the blot was then analysed by densitometry using Image J software (National Institutes of Health, USA). The density of the band under examination could be quantified as a ratio, relative to a control protein band.

2.2.6 Knockdown of miR-155 by liposomal transfection

Transient knockdown of miR-155 was achieved through liposomal transfection of antimiR-155. RNase contamination on surfaces within the laminar flow hood was minimised through liberal spraying with RNaseZap® decontamination solution. Rat PAFs were cultured using antibiotic-free DMEM containing 10% serum, in 6-well or 24-well plates, as necessary for the particular experiment, until they were 60-80% confluent.

AntimiR-155 and the negative control antimiR (both from mirVana, Life Technologies) were reconstituted with nuclease-free water per instructions supplied with the product, resulting in a 50μM (50pmol/μL) working stock solution. The transfection reagent used to introduce the antimiR into the cell was Lipofectamine 2000.

Lipofectamine 2000-antimiR complexes were prepared at a ratio of 1:1 in Opti-MEM reduced serum media. To achieve this, two separate tubes were prepared for each transfection. The first tube contained antimiR & Opti-MEM, mixed gently by pipetting, and the second tube contained Lipofectamine and Opti-MEM, mixed gently. Both tubes were incubated for five minutes before the contents were combined and incubated for a further 20 minutes.

Once the Lipofectamine 2000-antimiR complexes were prepared, the culture medium in each well was replaced with an appropriate quantity of fresh Opti-MEM. The Lipofectamine-antimiR complexes were then added to the cells, such that the final concentration of antimiR was 50nM. Cells were returned to the incubator, and 24 hours later the Opti-MEM was replaced with DMEM containing the desired amount of serum. Proliferation assays, protein analysis or migration studies could then be undertaken as above.

2.3 In vivo methods

MicroRNA-155 knockout (KO) mice and age-matched wild type (WT) controls were used throughout for in vivo experiments. These were kindly donated by Dr Charles McSharry and Dr Mariola Kurowska-Stolarska (Institute of Infection, Immunity and Inflammation, University of Glasgow). Mice were kept at the University of Glasgow animal house, in pathogen-free conditions managed by the biological services staff at the University. All animals were kept in strict accordance with guidelines set out by the United Kingdom Home Office Animal (Scientific Procedures) Act 1986. All procedures were carried out under project licence number PL60-4404, held by Professor M MacLean, Institute of Cardiovascular and Medical Sciences, University of Glasgow.

2.3.1 Chronic hypoxic animal model

2.3.1.1 Hypobaric chamber design

A hypobaric chamber was used to create a hypoxic environment. Transparent high resistance plexiglass was used to construct the chamber, which comprises of two separate compartments. Use of a vacuum pump lowers the pressure inside the chamber to a final pressure of 550mbar, equivalent to 10% O_2. A constant flow of air through the chamber prevented the accumulation of moisture and CO_2. The chamber was housed in a dedicated room, allowing the regulation of temperature (at 21°C) and humidity, and a regular 12-hour on/12-hour off light cycle.

2.3.1.2 Induction of PH & animal maintenance

Up to 12 animals in 2 cages were placed in the chamber at any one time. Pressure was gradually reduced to 550mbar over 48 hours to allow acclimatisation. Animals were subjected to this hypoxic environment for 14 days to allow PH to develop. Frequent checks were made throughout the day to ensure the animals were not distressed and the equipment was functioning optimally. When care for the animals was needed, the chamber was brought back to atmospheric pressure over 1 hour. Cleaning and replenishment of food and water was carried out and the

pressure was re-established over a further 1 hour. Control ("normoxic") mice were housed in the same room at normal atmospheric pressure, equivalent to 21% O_2.

2.3.2 Assessment of pulmonary hypertension

2.3.2.1 Anaesthesia

Administration of 3% Isoflurane (Abbott Laboratories, Berkshire, UK) in an anaesthetic box was used to induce anaesthesia in the mice. This was supplemented with oxygen 0.5L/min. The mice were then immediately weighed and transferred to a facemask which continuously delivered 1.5% isoflurane, supplemented with oxygen. The absence of hind-limb or tail reflexes was used to confirm that the required depth of anaesthesia had been achieved. This was monitored throughout the procedure, along with heart rate and respiratory rate, to ensure anaesthesia was neither too deep nor too shallow. At the end of the procedure, the mice were sacrificed using a terminal dose of anaesthesia.

2.3.2.2 Haemodynamics

Right ventricular pressure was measured by cannulating the right internal jugular vein with A Millar Mikro-Tip® PVR-1030 pressure-volume catheter, which was then slowly advanced into the right ventricle until a typical RV pressure trace with even oscillations was obtained. Recordings were made using the PowerLab data acquisition system (AD Instruments, Oxford, UK) and analysed using LabChart v7 software. Although not performed for this work, systemic pressures should be measured as a part of future research. This would then clarify whether any haemodynamic effect of miR-155 down-regulation was specific to the pulmonary circulation.

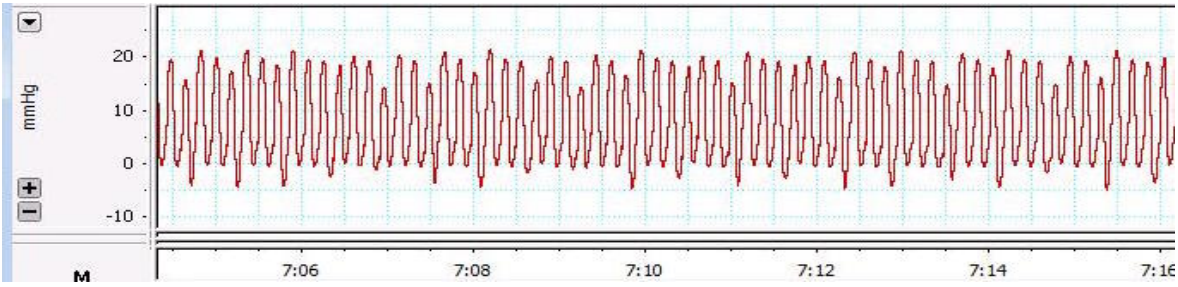


Figure 2-5 Typical right ventricular systolic pressure trace

2.3.2.3 Right ventricular hypertrophy

RVH was assessed using Fulton's Index [178]. The right ventricular free wall ("RV") was separated from the left ventricle and septum ("LV+S") using a dissecting microscope. The LV was subsequently opened flat, and all tissue was blotted dry and weighed. The ratio of the mass of the RV to LV+S was used to assess the degree of RV hypertrophy.

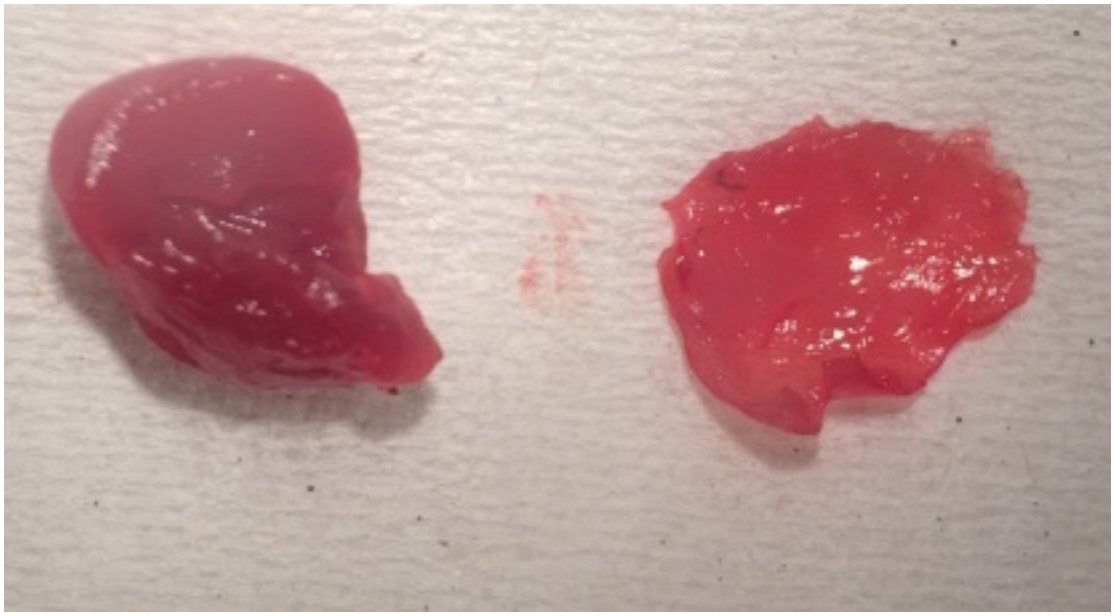


Figure 2-6 Right ventricle (right) dissected from left ventricle and septum

2.3.2.4 Pulmonary vascular remodelling

Solutions and reagents used for histology are summarised in table 2-2.

Solution/reagent	Composition/source
Dulbecco's PBS	Sigma, UK
10% neutral buffered formalin	Sigma, UK
Histoclear	ThermoFisher Scientific
0.5% potassium permanganate	1g $KMnO_4$ in 200ml distilled water
1% oxalic acid	2g oxalic acid in 200ml distilled water
Miller's elastic stain	Sigma, UK
Picro-sirius red	Sigma, UK
Acidified water	5ml Glacial acetic acid in 1L distilled water

Table 2-2 Details of specific reagents and solutions used for histology

Immediately following sacrifice, the lungs were gently flushed with Dulbecco's phosphate buffered saline solution. They were then gently inflated with 10% neutral buffered formalin solution (NBF), before being placed in NBF solution at least overnight. After fixation in formalin, the tissue was dehydrated through a water-Histoclear gradient and subsequently embedded in paraffin using a Citadel 100 tissue processor (ThermoScientific, UK). Tissue blocks were then stored in a -20°C freezer to solidify the paraffin. The tissue blocks were then sliced into 5µm sections using a microtome (Leica RM2125, Leica Microsystems, Milton Keynes, UK) and placed in a 37°C water bath to gently melt the paraffin. The sections were then transferred onto histology slides (VWR) and left to dry.

The slides were then treated with Histoclear and rehydrated through an ethanol-water gradient, before immersion in 0.5% potassium permanganate solution, followed by decolourisation using 1% oxalic acid. The sections were then stained with Miller's elastic stain and counter-stained with picro-sirius red. The sections were washed in acidified water, then rapidly dehydrated with ethanol before final washes with Histoclear. The slides were then mounted, ready for analysis.

Analysis was performed in a blinded fashion. Peripheral pulmonary vessels smaller than 80µm external diameter that were not in proximity to an airway were examined. A vessel was considered to be remodelled if greater than 50% of the circumference of the vessel had a double elastic lamina, which appeared black after staining. Remodelling was expressed as a percentage:

% pulmonary vascular remodelling = [remodelled vessels/total vessels] x100

2.4 Statistical analysis of data

For normally distributed values, data was reported as mean +/- SEM, and Students t-test was used. For multiple comparisons of means across different groups, one-way or two-way analysis of variance (ANOVA) was used as appropriate, with Bonferonni post-hoc analysis (Prism v7, GraphPad, California). In animal experiments, n = number of animals per condition. $p<0.05$ was accepted as significant throughout.

Chapter 3

Knockout of microRNA-155 influences remodelling processes and signalling pathways in mouse pulmonary artery fibroblasts

3.1 Introduction

As described in the general introduction chapter, changes in all three layers of the vessel wall contribute to the pulmonary vascular remodelling typically seen in pulmonary hypertension [18].

Endothelial cells are the predominant constituent of the intimal layer. In response to stimuli such as inflammation and shear stress they undergo proliferation, contributing to neointimal thickening and the formation of plexiform lesions, resulting in vessel remodelling [41, 42]. The intima is also directly influenced by various vasoactive mediators such as nitric oxide and endothelin. It is therefore important when it comes to the mechanism of action of most of the current treatments for PAH, which depend on these mediators [41]. Further vascular remodelling results from hypertrophy and muscularisation of the medial layer, at least in part due to the imbalance between proliferation and apoptosis of pulmonary artery smooth muscle cells seen in PH [35].

Adventitial fibroblasts are key orchestrators of the cellular response to hypoxia and the vascular remodelling seen in hypoxic pulmonary hypertension. In the adventitia, a combination of increased extracellular matrix deposition along with fibroblast proliferation leads to thickening of the vessel wall. Additionally, activated fibroblasts undergo transformation to myofibroblasts, the subsequent migration of which leads to the muscularisation of distal vessels which were previously non-muscularised [29, 34]. These processes indicate that the fibroblast is a valuable target for research into causative mechanisms and potential therapeutic interventions in hypoxic pulmonary hypertension.

Utilisation of the hypoxic model of PH and its effect on PAFs for the work in this thesis is justified by previous findings from both within the SPVU laboratory, where it is a well-established model, and from others. These findings include the proliferative response of PAFs to acute hypoxia [92], which has been shown to be selective for the pulmonary circulation (as it is not seen in fibroblasts from the systemic circulation) and to be preserved across animal species [87, 90]. Furthermore, the proliferative and signalling mechanisms activated in acutely hypoxic PAFs mirror those seen in PAFs from chronic hypoxic animals. p38 MAP

kinase, a stress-responsive protein, has been shown to play an essential role in the interplay between hypoxia and PAFs [89-91]. Upregulation of p38 MAPK has been shown in PAFs in response to hypoxia, and inhibition of p38 MAPK preventing or reversing the hyperproliferative effect of hypoxia on PAFs. The p38 MAPK pathway has also been shown to be activated in PASMCs with BMPR2 mutations. These mutations are a key genetic cause of PAH, and they result in ineffective Smad signalling through the TGF-β/BMP pathway [74].

MicroRNAs are non-coding strands of RNA which act as post-transcriptional regulators of gene expression, through the repression or degradation of the messenger RNA to which they bind [131]. Many thousands of microRNAs have been identified to date. Their actions are complex, as a single miRNA is capable of regulating many genes and a single gene may be regulated by multiple miRNAs [137]. Whilst many microRNAs are widely conserved across species, the extent of their expression, and therefore their effect, can vary between species and also between different stages of development within a species [147]. Despite this, their influence on gene expression makes them attractive tools for studying signalling pathways and disease aetiology. This can be done by manipulation of microRNA levels either pharmacologically or through the use of transgenic animals. Furthermore, pharmacological manipulation of microRNAs has also been explored as a therapeutic intervention, including in a human clinical trial on the treatment of Hepatitis C [149]. However, given their complex roles and interactions, and the potential for “off-target” effects it is unclear whether microRNA manipulation could become an established treatment strategy.

Many of the causative stimuli of PH can modify microRNA expression in vascular cells. This dysregulation impacts on signalling pathways and influences many of cellular processes behind vascular remodelling, including proliferation, apoptosis and migration [132]. Various microRNAs have been studied in PH, although the results are often complex and at times contradictory. MicroRNA-21 has been extensively studied. In vitro, upregulation of miR-21 in response to hypoxia is seen in human PAECs [155] and human PASMCs [156]. Upregulation is also seen in lung tissue from chronically hypoxic mice [157], as well as in the SU5416/hypoxia mouse model and in PAH patients [155]. Accordingly, downregulation of miR-21 results in reduced hypoxic proliferation and migration of human PASMCs [156],

and can prevent/reverse PH in mice exposed to chronic hypoxia [157]. This suggests that miR-21 contributes to the development of PH. However, evidence contradictory to this includes the finding that miR-21 is downregulated in the monocrotaline rat model [154]. Other noteworthy microRNAs studied in PH are outlined in the general introduction chapter.

MicroRNA-155 plays a role in numerous biological processes and diseases including inflammation, immunity, haematopoiesis and malignancy [165]. It has also been demonstrated to target many genes with links to pathways that are important in the pathogenesis of PH. Smad5, an intermediary in the TGF-β/BMP signalling family, has been shown to be a direct target of miR-155 [167]. An inverse correlation between Smad5 and miR-155 has been demonstrated in diffuse large B-cell lymphoma cells lines, with ectopic overexpression of miR-155 resulting in downregulation of Smad5 and miR-155 inhibition resulting in elevated Smad5 levels. More aggressive forms of DLBCL have been shown to overexpress miR-155, with such cells showing resistance to TGF-β1-mediated cell cycle arrest, and a decreased response to the growth-inhibitory effects of BMP 2 and 4 [170, 171]. This suggests miR-155 overexpression is linked to dysregulated cell growth.

The cellular response to hypoxia is also under the influence of miR-155, through links to HIF-1α, a master regulator of this response. A negative feedback loop between miR-155 and HIF-1α has been demonstrated in colonic epithelial cells where exogenous miR-155 overexpression decreases HIF-1α levels and activity, and downregulation via antimiR-155 restored HIF-1α activity [174]. Inflammation plays an important role in PAH pathogenesis, and microRNA-155 has been linked to inflammatory diseases including rheumatoid arthritis [172], with evidence that it promotes production of IL-6 and other cytokines in the disease [173]. Finally, in the systemic circulation miR-155 overexpression has been demonstrated to decrease endothelial production of nitric oxide, a potent vasodilator, through its control of endothelial NO synthase (eNOS) [175]. This suggests that there could be a role for miR-155 influencing vascular tone in the pulmonary circulation, although this has not been proven.

The aim of this thesis is to show that manipulation of microRNA-155 is a useful tool to enhance understanding of the mechanisms and signalling pathways behind

pulmonary vascular remodelling in PH. This greater understanding should contribute to the development of new treatments which more effectively target this remodelling and improve patient survival. Within this chapter, the aim is to establish the *in vitro* effect of miR-155 knockout on mouse PAFs with regards remodelling processes, including cell migration and proliferation, and to establish whether p38 MAPK and Smad5 signalling may be involved in these remodelling processes.

As miR-155 has been shown to promote dysregulated cell growth and inflammation, and to affect the action of HIF-1α, I hypothesised that miR-155 downregulation would counteract hypoxia-driven proliferation and migration of PAFs. Upregulation of Smad5 was also predicted, given the previously demonstrated inverse correlation between miR-155 expression and Smad5 levels, which could be a possible pathway by which manipulating miR-155 would lead to an effect on migration and proliferation. Given the link between p38 MAPK and BMPR2 mutations, and therefore the TGF-ß/BMP signalling pathway, p38 MAPK was also predicted to be another possible pathway through which miR-155 may act, with reduced p38MAPK activity predicted when miR-155 was downregulated. Overall this led to a hypothesis that miR-155 knockout would be protective against PH. The experiments in this chapter were conceived to clarify these hypotheses.

3.2 Relevant methods

Detailed methods pertaining to proliferation and migration assays, as well as protein detection and quantification are outlined in Chapter 2. Briefly, the rate of incorporation of [^{3}H]-Thymidine by PAFs under specific conditions was used as an indirect measure of proliferation. Migration was measured by the degree of closure of a scratch in a monolayer of PAFs when experimental conditions were applied.

The Western blot technique was employed for protein detection and quantification. The primary antibodies used for immunoblotting, along with their specific dilutions were as follows:

- Phosphorylated p38 MAPK (1:500)
- Total p38 MAPK (1:500)
- Phosphorylated Smad5 (1:1000)

3.3 Results

3.3.1 MicroRNA-155 knockout ameliorates the proliferation of PAFs in acute hypoxia

To examine the effect that miR-155 knockout in mice would have on hypoxia-driven PAF proliferation, [^{3}H]-Thymidine assays were performed, comparing wild type MPAFs with KO MPAFs. The results are displayed in figure 3-1.

When cultured with increasing doses of serum in normoxic conditions, a progressive increase in proliferation of wild type mouse PAFs was noted, as indicated by higher radioactivity counts. This effect was augmented when the WT MPAFs were exposed to hypoxia, with statistially significant differences first noted at 5% serum. The additional proliferative response was masked at the highest serum dose (10%), likely due to cellular contact inhibiting further proliferation.

When the proliferation assay was repeated with PAFs from miR-155 KO mice, the additional hyperproliferative response of the PAFs to hypoxia was altered. Whilst the response to serum in a dose-dependent fashion was maintained, the additional effect of hypoxia on DNA synthesis was not seen. This indicates that the proliferative response to hypoxia was abrogated in the knockout PAFs.

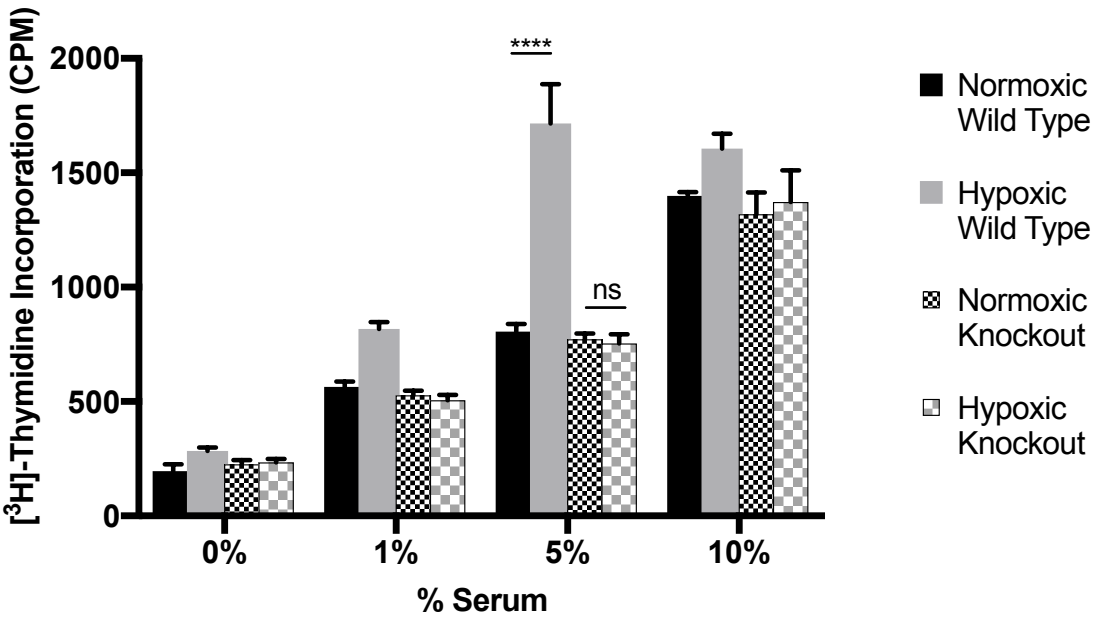


Figure 3-1 The hypoxia-enhanced proliferative response of Wild Type MPAFs is not seen in miR-155 knockout MPAFs.

WT MPAFs were cultured in normoxic conditions with the addition of [^{3}H]-Thymidine for the final 4 hours. The effect of stimulation with increasing doses of serum was observed. Increased proliferation with higher doses of serum was noted, as indicated by increased incorporation of [^{3}H]-Thymidine with radioactivity measured as CPM. When repeated with WT MPAFs in hypoxia, a trend towards an additional proliferative effect was seen, above that of the serum dose alone. This reached statistical significance at 5% serum, although the signal was lost at 10% serum, possibly due to contact inhibition. When repeated with miR-155 knockout MPAFs, the dose-dependent proliferative response to serum was maintained. However, KO MPAFs did not demonstrate any additional response to hypoxia. Four experiments per condition were conducted, with values expressed as mean +/- SEM. Results were analysed using 2-way ANOVA with Bonferroni post-hoc analysis. ****$p<0.0001$.

3.3.2 Hypoxia-induced migration of PAFs is reduced in microRNA-155 knockout

The scratch assay was utilised to evaluate cell migration. A vertical scratch was made through a cellular monolayer of both WT and KO MPAFs and, after further incubation in either normoxic or hypoxic conditions, the degree of closure of the scratch during this incubation was assessed.

After 24 hours in normoxic conditions, the scratch created in the WT MPAFs had narrowed only marginally, to 96% of its original width. However, after 24 hours in hypoxia the WT PAFs had migrated to such an extent that the scratch had closed completely, $p<0.01$ (figure 3-2A).

The knockout MPAFs however did not demonstrate this migratory response to hypoxia. After 24 hours, there was no statistically significant difference between the change of the hypoxic and normoxic KO MPAF scratches (figure 3-2B).

The width of each scratch was measured at 4 points and the results were displayed graphically (figure 3-2C).

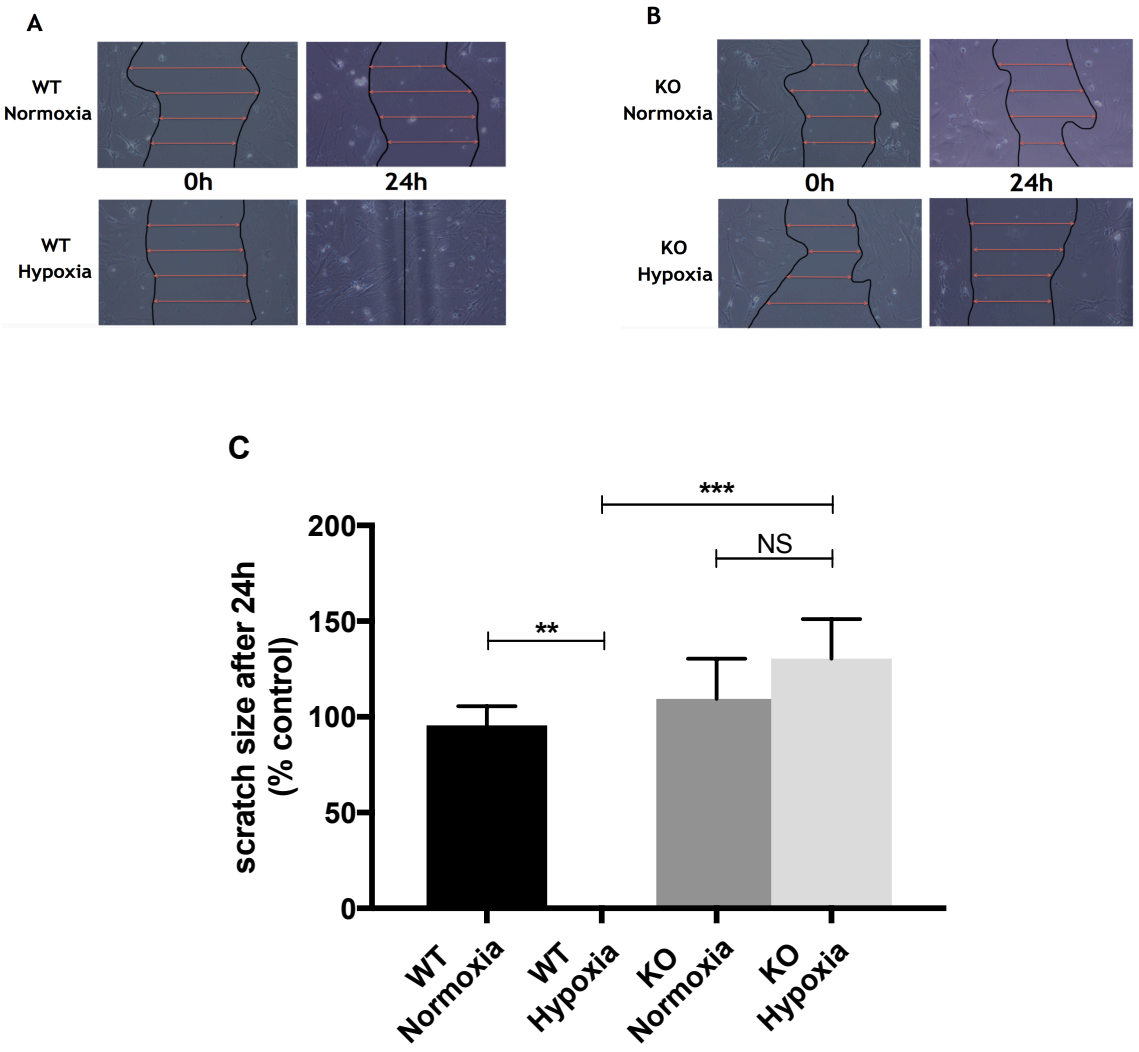


Figure 3-2 Migration of MPAFs in response to hypoxia is diminished in miR-155 knockout compared with wild type.
(A) Wild type MPAFs were cultured until there was a fully confluent monolayer of cells. A scratch was then drawn down through the layer of cells and photographed. Additional incubation in hypoxic or normoxic conditions for another 24 hours was carried out. After this time, the width of the remaining scratch was compared to the scratch at baseline i.e. 0h. Four measurements were taken at equal distances along the length of each scratch. In normoxic conditions, there is negligible change in the size of the scratch. However, in hypoxia, migration of the WT MPAFs results in closure of the scratch after 24h. (B) The scratch assay was repeated using miR-155 KO MPAFs. In contrast to the WT MPAFs, hypoxia does not induce a migratory response after 24h, with no reduction in the width of the scratch. (C) Graphical representation of the change in scratch size after 24h in each condition, expressed as a percentage of the original scratch size. Data presented as mean +/- SEM, analysed by 2-way ANOVA with Bonferroni post-hoc analysis, **p<0.01, ***p<0.001.

3.3.3 Knockout of microRNA-155 leads to a greater expression of Smad5 in hypoxia

The literature suggests that miR-155 controls expression of Smad5, with an inverse correlation demonstrated between the two. The combined effects of miR-155 KO and hypoxia on Smad5 levels in MPAFs were investigated by culturing WT and KO cells with increasing doses of serum in either normoxic or hypoxic conditions. Immunoblotting for phosphorylated Smad5 was performed, with total p38 MAPK used as a control. Protein levels were then quantified by densitometry.

Figure 3-3 displays the results. In MPAFs under normoxic conditions, the inverse correlation between miR-155 and Smad5 was not seen. In fact, there was more of a signal to suggest that Smad5 levels were lower in the KO cells in normoxia.

However, under hypoxia conditions this inverse relationship was demonstrated. Whilst there was some increase in Smad5 levels in the WT MPAFs in hypoxia, this was significantly enhanced in the KO MPAFs, $p<0.0001$. This was seen in all of the concentrations of serum used.

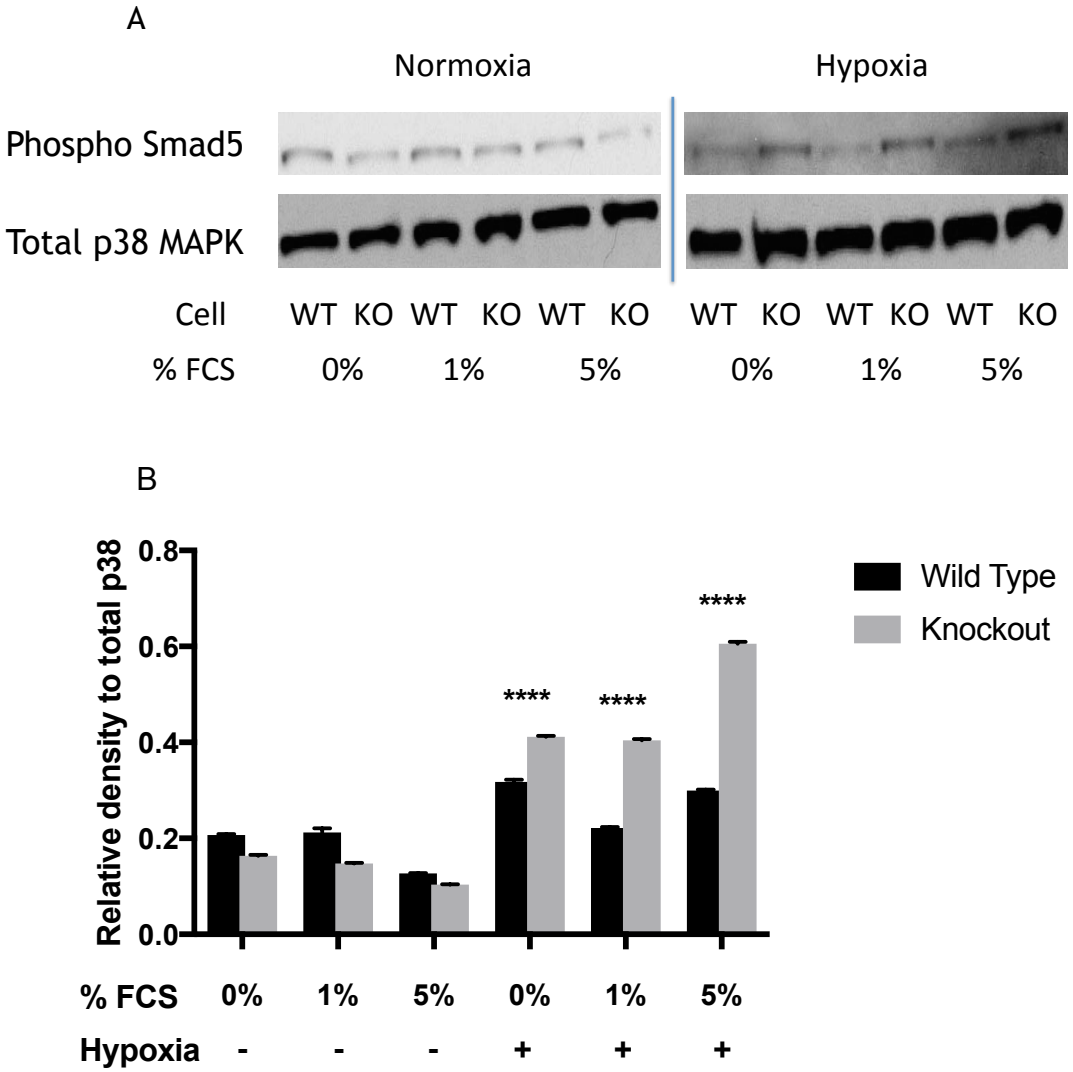


Figure 3-3 Phosphorylated Smad5 levels are upregulated in hypoxic conditions, especially in miR-155 knockout MPAFs.

(A) PAFs were isolated from both wild type and knockout mice and stimulated with increasing doses of serum and cultured for 24 hours in either normoxic or hypoxic conditions. Cell lysates were immunoblotted for phosphorylated Smad5 and total p38 MAPK. In normoxic conditions, the inverse correlation between miR-155 and Smad5 levels was not seen. However in hypoxia, whilst both cell types show increased phosphorylated Smad5 levels, this is especially the case with the KO MPAFs. Molecular weights: phosphorylated Smad5 52kDa, total p38 MAPK 43kDa. (B) Results quantified, using densitometry, as density relative to total p38 MAPK. Data presented as mean +/- SEM. Analysed by 2-way ANOVA with Bonferroni post-hoc analysis, ****$p<0.0001$

3.3.4 Hypoxia-induced phosphorylation of p38 MAPK in PAFs is lost in microRNA-155 knockout

Activation of the p38 MAPK signalling pathway is seen when PAFs hyperproliferate in response to hypoxia. It was hypothesised that the lack of hypoxia-driven proliferation in KO MPAFs demonstrated earlier in this chapter may be due to miR-155 downregulation preventing hypoxic activation of p38 MAPK. To investigate, WT and KO MPAFs were cultured with increasing doses of serum in hypoxia or normoxia. Immunoblotting for phosphorylated and total p38 MAPK was performed.

Figure 3-4 shows these immunoblots along with a graphical quantification of phosphorylated p38 MAPK. WT MPAFs cultured in hypoxia demonstrate enhanced p38 MAPK phosphorylation compared with the WT cells under normoxic conditions. This is the expected result, based on previous work.

When this was repeated using the miR-155 KO MPAFs, the ability of hypoxia to activate the p38 MAPK signalling pathway was lost. A signal was also detected which suggested that even without hypoxic stimulation (i.e. under normoxic conditions) the WT MPAFs showed greater levels of phosphorylated p38 MAPK than the KO MPAFs.

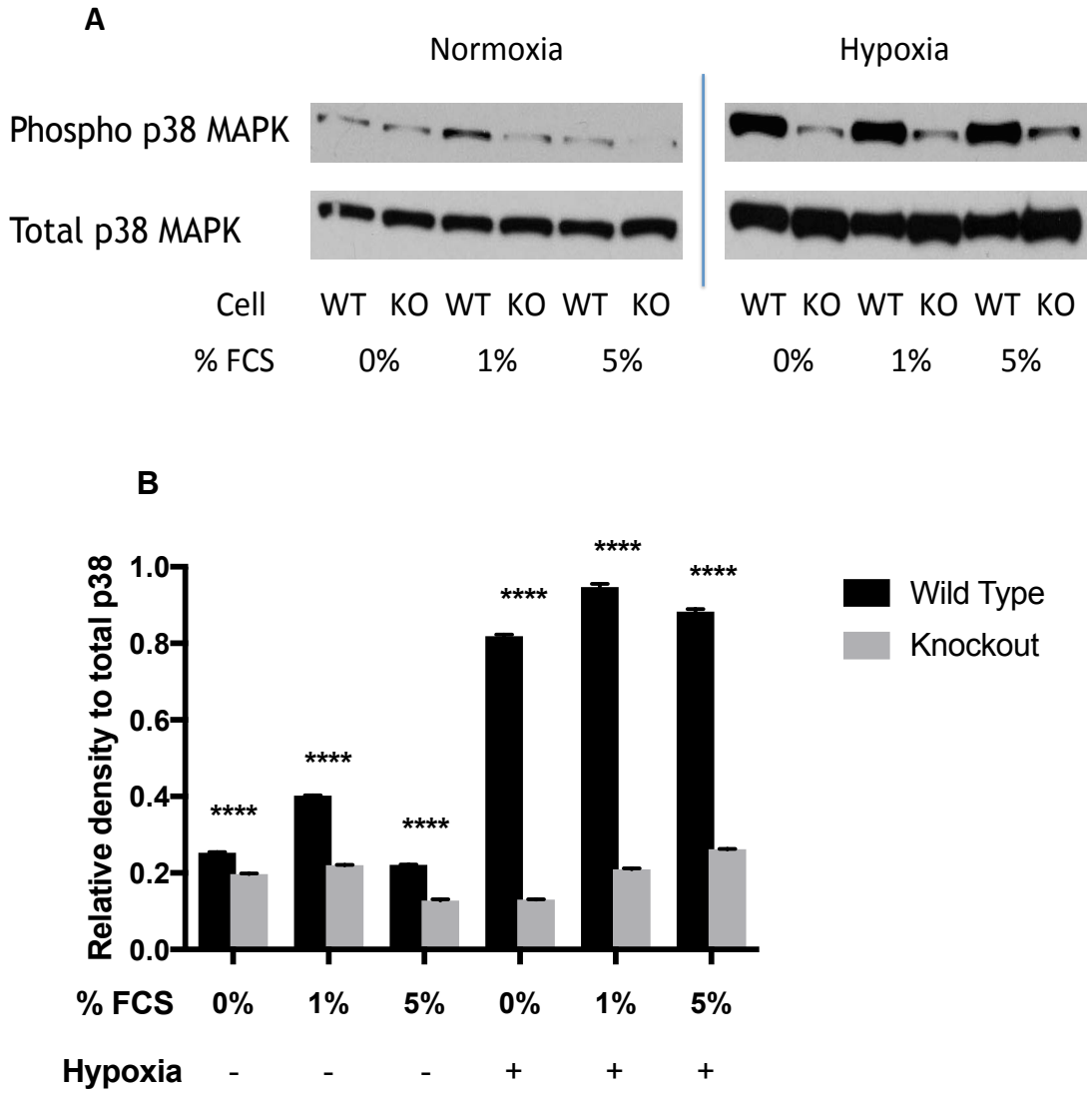


Figure 3-4 The effect of hypoxia on the phosphorylation of p38 MAPK is abrogated in miR-155 knockout MPAFs compared with wild type MPAFs.
(A) Wild type and knockout MPAFs were stimulated with increasing quantities of serum. They were then cultured in normoxia or hypoxia for 24 hours. Cell lysates were subsequently immunoblotted for total p38 MAPK and phosphorylated p38 MAPK. When cultured in hypoxia, the WT MPAFs showed the expected response, a rise in phosphorylated p38 MAPK levels. However, this response to hypoxia was not seen in the KO MPAFs. Molecular weights: both 43kDa. (B) Results quantified, using densitometry, as density relative to total p38 MAPK. Data expressed as mean +/- SEM, Analysed by 2-way ANOVA with Bonferroni post-hoc analysis, ****p<0.0001.

3.4 Summary

The results from this chapter have shown that miR-155 knockout can be used to understand PH pathobiology through the mechanisms and pathways which contribute to pulmonary vascular remodelling.

Fibroblasts play a key early role in the cellular response to hypoxia and subsequent vascular remodelling. The proliferation of PAFs in response to hypoxia leads to adventitial thickening, which contributes to PH [22, 23]. Results from this chapter have shown the expected proliferative response of WT MPAFs to hypoxia. However, this response to hypoxia is clearly lost in miR-155 KO MPAFs. These results are in-keeping with the predicted outcome, based on the original hypothesis that miR-155 KO would be protective against PH.

The transformation of PAFs into myofibroblasts and their migration from the adventitia leads to muscularisation of distal vessels and further drives pulmonary vascular remodelling [28, 29]. However, knockout of miR-155 in this mouse model prevented the migration of PAFs in response to hypoxia, with a clear persistence of the scratch in the migration assay. This therefore appeared to be a successful result as it is also in-keeping with the original hypothesis.

In order to elucidate possible pathways through which miR-155 KO may be exerting these effects on PAFs, a relationship with Smad5 was investigated. Smad5 is a key component of the TGF-β/BMP signalling pathway, which is important in PH. Smad5 is also an established target of miR-155, and an inverse correlation between the two has been illustrated previously [167]. Under basal normoxic conditions, the KO MPAFs showed generally lower levels of phosphorylated Smad5, compared with their wild type counterparts. When repeated in hypoxia, the result was an increase in the levels of phosphorylated Smad5 in both cell types. This was however to a much greater extent in the knockout PAFs. This inverse relationship was therefore only seen under hypoxic conditions.

The Smad5 experiments appeared to be generally successful in so far as they confirmed a link between miR-155 and Smad5, at least in hypoxia. Hypoxia itself may lead to an increase in Smad5 phosphorylation, and hence the increased levels

in the WT cells in hypoxia. However, it's clear that miR-155 KO enhances this effect. There may be several explanations for not seeing the anticipated inverse correlation between miR-155 and Smad5 in normoxia. Firstly, it may be that an additional stimulus such as hypoxia is necessary for this correlation to be manifested in PAFs. Alternatively, the choice of primary antibody may be relevant. Total p38 MAPK was used as the control for the Smad5 experiments. It would be valuable to repeat the experiment and using a Total Smad5 antibody, to evaluate if the inverse correlation was only present in normoxia with regards the total levels of Smad5.

As the links between hypoxia, PAFs and p38 MAPK are a core interest in the SPVU laboratory, a possible link between p38 MAPK and miR-155 was also explored. The literature shows that upregulation of p38 MAPK is essential in the proliferation of PAFs, and that phosphorylation of p38 MAPK is increased in PAFs exposed to hypoxia. Furthermore, hypoxic hyperproliferation of PAFs can be prevented by inhibition of p38 MAPK [87, 89-91].

Given the failure of KO MPAFs to hyperproliferate in hypoxia seen in this chapter, it was hypothesised that miR-155 KO would prevent p38 MAPK activation. This was indeed the observed outcome. The expected proliferative response to hypoxia of the WT MPAFs was confirmed. However, hypoxic phosphorylation of p38 MAPK was suppressed in the KO cells. Interestingly, even in normoxia, the KO cells appeared to have lower levels of phosphorylated p38 MAPK than their WT controls.

These results proved promising not only in terms of demonstrating that microRNA-155 manipulation could be used as a tool to better understand the pathobiology of pulmonary vascular remodelling, but also that miR-155 downregulation appears to confer a protective effect against the development of PH.

As microRNAs are not universally conserved across species, it remained unknown whether the effect of miR-155 downregulation in mice could be replicated in another species. Furthermore, exploring whether the effects could be achieved pharmacologically would be necessary to determine if microRNA-155 downregulation could have any therapeutic application. The experiments conceived to answer these problems formed the basis for the next chapter.

Chapter 4

Exploration of pulmonary hypertension mechanisms and pathways after pharmacological knockdown of microRNA-155 in an in vitro rat model

4.1 Introduction

The use of miR-155 manipulation as a tool to understand the mechanisms and pathways contributing to pulmonary vascular remodelling was demonstrated in a KO mouse model in Chapter 3. Knockout MPAFs failed to hyperproliferate in response to hypoxic stimulus, and hypoxia-mediated cellular migration was also abrogated by miR-155 knockout. Suppression of the hypoxia-driven p38 MAPK phosphorylation in PAFs was seen in the miR-155 KO cells, and there were enhanced levels of phosphorylated Smad5 in KO MPAFs in response to acute hypoxia. Overall this suggests that miR-155 downregulation protects against pulmonary vascular remodelling, possibly by suppressing activation of p38 MAPK signalling, and by restoring Smad5 levels.

As described previously, the actions of microRNAs are complex. A single microRNA is capable of regulating many genes, and conversely a single gene can be influenced by many microRNAs [137]. Many thousands of microRNAs have been identified in humans alone, and whilst conservation of many of these (and their targets) across species is common, it is not universal. Even if a microRNA is widely conserved across species, the degree of expression can vary from species to species, and at various time points in the development of an organism [147]. Determining whether miR-155 was conserved across species would be important to determine if there was any potential human application for these results.

Transgenic knockout is just one method of manipulating microRNA expression. Pharmacological alteration of microRNA levels has also been demonstrated. Enhanced expression of a microRNA can be achieved through use of a miR-mimic, which acts as a substitute for a downregulated microRNA. Suppression of microRNA levels can also be achieved by an antimiR, an oligonucleotide with a sequence complimentary to the microRNA being targeted. This therefore binds to the microRNA causing downregulation/knockdown.

The potential therapeutic application of manipulating microRNA levels has been explored. Amongst the potential benefits of miRNAs is that their ability to target several genes within a network could enhance the therapeutic effect. Their relatively small size also lends them to ease of delivery into tissues. However,

several challenges also exist. Targeting of the specific tissue where the microRNA imbalance exists can prove difficult, and issues therefore exist with “off-target” effects. Amplification of these “off-target” effects could also be caused by the ability of a single miRNA to suppress many genes. Additional concerns are also raised by the possibility of an immune response to the miR-mimic or antimiR in question, or the vector used to deliver it to its target tissue.

Despite these potential challenges, several microRNAs are being investigated in human clinical trials. MicroRNA-122 is a liver specific miR that binds to the 5’-UTR of the hepatitis C virus genome and promotes the stability and accumulation of HCV RNA. Administration of Miravirsen, an antimiR-122 oligonucleotide, results in a prolonged decrease in HCV RNA in patients with chronic hepatitis C [179]. MIcroRNA-155’s association with haematological malignancy [170, 171] has also led to a clinical trial in humans. A phase 1 trial involving Cobomarsen, an animiR-155 oligonucleotide, is currently underway to evaluate the effect of the antimiR on a form of Cutaneous T Cell Lymphoma [180].

Whatever the potential therapeutic uses of microRNA manipulation, their primary application in this thesis is as an exploratory tool. The key aims of this chapter are to validate and corroborate the results demonstrated in chapter 3, and to explore if they could be replicated both across species and by using a different technique for miR-155 downregulation. The hypothesis central to this is that an antimiR will be a successful alternative way to explore the effects of miR-155 downregulation on pulmonary vascular remodelling, and that it will provide a protective effect against PH.

The hypoxic model of PH will once again be utilised, however PAFs from rats will be used, and downregulation of miR-155 will be achieved through pharmacological knockdown with antimiR-155.

4.2 Relevant methods

The techniques used in this chapter to evaluate the migration and proliferation of cells, and to examine protein expression are described in chapter 2.

MicroRNA-155 knockdown was achieved through liposomal transfection of antimiR-155. Lipofectamine 2000 was used as the transfection reagent and, after optimisation experiments were performed, a volume of 0.5µl per well in a 24 well plate (500µl total/well) or 3µl/well in a 6 well plate (3ml total/well) was used. AntimiR-155 was reconstituted with nuclease-free water per instructions supplied with the product, resulting in a 50µM working stock solution. Lipofectamine/antimiR-155 complexes were prepared at a 1:1 ratio using OptiMEM culture medium and an appropriate volume was then added to each well, such that the final concentration of antimiR-155 was 50nM. After 24 hours, the transfection process was halting by replacing the OptiMEM/antimiR-155 medium with standard DMEM culture medium containing the desired amount of serum. Experiments were then carried out as per standard protocols.

To account for any potential impact on the experiment due to the actions of either the transfection reagent or the transfection process itself, experiments were also performed using a "Lipofectamine-only" control, and a random sequence "Negative Control" antimiR.

The primary antibodies used for immunoblotting, along with their specific dilutions were as follows:

- Phosphorylated p38 MAPK (1:500)
- Total p38 MAPK (1:500)

4.3 Results

4.3.1 Pharmacological knockdown of microRNA-155 abrogates the hypoxic proliferative response in RPAFs.

Abrogation of the hypoxic hyperproliferation of MPAFs by miR-155 knockout was demonstrated in Chapter 3. It was hypothesised that this could be replicated by pharmacological knockdown of miR-155 in rat PAFs. [^{3}H]-Thymidine proliferation assays with increasing doses of serum were performed in normoxia and hypoxia using RPAFs treated with either antimiR-155, a Negative Control antimiR or Lipofectamine reagent alone.

Figure 4-1 displays the results from these proliferation assays.

Under normoxic conditions, the expected pattern of increasing proliferation with increasing doses of serum was seen. This was apparent not only in the cells treated with Lipofectamine alone, but also in the RPAFs transfected with the Negative Control antimiR, and also those treated with antimiR-155.

The expected further proliferative response due to hypoxia was noted in the RPAFs exposed to Lipofectamine alone. This reached significance with the addition of both 1% and 5% FCS.

Similarly, the hyperproliferative response to hypoxia was also seen in the RPAFs that had been transfected with the Negative Control antimiR. Once again, this was noted with 1% and 5% FCS. At the highest serum dose (10%) there was no significant difference between the normoxic and hypoxic Negative Control RPAFs, possibly due to cell contact inhibition.

However, when the RPAFs that had been transfected with antimiR-155 were exposed to hypoxia, there was no additional proliferative response beyond the expected dose-dependent response to serum. This closely mirrored the results seen in the knockout MPAFs in chapter 3 and demonstrated that pharmacological downregulation has a similar effect to transgenic knockout.

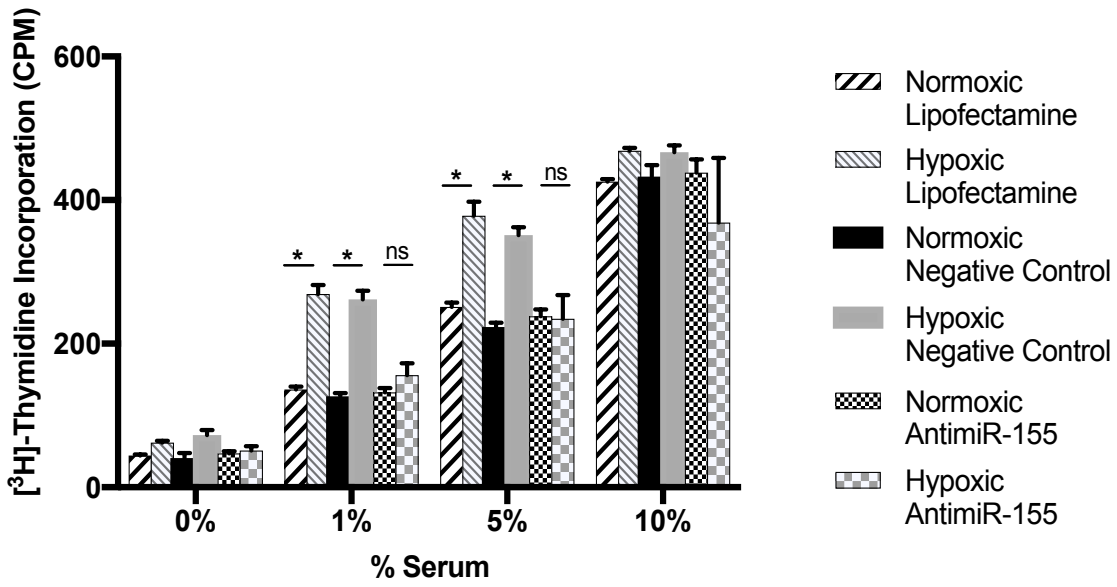


Figure 4-1 Pharmacological knockdown of miR-155 ameliorates the enhanced proliferative response of RPAFs to hypoxia

Normal RPAFs were treated with either Lipofectamine transfection reagent or a negative control antimiR and then cultured in normoxic conditions with the addition of [^{3}H]-Thymidine for the final 4 hours. The effect of stimulation with increasing doses was observed. Increased proliferation with higher doses of serum was noted, as indicated by increased incorporation of [^{3}H]-Thymidine with radioactivity measured as CPM. When repeated in hypoxia an additional proliferative effect was noted, which reached significance at 1% and 5% serum, although the signal was lost at 10% serum, possibly due to contact inhibition. When the proliferation assay was repeated with RPAFs which had been treated with antimiR-155, the dose-dependent proliferative response was maintained. However, these antimiR-155 treated RPAFs did not demonstrate any additional response to hypoxia. Four experiments per condition were conducted, with values expressed as mean + SEM. Results were analysed using 2-way ANOVA with Bonferroni post-hoc analysis. *$p<0.05$.

4.3.2 Knockdown of miR-155 eliminates the migratory response of RPAFs to hypoxia

Prevention of hypoxia-induced MPAF migration by miR-155 knockout was shown in Chapter 3. Transient downregulation of miR-155 in rat PAFs was hypothesised to produce the same effect. Scratch assays were performed on RPAFs that had been treated with either antimiR-155, a Negative Control antimiR, or Lipofectamine reagent alone, and subsequently exposed to either normoxia or hypoxia.

Figure 4-2 displays the results.

After 24 hours in normoxic conditions, the scratch created in the RPAFs treated with Lipofectamine alone persisted, with no degree of closure identified. When exposed to hypoxia however, migration of the Lipofectamine control RPAFs led to complete closure of the scratch, $p<0.0001$ (figures 4-2A).

Similar findings were seen with the Negative Control antimiR (figures 4-2B). After 24 hours in normoxic conditions, the scratch created in the NC RPAFs was still 93.7% of its original width, showing minimal migration of the cells. However, after 24 hours in hypoxia, migration of the NC RPAFs resulted in complete abolition of the scratch.

However, knockdown with antimiR-155 prevented this hypoxia-driven migratory response of the RPAFs, with no significant difference in the width of the scratch after 24 hours, compared with the normoxic experiment (figure 4-2C).

The width of each scratch was taken at 4 points. The graph in figure 4-2D summarises the results.

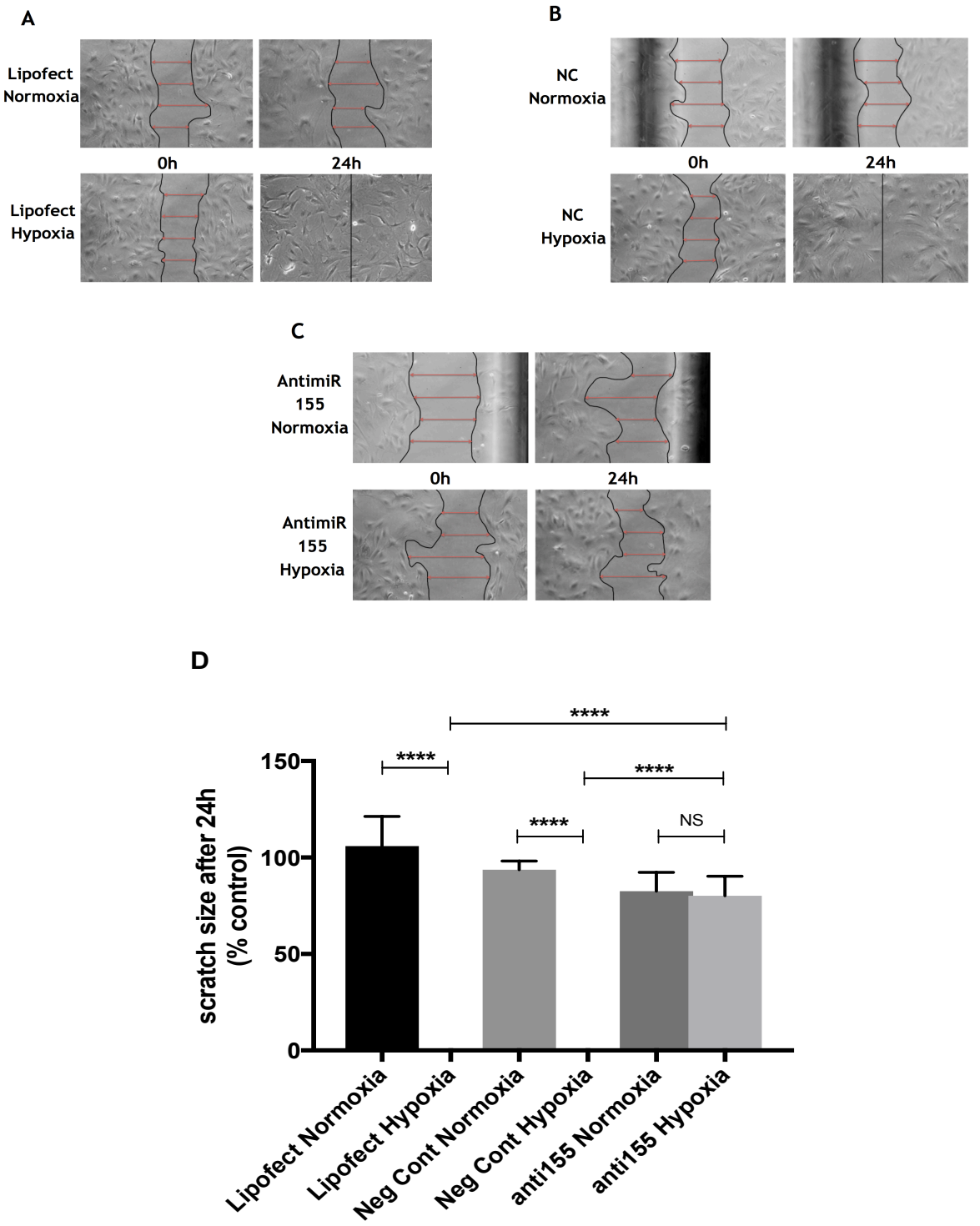


Figure 4-2 Pharmacological knockdown of miR-155 abrogates hypoxic migration of RPAFs. (A) RPAFs were cultured until they were near 100% confluent and the incubated for 24 hours with Lipofectamine 2000 transfection reagent. A vertical scratch was drawn through the monolayer of cells and photographed. The RPAFs were cultured for another 24 hours in either normoxia or hypoxia with fresh culture medium. The size of the residual scratch was then compared to the scratch at baseline. Measurements were taken at 4 equally-spaced points for each scratch. Whilst in normoxic conditions the scratch remains, the RPAFs exposed to hypoxia migrate to close the scratch entirely. (B) Scratch assay repeated with a Negative Control antimiR, introduced into RPAFs by liposomal transfection utilising Lipofectamine 2000. Once again, the scratch remains largely unchanged in normoxic conditions, but hypoxia results in closure of the scratch (C) RPAFs transfected with antimiR-155. After 24hrs in hypoxia, there is no significant difference in the degree of closure of the scratch compared with the cells exposed to normoxia. (D) Graphical representation of the change in scratch size for each condition after 24 hours, expressed as a percentage of the scratch size at 0 hours. Data presented as mean +/- SEM, analysed by 2-way ANOVA with Bonferroni post-hoc analysis, ****$p<0.0001$.

4.3.3 Hypoxia-induced p38 MAPK phosphorylation in RPAFs is ameliorated by treatment with antimiR-155.

In Chapter 3 it was demonstrated that the hypoxia-induced phosphorylation of p38 MAPK is lost in miR-155 KO MPAFs. It was hypothesised that this could be replicated by pharmacological knockdown of miR-155 in RPAFs. Transfection of RPAFs with either antimiR-155 or negative control antimiR, or treatment with Lipofectamine alone, was carried out. Incubation in either normoxia or hypoxia was undertaken, both with and without serum. Immunoblotting of cell lysates for total and phosphorylated p38 MAPK was performed.

Figure 4-3 shows these immunoblots along with a graph demonstrating the relative density of phosphorylated p38 MAPK compared with total p38 MAPK.

When exposed to hypoxia, both the Lipofectamine-only RPAFs and those transfected with the Negative Control antimiR demonstrated a marked increase in the levels of phosphorylated p38 MAPK, compared to the normoxic controls.

However, treatment of RPAFs with antimiR-155 ameliorated the effect of hypoxia, with significantly less p38 MAPK phosphorylation in the antimiR-155 RPAFs, compared to either of the control RPAF groups in hypoxia, $p<0.0001$.

Densitometry was used to quantify these results (figure 4-3B).

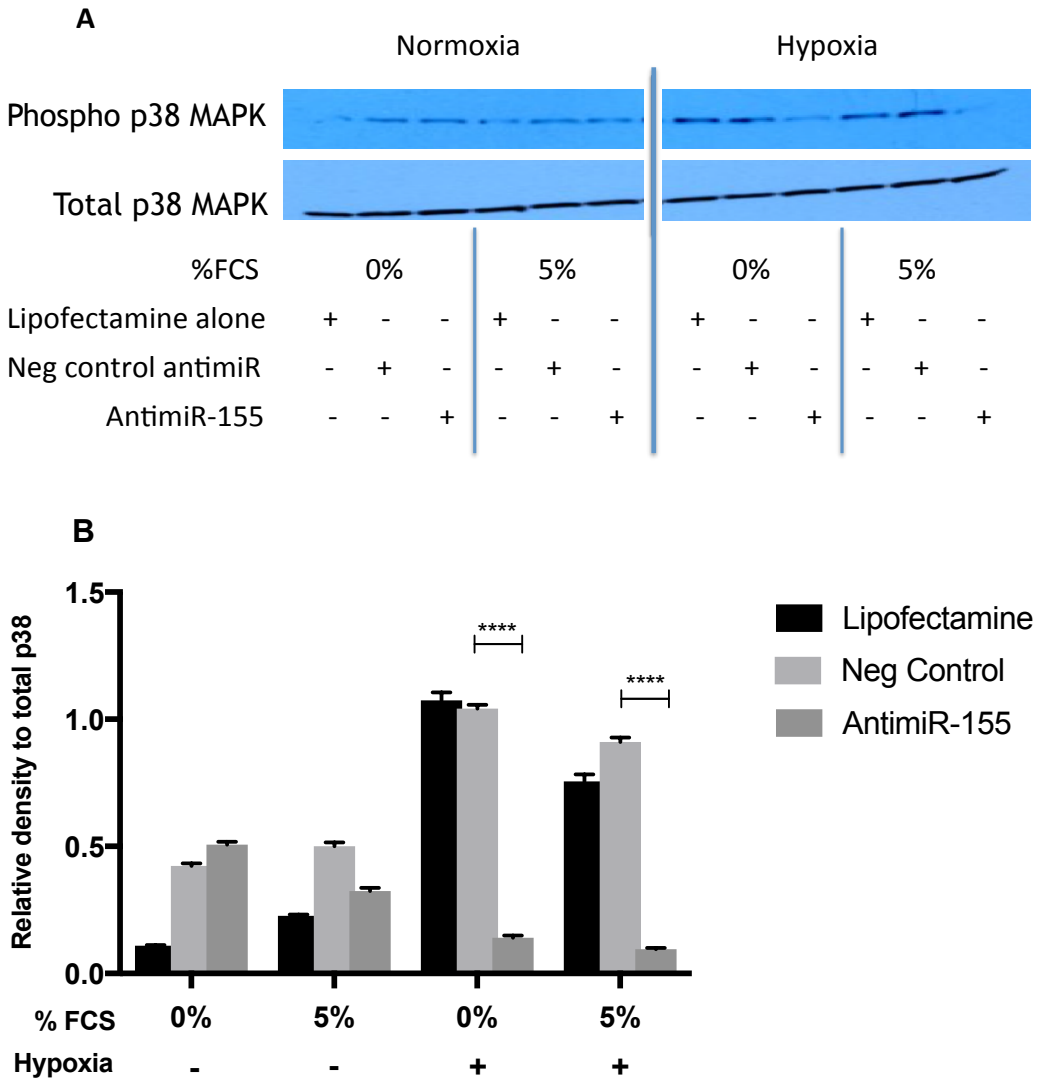


Figure 4-3 Treatment with antimiR-155 abrogates the hypoxic phosphorylation of p38 MAPK in RPAFs

(A) Liposomal transfection of RPAFs with antimiR-155 or a negative control antimiR using Lipofectamine 2000 transfection reagent was performed. A third group of RPAFs was treated with Lipofectamine transfection reagent alone. Half of the wells for each condition were then additionally stimulated with 5% FCS. Culture in either normoxia or hypoxia subsequently took place for 24 hours. Cell lysates were then immunoblotted for phosphorylated p38 MAPK or total p38 MAPK. In hypoxic conditions, the pharmacological knockdown of miR-155 resulted in downregulation of phosphorylated p38 MAPK levels, compare to RPAFs treated with either the negative control antimiR or transfection reagent alone. Molecular weights: phosphorylated p38 MAPK and total p38 MAPK both 43kDa. (B) Results quantified using densitometry, as density relative to total p38 MAPK. Data expressed as mean +/- SEM, Analysed by 2-way ANOVA with Bonferroni post-hoc analysis, ****$p<0.0001$.

4.4 Summary

This chapter has demonstrated that the effects of miR-155 downregulation on mechanisms of pulmonary vascular remodelling and the p38 MAPK pathway can be replicated when an alternative method of manipulating microRNA levels is applied to a different species. This implies that the actions of miR-155, at least within the experiments carried out here, are conserved between mice and rats. Treatment of rat PAFs with antimIR-155 protects against the development of features seen in PH.

As outlined in the general introduction, the proliferative response to hypoxia by PAFs is a key early development in pulmonary vascular remodelling [22, 23]. In this chapter, the expected hyperproliferative response to hypoxia was seen in the Lipofectamine-control RPAFs and the Negative Control antimiR RPAFs. However, this additional proliferation due to hypoxia was prevented in the RPAFs treated with antimiR-155. These results successfully show that transient knockdown with antimiR-155 can achieve the same results as those seen with knockout mice PAFs in Chapter 3, conveying a protective effect against PH.

It was noted however that the radiation counts (i.e. the CPM) were generally lower in the RPAFs used in the knockdown experiments, compared with the KO MPAFs in Chapter 3. The exact reason for this is unclear. One potential explanation is that the RPAF cells may have been older, in terms of their passage stage, than the MPAFs from Chapter 3. They may also have been previously stored in liquid nitrogen and this could have had an influence on their proliferation. A further possibility is that the transfection reagent is causing a degree of cellular toxicity. This could perhaps have been clarified if the proliferation assay was also performed on a separate control group of RPAFs, in the absence of Lipofectamine.

Muscularisation of previously non-muscularised vessels through the transformation of PAFs and their subsequent migration contributes to remodelling [28, 29]. Whilst the lipofectamine and negative control RPAFs migrated in response to hypoxia as expected in the scratch assays, this response was abrogated in the RPAFs transfected with antimiR-155. Once again, the results of Chapter 3 were

replicated in a different species using a different technique for miR-155 downregulation.

The Lipofectamine reagent did not obviously appear to negatively impact on the migration process in a way that would support the theory that it potentially contributed to the decreased radiation counts seen in the proliferation assays. However, it may be that the 24hr timeframe was still sufficient for the RPAFs which hadn't been treated with antimiR-155 to overcome any impediment to their migration caused by the transfection reagent. Migration assays assessed at various time points between 0 and 24 hours may help clarify this.

Upregulation of p38 MAPK pathways is seen in RPAFs following exposure to hypoxia, resulting in their proliferation. This hypoxic proliferation can be prevented by p38 MAPK antagonism [87, 89-91]. Results from Chapter 3 show that miR-155 KO prevents the hypoxic upregulation of p38 MAPK in MPAFs. Corroboration was therefore sought using this second model. Higher levels of phosphorylated p38 MAPK were detected in the Lipofectamine RPAFs and the Negative Control RPAFs as a result of acute hypoxia. This was prevented when RPAFs were treated with antimiR-155, mirroring results from the previous chapter. This once again suggests that the inhibition of PAF proliferation caused by miR-155 downregulation is mediated by p38 MAPK.

The interactions between hypoxia, PAFs and p38 MAPK are well-established and a key theme of the work carried out in our lab. The evidence obtained from these two *in vitro* models is that miR-155 also links into this relationship. MicroRNAs are known to be capable of influencing multiple genes in the same disease network. This raises the question as to what other PH-relevant genes might be influenced by miR-155 downregulation.

The previously described links between Smad5 and miR-155 in haematological malignancy [167], combined with the involvement of Smad5 in TGF-β/BMP signalling, encouraged the investigation into a potential relationship between the two in PH. Chapter 3 confirmed the expected inverse correlation between Smad5 and miR-155, with increased levels of phosphorylated Smad5 seen when MPAFs are exposed to acute hypoxia.

Attempts to replicate these results using RPAFs transfected with antimiR were made alongside the other work from this chapter, but no interpretable results could be obtained. Non-specific binding of the primary antibody was repeatedly seen when the immunoblot was being probed. It was confirmed that the antibody in use was suitable for Western blots with mouse cells, but despite repeated efforts to optimise antibody concentrations an interpretable blot could not be produced. Similar results were noted with an antibody from a second manufacturer. Once again, the possibility of interference in the process by the transfection reagent or some other aspect of the transfection process is a potential explanation for these difficulties.

Despite the issues experienced with linking miR-155 and Smad5 in the knockdown RPAFs, the results overall arguably fulfil the aims set out at the beginning of this chapter. The actions of miR-155, at least with regards to the measures of pulmonary vascular remodelling assessed here, have been shown to be conserved across at least two species. The use of pharmacological knockdown appears to have succeeded in that results in-keeping with the hypothesised effects of miR-155 downregulation were achieved. These results therefore also provide further evidence for the hypothesis that miR-155 downregulation protects against PH. When combined with the results from the KO MPAFs, a compelling case for miR-155's role as a tool to understand pulmonary vascular remodelling *in vitro* has been built.

The translation of *in vitro* findings into *in vivo* results is an essential next step in understanding disease pathobiology, and therefore establishing if research findings have any potential clinical application or therapeutic role in humans. Exploring the phenotypic effect of miR-155 knockout on mice exposed to chronic hypoxia was the natural progression of this project. Whether the cellular findings of Chapters 3 &4 would result in the prevention of PH in vivo was a core question. The experiments devised to answer this question are outlined in Chapter 5.

Chapter 5

In vivo effects of microRNA-155 knockout on indices of pulmonary hypertension in an experimental mouse model

5.1 Introduction

Chapters 3 and 4 have demonstrated that manipulation of microRNA-155 in two different *in vitro* models facilitates the investigation of processes and pathways that lead to pulmonary vascular remodelling.

The proliferative and migratory effects of acute hypoxia on PAFs were lost in miR-155 knockout mice. Similar results were shown by pharmacological knockdown of miR-155 in rat PAFs. Manipulation of miR-155 influenced signalling pathways that are known to be important in the development of pulmonary hypertension. In PH, the hypoxic phosphorylation of p38 MAPK is a key driver of fibroblast proliferation. This was ameliorated in the KO MPAFs and in RPAFs treated with antimiR-155. miR-155 knockout mouse PAFs in hypoxia also had enhanced levels of phosphorylated Smad5, compared with wild type counterparts.

Therefore, downregulation of microRNA-155 *in vitro* appears to protect against pulmonary vascular remodelling by preventing PAF migration and proliferation, which may be due to ensuring adequate Smad5 levels (and therefore preserving normal TGF-β/BMP signalling) and by preventing the activation of p38 MAPK signalling. Overall, the apparent implication of this is that miR-155 downregulation may prevent PH.

Investigating whether *in vitro* findings translate to an effect *in vivo* is a key part not only of understanding the pathogenesis of a disease, but also in determining whether those findings may lead to the development of a potential treatment. The choice of animal model to use for an *in vivo* study is pivotal to help bridge the translation of a treatment "from bench to bedside". The model must be validated and fit-for-purpose in order to address the clinical question.

Several of the most commonly utilised animal models of PH have been described in the general introduction. The chronic hypoxic animal model is one of the most well recognised, and is firmly established in the SPVU laboratory. The basis behind this model is the finding that elevated pulmonary pressures and RVH develop in humans and animals that reside at high altitude [115, 116]. Acute hypoxia is a potent vasoconstrictor in the pulmonary circulation, however when hypoxia is

chronic it results in vascular remodelling [104], with HIF-1α mediating many of the cellular changes seen in response to hypoxia. The timeframe for this remodelling can vary between animals. After exposure to hypobaric hypoxia for 2 weeks, rats can develop elevated PA pressures, RV hypertrophy, muscularised and thickened distal vessels, and vascular pruning relatively quickly [117]. In mice however, hypoxia results in haemodynamic changes but remodelling can be less marked [118, 119].

Monocrotaline is a plant-derived toxin usually administered to rats to induce PH. Its effect is thought to be mediated by the direct toxic effect of its metabolites on lung tissue [122] with activation of pro-proliferative and anti-apoptotic factors, combined with dysregulated NO production [123, 124]. Neither the monocrotaline rat model nor the chronic hypoxic model result in formation of the plexiform lesions that are the hallmark of human IPAH. The Sugen/hypoxia model is a newer animal model which more closely reflects the human disease. This is not only because of the development of more extensive remodelling, including the presence of these plexiform lesions [129, 130], but also in the mechanism by which PH is induced. It involves the administration of a VEGF receptor-2 inhibitor (Sugen), followed by exposure to chronic hypoxia. This therefore fits with the current "multiple-hit" hypothesis thought to be important in the development of PH.

Although there are limitations to its use, the simplicity of the chronic hypoxic model led to its application in this thesis. The aim of this chapter is to assess the effects *in vivo* of miR-155 knockout on the development of PH in the chronic hypoxic mouse model, the hypothesis being that miR-155 KO mice would be protected against hypoxic pulmonary hypertension and its consequences.

5.2 Relevant methods

Hypoxic conditions were created using a hypobaric chamber. Mice were housed in the chamber for 14 days at a pressure of 550mbar, after a gradual reduction in atmospheric pressure over an initial 48-hour period. These conditions resulted in a reduction in oxygen concentration from approximately 21% to 10%. Temperature and humidity were maintained at constant levels, food and water were freely available, and a 12-hour light/dark cycle was imposed. Normoxic control animals were maintained adjacent to the chamber under the same conditions, but without depressurisation.

An initial pilot study was undertaken. WT and KO mice were exposed to chronic hypoxia; however, comparison was only made to WT animals in normoxia due to the limited availability of KO animals.

A larger study was subsequently performed, when KO animals under normoxic conditions were also included. Table 5-1 below displays the numbers of mice used in the main study. It outlines their characteristics based on whether they were WT or KO, whether they were exposed to hypoxia or normoxia, and their gender. It should be noted that the disparity between the number of animals used and the number of results obtained was occasionally influenced by technical issues.

Wild Type n = 18	Normoxia n = 7	Male n = 3
		Female n = 4
	Hypoxia n = 11	Male n = 6
		Female n = 5
Knockout n = 13	Normoxia n = 7	Male n = 4
		Female n = 3
	Hypoxia n = 6	Male n = 3
		Female n = 3

Table 5-1 Characteristics of the mice used in the main in vivo study

The methods for measuring haemodynamics and assessing RVH and vascular remodelling have been outlined already in Chapter 2.

5.3 Results

5.3.1 Pilot study

5.3.1.1 Right ventricular systolic pressure

The right ventricle was catheterised via the right internal jugular vein using a Millar Mikro-Tip® PVR-1030 pressure-volume catheter. Figure 5.1 displays the RVSP results from the pilot study.

The mean right ventricular systolic pressure of the WT mice kept in normoxia was 23.62mmHg. When exposed to hypoxia, the mean RVSP rose to 38.64mmHg, a significant increase compared with the normoxic controls, $p<0.0001$.

However, the mean RVSP of the knockout animals in hypoxia was significantly lower than the hypoxic WT controls at 31.91mmHg, $p<0.005$.

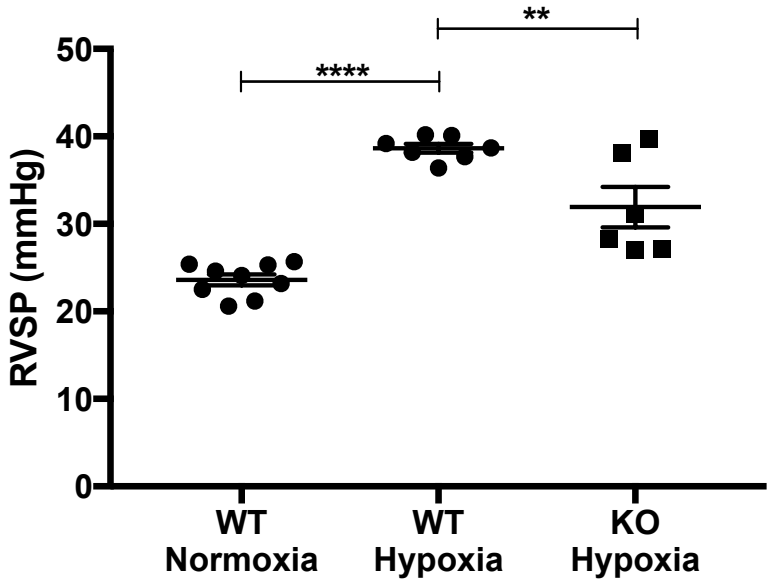


Figure 5-1 MicroRNA-155 knockout reduces the effect of chronic hypoxia on RVSP in pilot data from an experimental mouse model.
Wild type and knockout mice were exposed to hypobaric hypoxia for two weeks. Right ventricular systolic pressure was then measured. Wild type controls in normoxic conditions were also studied. MicroRNA-155 knockout conveys a protective effect against hypoxia, with lower RVSP than wild type animals in hypoxia (31.91mmHg vs 38.64mmHg). Data points indicate individual animals. Values are mean +/- SEM, analysed by one-way ANOVA with Bonferroni post hoc analysis, **p<0.005, ****p<0.0001

5.3.1.2 Right ventricular hypertrophy

Hearts from the WT and KO mice that had been exposed to hypoxia were obtained. Right ventricular hypertrophy was assessed by Fulton's Index, the ratio of the mass of the RV free wall to the LV plus septum.

The mean Fulton's Index of the WT mice after hypoxic exposure was 0.387. However, this was significantly lower in the hypoxia-exposed KO mice (0.345, $p<0.05$).

The knockout mice therefore developed less RVH, as shown in figure 5-2.

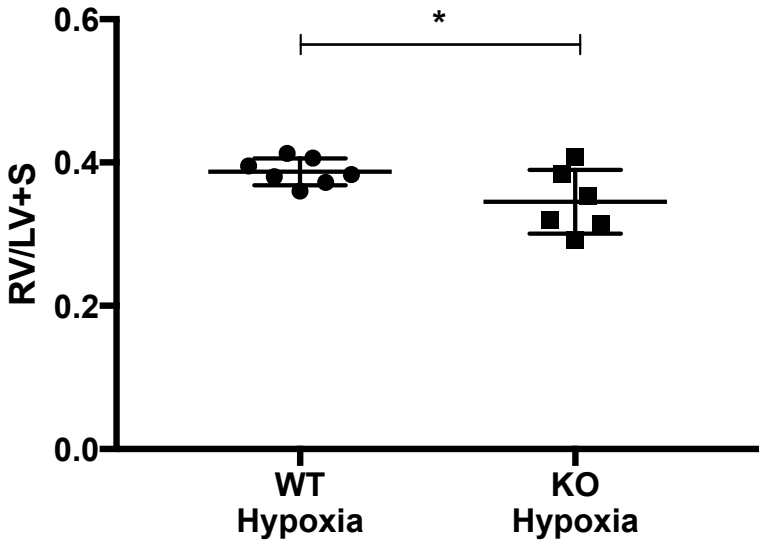


Figure 5-2 RVH is reduced in KO mice exposed to hypoxia versus WT controls in pilot data from an experimental mouse model.
Heart were isolated from the mice. The right ventricular wall was separated from the left ventricle and septum. The ventricles were weighed after being blotted dry. The ratio of the RV to LV+S was calculated (Fulton's Index). After hypoxic exposure, the KO mice had significantly less RVH than the WT mice, as demonstrated by a lower Fulton's Index of 0.345 vs 0.387. Individual values are plotted and expressed as mean +/- SEM, analysed by unpaired t test. *$p<0.05$

5.3.2 Main study

5.3.2.1 Right ventricular systolic pressure

As in the pilot study, RVSP was measured by cannulating the right internal jugular vein and advancing a pressure-volume catheter into the right ventricle.

Right ventricular pressure traces such as those shown in figure 5-3 were obtained.

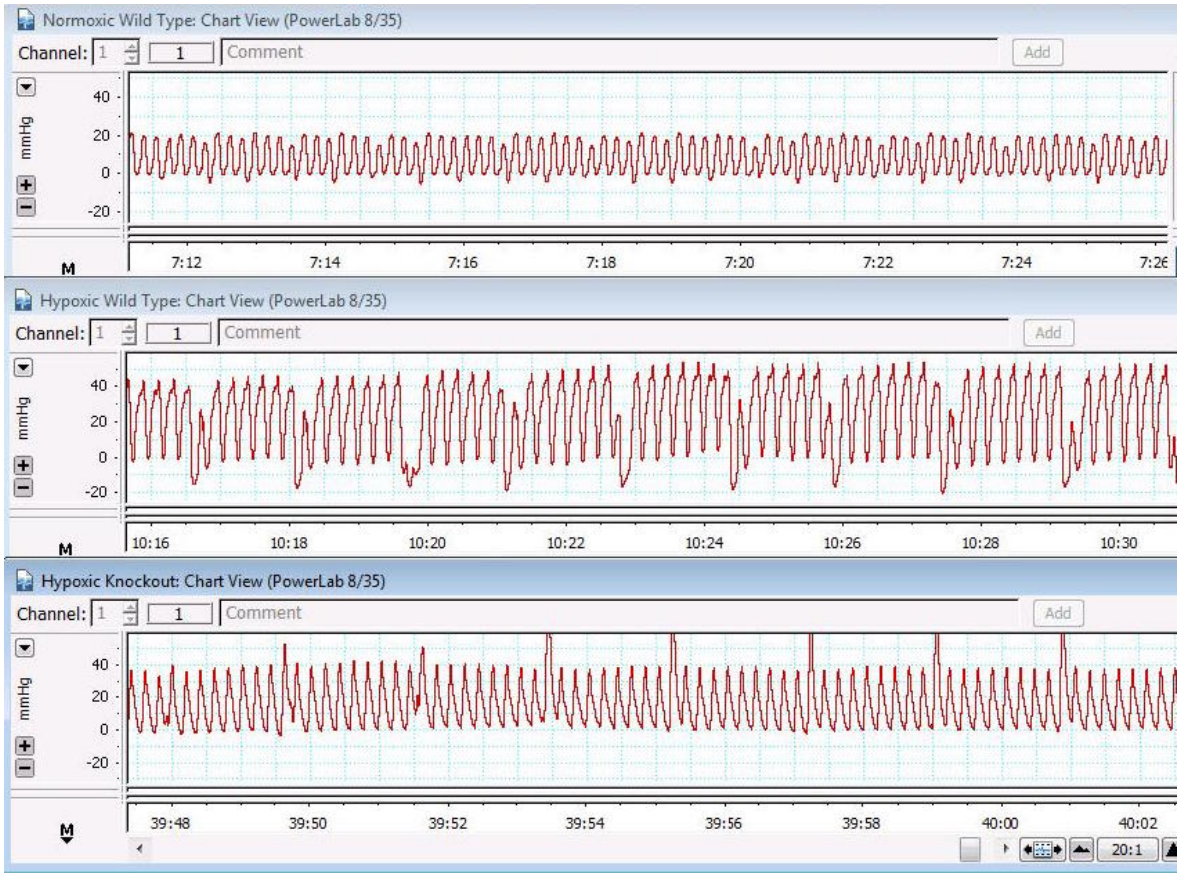


Figure 5-3 Right ventricular pressure traces obtained from mice
The right ventricle was catheterised via the right internal jugular vein using a Millar Mikro-Tip® PVR-1030 pressure-volume catheter. Once a typical RV trace was obtained the readings were allowed to settle. Traces from top to bottom are normoxic WT, hypoxic WT and hypoxic KO.

Figure 5-4 shows the RVSP results from the main study.

Under baseline normoxic conditions, no statistically significant difference in RVSP was demonstrated between the wild type and knockout mice. Mean RVSP in the normoxic WT mice was 23.01mmHg versus 19.85mmHg in the KOs.

When the wild type animals were exposed to hypoxia, mean RVSP was significantly higher than in the normoxic WT controls (38.89mmHg vs 23.01mmHg, $p<0.0001$). The knockout animals also demonstrated a rise in mean RVSP when exposed to hypoxia (31.61mmHg vs 19.85mmHg in the normoxic KOs, $p<0.0005$).

When comparing the effect of hypoxia on the WT and KO mice, miR-155 appeared to convey a protective effect, as the mean RVSP of the KO hypoxic animals was significantly lower than that of the WT hypoxic animals (31.61mmHg vs 38.89mmHg, $p<0.05$).

When calculated as a percentage change in mean RVSP due to hypoxic exposure, the mean RVSP in the WT animals rose by 69%, vs a 59% increase in RVSP in the KO animals after hypoxia. This was calculated using the following equation:

$$\% \text{ change in mean RVSP} = \frac{[\text{mean hypoxic RVSP} - \text{mean normoxic RVSP}]}{\text{Mean normoxic RVSP}} \times 100$$

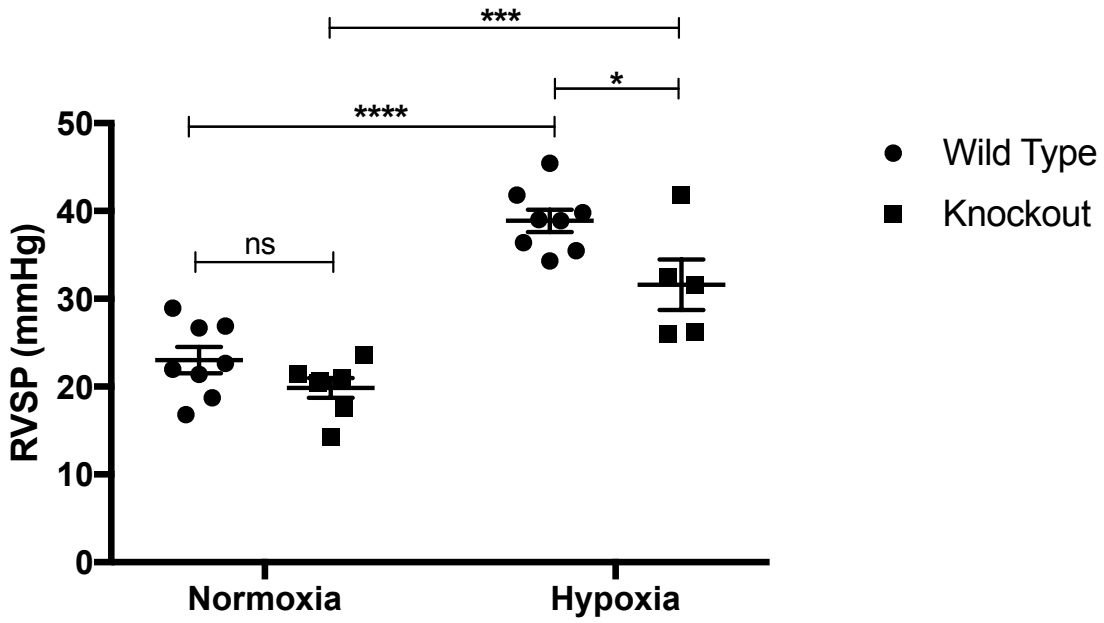


Figure 5-4 MicroRNA-155 knockout mice exposed to hypoxia have a lower right ventricular systolic pressure than wild type controls.
Wild type and knockout mice were either exposed to hypobaric hypoxia for two weeks, or maintained in normoxic conditions for the same duration. RVSP was then measured. There was no significant difference in RVSP between the WT and KO animals in normoxia (23.01mmHg vs 19.85mmHg). Both the WT and KO animals showed an increase in mean RVSP when exposed to hypoxia (from 23.01mmHg to 38.89 mmHg, and 19.85mmHg to 31.61mmHg respectively). After exposure to hypoxia however, there was a significantly lower RVSP in the KO mice compared to the WT controls in hypoxia (31.61mmHg vs 38.89mmHg). Mean values for individual animals are plotted +/- SEM, n = 5-8 animals. Analysis by two-way ANOVA with post hoc Bonferroni test, *$p<0.05$, ***$p=0.0005$, ****$p<0.0001$

5.3.2.2 Right ventricular hypertrophy

Fulton's Index, the ratio of the mass of the RV free wall to the LV plus septum, was again used to assess RVH. Hearts were obtained from both WT and KO mice, after exposure to either normoxia or hypoxia. The results are shown in figure 5-5.

There was no significant difference between the mean Fulton's Index of the WT and KO mice that had been kept in normoxic conditions, 0.292 and 0.243 respectively.

Fulton's Index was higher in the WT mice after hypoxic exposure, compared with their normoxic controls, demonstrating the development of RVH (0.364 vs 0.292, $p<0.01$). The KO animals however did not show a significant increase in Fulton's Index after hypoxic exposure (0.303 vs 0.243 for the KO animals in normoxia). The development of RVH in these mice after two weeks of hypoxia therefore appeared to be attenuated by miR-155 knockout.

Finally, whilst there appeared to be a trend towards a lower mean Fulton's Index in the KO mice in hypoxia compared with the WT mice in hypoxia, this did not amount to a statistical significance (0.303 vs 0.364).

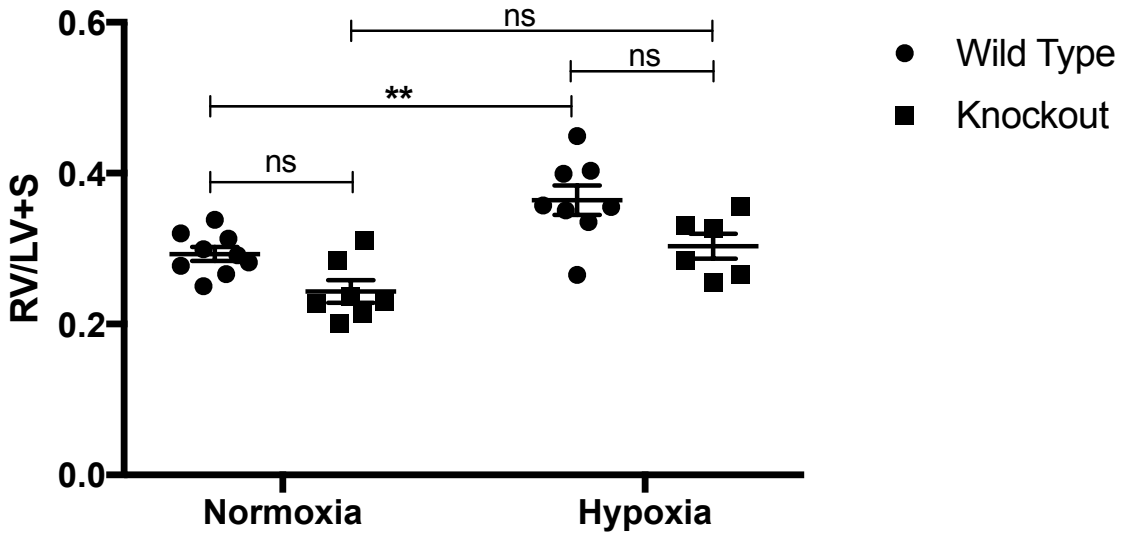


Figure 5-5 The effect of hypoxia on the development of RVH in mice is attenuated in microRNA-155 KO, compared to WT controls.
Knockout mice and wild type controls were exposed to either hypoxia or normoxic conditions for two weeks. Hearts were then isolated from the mice. The right ventricular wall was dissected from the left ventricle & septum, and after being blotted dry, the ventricles were weighed, and the ratio of RV/LV+S (Fulton's Index) was calculated. Fulton's index was not statistically different between the WT and KO mice in normoxia (0.292 vs 0.243). Exposure of the WT mice to hypoxia lead to the development of RVH, as demonstrated by a Fulton's Index of 0.364 vs 0.292 in WT normoxic animals. Two weeks of hypoxic exposure did not cause significant RVH in the KO animals (Fulton's Index 0.303 vs 0.243 in the KO normoxic controls). There appeared to be a lower Fulton's Index in KO hypoxic mice compared with WTs in hypoxia, but this was not significant. Data points represent individual animals, n = 6-9 animals, mean +/- SEM. Two-way ANOVA with post hoc Bonferroni test was performed, **$p<0.01$

5.3.2.3 Pulmonary vascular remodelling

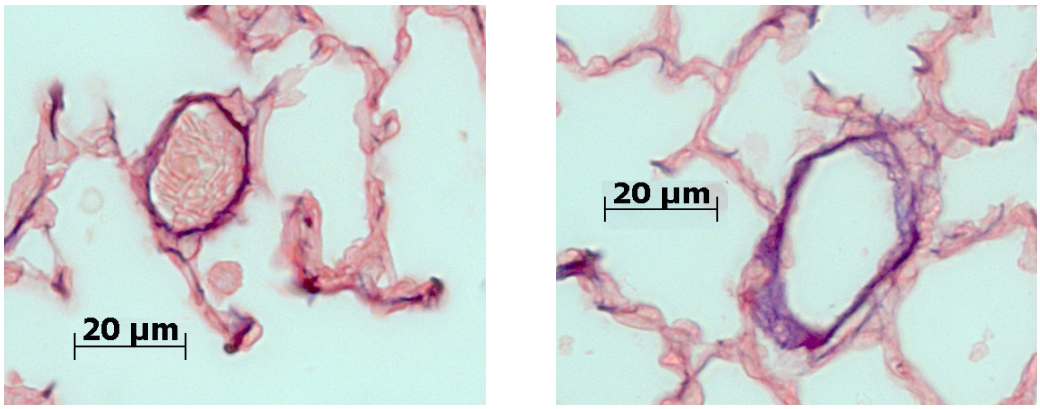
The method for evaluating pulmonary vascular remodelling has been described in chapter two. Vessels from WT and KO mice were obtained, after exposure to either normoxia or hypoxia. Pulmonary vascular remodelling was deemed as present if a vessel less than 80μm had a double elastic laminae for >50% of its wall's circumference. The results are summarised in figure 5-6.

Under normoxic conditions, there was no difference between the percentage of remodelled vessels seen in WT animals and the KO animals (6.71% vs 6.51%, respectively).

Pulmonary vascular remodelling however was clearly demonstrated in the lungs of wild type animals exposed to hypoxia, with a mean of 13.75% of vessels remodelled, compared with 6.71% in the WT normoxic animals.

Interestingly, only 7.06% of the KO vessels demonstrated remodelling after hypoxic exposure, similar to the results seen in the KO mice in normoxia, and significantly lower than the 13.75% of vessels in the WT hypoxia, $p<0.0001$. Knockout of miR-155 therefore appeared to protect against hypoxic vascular remodelling.

A



Non-remodelled Remodelled

B

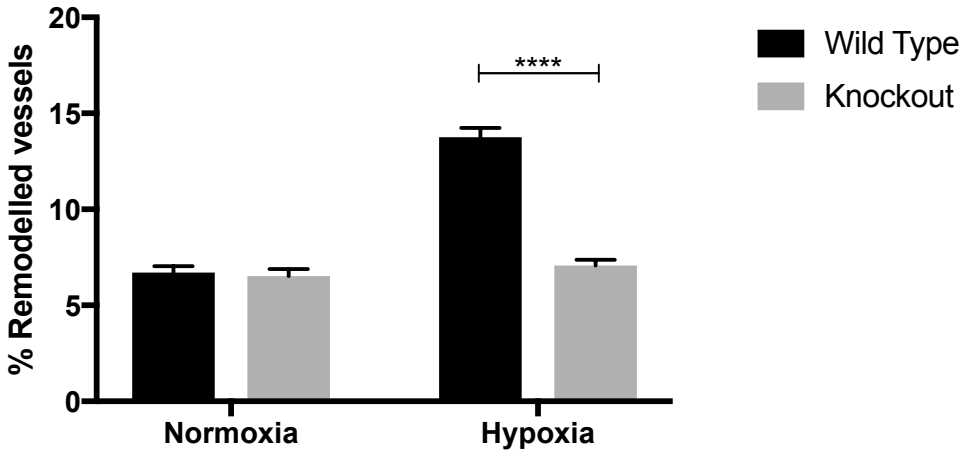


Figure 5-6 MIcroRNA-155 knockout prevents remodelling of pulmonary vessels in an experimental mouse model

(A) Lungs were removed and 5µm sagittal sections were cut. These were stained with Miller's Elastic stain and assessed microscopically in a blinded fashion. Vessels less than 80µm and not associated with an airway were analysed for remodelling, defined as the presence of a double elastic laminae for >50% of the vessel wall circumference. Representative examples of remodelled and non-remodelled vessels are displayed. Hypoxia promoted the remodelling of small non-muscularised vessels in the wild type mice. miR-155 KO prevented this hypoxic vascular remodelling. Lung sections from three mice per group were studied, with three sections counted per mouse, and approximately 90 vessels per section. (B) Results presented graphically. Analysis by two-way ANOVA with post hoc Bonferroni test was performed, ****$p<0.0001$

5.4 Summary

This aim of this chapter was to explore whether the encouraging *in vitro* results from Chapters 3 and 4 would translate to a demonstrable impact on *in vivo* indices of PH including haemodynamics and vascular remodelling.

Downregulation of miR-155 *in vitro* resulted in attenuation of the hypoxia-driven proliferation and migration of PAFs, key contributors to vessel remodelling in PH [23, 29]. The p38 MAPK signalling pathway is a key driver of the PAF response to hypoxia, including proliferation [90, 91]. Activation of this pathway was prevented by miR-155 downregulation. Smad5 is a proven target of miR-155 and is essential for normal TGF-β/BMP signalling. The literature suggests a negative correlation between miR-155 and Smad5 [167], and therefore abnormal signalling through Smad5 due to the action of miR-155 would be anticipated. Chapter 3 showed elevated levels of Smad5 in miR-155 knockout MPAFs exposed to hypoxia, suggesting that miR-155 downregulation aids normal TGF-β/BMP signalling.

This chapter's results are in-keeping with the *in vitro* results and support the core hypothesis that miR-155 downregulation protects against PH. An initial pilot study demonstrated that miR-155 KO mice had lower RVSP after two weeks in hypoxic conditions, compared to wild type controls. The KO mice also had less hypertrophy of their right ventricles. These results, whilst promising, were limited by the lack of assessment of the baseline features of the KO mice, as there was no group of KO animals that had been kept in normoxic conditions.

A further study was subsequently carried out, this time with analysis including KO mice in normoxia. The KO mice at baseline had an RVSP that was not significantly different to the WT controls, 19.85mmHg vs 23.01mmHg. In hypoxia, whilst both KO and WT animals developed a degree of pulmonary hypertension, the RVSP in the KO animals was lower than the WT animals (31.61mmHg vs 38.89mmHg, $p<0.05$) indicating a partially protective effect of miR-155 KO against the development of PH in mice.

As would be expected, WT mice in the main study developed RVH as a result of hypoxic exposure. The KO mice in hypoxia however had a Fulton's Index that was

not significantly different to their normoxic controls. This may indicate some degree of protection, although it's also possible that a more prolonged exposure to hypoxia would have led to more significant remodelling of the right ventricle.

Finally, the degree of pulmonary vessel remodelling was also assessed. The wild type animals developed clear evidence of remodelling after two weeks in hypoxic conditions. However, after two weeks in hypoxia, the KO animals had not developed any additional evidence of remodelling beyond their normoxic counterparts, which again suggests a protective effect against the development of PH and its consequences.

Whilst the lack of remodelling in the KO hypoxia animals is in-keeping with the core hypothesis, the degree of remodelling seen in the WT mice in hypoxia is perhaps surprising to some degree. The generalised consensus from the literature is that whilst chronic hypoxic mice develop haemodynamic changes consistent with PH, the degree of vascular remodelling is less marked at two weeks than that seen in other animals, for example, chronic hypoxic rats [118, 119]. Therefore, whilst the detected difference in remodelling is encouraging, the reported literature should be borne in mind.

It should be noted that in terms of absolute percentages, the difference in remodelled vessels in the hypoxic WT versus hypoxic KO mice from this study was 13.75% versus 7.06%. Therefore 86.25% of vessels in the hypoxic WT mice were not remodelled. The literature also reports that different mouse strains respond differently to hypoxic stimulus. For example, the C57BL6 strain responds moderately, whilst other strains exhibit very little remodelling [181]. The degree of remodelling can also depend upon the age of the animal, with the lungs of younger animals being more susceptible to the effects of chronic hypoxia [35]. These factors should be considered when interpreting the results presented.

In summary, use of the chronic hypoxic mouse model has demonstrated that the in vitro results from Chapters 3 & 4 could be translated to an in vivo effect. The results from this chapter have shown that miR-155 knockout has a protective effect against the development of pulmonary hypertension in a chronic hypoxic mouse model.

Chapter 6

Discussion

6.1 Discussion of findings

The impact of miR-155 downregulation on signalling pathways and pathological processes relevant to pulmonary vascular remodelling, and the subsequent in vivo effects, have been studied for the first time. Overall, a protective effect against the development of pulmonary hypertension appears to be conveyed by downregulation of miR-155. Manipulation of the levels of this microRNA has proven to be a useful tool in understanding the pathobiology of PAH.

6.1.1 Downregulation of microRNA-155 influences signalling pathways important in pulmonary hypertension

The TGF-β/BMP signalling superfamily regulates cardiovascular development and disease. In PH, it influences the proliferation, differentiation and migration of PAECs and PASMCs [36, 71, 72]. Mutations of receptors from this family are also key pathological influences in hereditary PAH and PAH related to HHT, for example BMPR2 mutations [77, 80, 81]. Links between miR-155 and TGF-β/BMP signalling exist, and a prominent example is the effect of miR-155 on Smad5, a key signalling intermediary of the superfamily. This has been explored in the context of Diffuse Large B-Cell Lymphoma, where miR-155 was found to directly control Smad5 [167] and promote dysregulated cell growth. The interaction between miR-155 and Smad5 in PH was therefore examined.

Results from chapter 3 illustrate that under basal normoxic conditions the predicted inverse correlation between miR-155 and Smad5 was not seen. In fact, there was a signal that the KO MPAFs in normoxia had lower levels of phosphorylated Smad5. Hypoxia however had the effect of increasing Smad5 levels in both the WT and KO MPAFs, but this was significantly greater in the KO cells, which is in-keeping with the predicted inverse correlation.

It is unclear exactly why the anticipated inverse correlation between miR-155 and Smad5 was not seen in normoxia. Perhaps a further stimulus such as hypoxia is required for this relationship to be manifested. Importantly, these results tell us about the activated state of Smad5 in these conditions rather that the total levels,

and perhaps using an additional primary antibody which detected total levels of Smad5 would clarify the picture. Given that miR-155 controls Smad5, I would expect to see increased levels of total Smad5 in the KO in both hypoxia and normoxia. Whether increased activation of Smad5 in the knockout model is protective against PH requires further clarification.

Evidence of an effect on Smad5 levels in RPAFs treated with antimiR-155 was also sought alongside the work carried out for Chapter 4. However, an interpretable result could not be produced. The quality of the immunoblots for phosphorylated Smad5 in RPAFs treated with antimiR-155 was generally disappointing, with non-specific binding of the primary antibody repeatedly seen. This was despite multiple attempts to optimise the experiment conditions and antibody concentrations, including using an antibody from a second manufacturer. The possibility of interference from the transfection reagent, or some other aspect of the transfection process, may be a potential explanation for this lack of results.

The role of p38 MAPK as a key regulator of pulmonary vascular remodelling has been established. p38 MAPK is essential to the proliferation of PAFs in acute hypoxia, and is upregulated in PAFs from chronic hypoxic animals. Inhibition of p38 MAPK can prevent or reverse the hyperproliferative effect in these situations [89-91]. Endothelial dysfunction is also caused by hypoxic upregulation of p38 MAPK, but this too can be reversed by p38 MAPK inhibition [93]. Signalling through p38 MAPK is activated in the context of PASMCs with BMPR2 mutations, due to ineffective Smad signalling. The effect of miR-155 knockout on p38 MAPK signalling was therefore an important area to consider.

Results from this thesis reconfirm the activation of the p38 MAPK pathway in normal PAFs exposed to hypoxia. However, the downregulation of miR-155 abrogates this hypoxic phosphorylation of p38 MAPK. This is the case in both KO MPAFs and RPAFs treated with antimiR-155. Given that p38 MAPK controls the hypoxic proliferation of PAFs and causes endothelial dysfunction, it can be inferred that miR-155 downregulation confers a protective effect against hypoxia-driven remodelling via p38 MAPK. Once further insight had been gained into some of the influence miR-155 had on signalling pathways, it was relevant to examine

the effect that this would have on some of the cellular mechanisms which lead to pulmonary vascular remodelling.

6.1.2 Mechanisms of pulmonary vascular remodelling are controlled by microRNA-155

The pathobiology of pulmonary hypertension involves vasoconstriction, vessel remodelling, vascular pruning and thrombosis in situ [18]. The current treatments for PAH are aimed primarily at treating vasoconstriction and have minimal effect on remodelling. They offer functional benefit but improved survival is seen only with epoprostenol [14] and macitentan [15]. Therefore, treatments which target remodelling are vital for the future management of PAH.

Dysfunctional cell proliferation, migration and apoptosis drives remodelling. Evidence of early adventitial remodelling and fibroblast proliferation has been noted in hypoxic animal models of PH [22], and hypoxic proliferation of PAFs has been shown to be greater than in other cell types [23]. The transformation of PAFs towards a myofibroblast phenotype occurs in hypoxia [27, 28], and these myofibroblasts migrate through the vessel wall resulting in the distal muscularisation of previously non-muscularised vessels [29-31]. Hypoxia-driven proliferation of PAFs has been proven to be conserved across species [86, 87].

In this thesis, the effect of miR-155 downregulation on proliferation was examined in MPAFs and RPAFs. In both models, there was a dose-dependent proliferative response to serum, and normal PAFs had a hyperproliferative response when exposed to hypoxia. However, both genetic and pharmacological downregulation of miR-155 ameliorated the hyperproliferative response to hypoxia.

Migration was assessed using scratch assays. Normal MPAFs and RPAFs responded as expected, with closure of the scratch confirming the migratory response of the PAFs to hypoxia. However, miR-155 knockout in mice or knockdown with antimiR-155 in rat PAFs prevented this hypoxia-driven migratory response.

Taken together, these results indicate that downregulation of miR-155 reduces pulmonary vascular remodelling by ameliorating the hypoxic proliferation and

migration of PAFs, and hence offers a protective effect against PH. This may be in part due to decreased p38 MAPK activity, given the results discussed above and by maintenance/restoration of Smad5 levels and therefore normal TGF-β/BMP signalling. The *in vivo* effect of altering these mechanisms was examined next.

6.1.3 Pulmonary hypertension and miR-155: *in vivo* translation

Compelling evidence for a beneficial effect of miR-155 downregulation *in vitro* has been revealed. Furthermore, given the different *in vitro* models utilised, the action of miR-155, at least with regards the signalling pathways and remodelling mechanisms outlined in this thesis, appears to be conserved across species. However, for the findings of biomedical research to have relevance to patient care, the translation of results from "bench to bedside" much be demonstrable. Evaluating the *in vivo* effects of miR-155 downregulation on indices of PH and pulmonary vascular remodelling was the vital next step in establishing the role of miR-155 in PH. The merits and disadvantages of various animal models of PH have been discussed in the introduction. The chronic hypoxic model was employed in this study.

An initial pilot study was performed. Encouraging results were obtained, with a significantly lower RVSP found in the hypoxic knockout mice compared to their hypoxic wild type controls. RV hypertrophy was less marked in the hypoxic KO mice. However, understanding the full implications of miR-155 KO was limited by the lack of assessment of KO animals in normoxic conditions.

A larger study was therefore undertaken, in order to corroborate results from the pilot, but also to evaluate baseline characteristics of the KO mice in normoxia. Once again, the effect of hypoxia on the KO mice was less marked than in their WT controls, as the KO mice had a significantly lower RVSP. At baseline, there was no significant difference between the WT and KO mice in terms of RVSP, suggesting that the protective effect of miR-155 KO against hypoxia is a true effect.

When RV hypertrophy was assessed in the animals from the main study, the WT mice responded as expected to chronic hypoxia, with RVH evident at 2 weeks. In

the KO mice, significant RVH in response to hypoxia was not observed. This could suggest a protective effect against hypoxia or it could reflect the deficiencies of the chronic hypoxic model. It is unclear whether a more prolonged exposure to hypoxia may induce greater RVH in the KO animals.

With regards to vascular remodelling *in vivo*, there was a clear difference in response to hypoxia between the WT and KO animals. Hypoxia resulted in increased vascular wall thickening after two weeks in the wild type animals, whereas this effect was not seen in the knockout mice, which demonstrated a similar degree of vascular remodelling as their normoxic controls. This finding is in-keeping with the other in vivo results and corroborates the main hypothesis. Intriguingly, the degree of remodelling in the WT animals was perhaps a little unexpected, as the consensus from the literature suggests that remodelling in less prominent in mouse models than other species. However, it has been noted that the degree of remodelling can vary depending on the mouse strain, as well as with the age of the animals [35, 181].

6.2 Limitations

There are several limitations to the studies that were undertaken.

Successfully examining the effect of miR-155 downregulation on Smad5 proved challenging, and this appeared to be due to problems with the primary antibody. Acceptable results were seen with the KO MPAFs, but obtaining interpretable results from the RPAFs treated with antimR-155 proved largely unsuccessful. Various antibodies from different companies were trialled at various concentration. Despite this non-specific binding remained a problem. A possible interaction between the primary antibody and the transfection reagent is among the possible explanations for this.

With regards to signalling pathways, attempts were only made to evaluate Smad5 and p38 MAPK. The examination of more pathways or identified targets of miR-155 that are known to be instrumental in PAH would add weight to this initial

data. Proving the existence of links to the likes of eNOS and IL-6, for example, would help corroborate my hypothesis.

A general criticism of the in vitro work may come from the use of PAFs obtained from proximal pulmonary vessels. As PAH is generally a disease of the smaller distal vessels, results which more closely reflect the human disease may be seen if PAFs from distal vessels were used for the in vitro studies.

In terms of the techniques used to examine remodelling processes, the scratch assay is relatively crude method of assessing migration, although it's ease is attractive. A more robust technique may be the Transwell migration assay, where the ability of cells to pass through a membrane clarifies migratory from non-migratory cells [182].

The advantages and disadvantages of different animal models of PH has been discussed. A limitation of this work might be the choice of animal model used. The hypoxic mouse model develops haemodynamic changes but often lacks a significant degree of vascular remodelling. Chronic hypoxic rats develop a greater degree of remodelling, but still lack the classical plexiform lesions seen in human PAH. Use of a model such as SU5416/hypoxia in mice, which fits with the 'multi-hit' hypothesis of PH, would perhaps better replicate human disease as exemplified by the presence of plexiform lesions [130]. Nonetheless, the fact that corroborating results from two different *in vitro* models was identified, with further confirmatory *in vivo* findings, suggests that the results obtained are valid and important.

A further criticism of the in vivo work is that genetic knockdown is akin to a prevention study, examining if the development of PH could be avoided. Whilst this allowed exploration and understanding of the clinical phenotype of the KO mice, it's relevance or application to patients is less relevant. A treatment study, where WT mice were allowed to develop chronic hypoxic PH and then treated with antimiR-155, would perhaps bear more similarity to the real-life experience of treating patients who have developed the disease.

6.3 Potential clinical application

The purpose of biomedical research is ultimately to aid in the understanding of human disease and their treatments. The findings within this thesis, including the successful translation from cellular results to an in vivo response, are encouraging. It is obvious, however, that this does not necessarily guarantee that the results will translate to an effect in humans. An important first step will be exploring whether the results obtained in rodent cells can be replicated in human PAFs.

As outlined previously, the biology of microRNAs can be complex. When it comes to their therapeutic application, there is the potential for great benefit. However, there are several anticipated/proven downsides. The actions of microRNAs are often not specific to the intended target tissue, creating off-target effects. This is especially in light of the ability of one microRNA to influence multiple genes. Problems with toxicity or immune responses may also arise either due to the therapeutic agent itself (e.g. an antimiR) or the vector used to get it to its target.

Despite these issues, several clinical trials involving microRNAs in humans are underway. These include miR-122 and hepatitis C [149]; and miR-155 and cutaneous T-cell lymphoma [180]. Whilst the use of miR-155 as a therapy in pulmonary hypertension is many large steps away from being a possibility, the promising results seen in this thesis do indeed suggest the usefulness of this microRNA in PH research. Manipulation of miR-155 (and indeed several others) is likely to have further potential as a tool with which to explore PH pathobiology.

6.4 Outstanding questions & future work

The research outlined in this thesis has led to further important questions that should be addressed in order to further advance the understanding of miR-155's role in PH and pulmonary vascular remodelling. The paramount question concerns the role of miR-155 in human PAH. Measurement of miR-155 levels in serum and whole lung tissue from PAH patients, and identifying if it was upregulated in small pulmonary vessels would indicate whether there was a potential translation from animal models to the human disease.

With regards to further *in vitro* work, replication of the experiments from chapter 4 using normal human PAFs would be considered important. Examining the effect of antimiR-155 on the hypoxia-driven proliferation and migration of normal human PAFs would hopefully produce results in-keeping with the effects seen in Chapters 3 and 4. Alongside this, exploring the effects of antimiR-155 on p38 MAPK and Smad5 in human PAFs would perhaps augment the evidence that these two pathways were implicated.

Furthermore, human PAH-PAFs could be treated with a antimiR-155, with evaluation of signalling pathways and proliferative and migratory response. This would mark a step forward in understanding the role of miR-155 in PH by using a "reversal of disease" approach.

Although only p38 MAPK and Smad5 have been examined in this work, the influence of miR-155 on other signalling pathways or causative stimuli should be addressed. In the general introduction, potential links between miR-155 and IL-6 and eNOS were identified. These would be good starting points. However, further examination of miRbase or similar microRNA databases would likely identify other potential targets of miR-155 linked to PH that could be examined.

Taken together, the above experiments should prove to be interesting lines of enquiry to pursue in order to develop this work further.

6.5 Final comment

This thesis has shown for the first-time direct links between miR-155 and pulmonary hypertension. Downregulation of miR-155 both in cells and animals conveyed a protective benefit, with evidence of less vascular remodelling and improved haemodynamics. Several potential signalling pathways are likely to be involved. The manipulation of miR-155 levels has proven to be a useful tool in elucidating these pathways and remodelling processes. Further research into pulmonary hypertension through microRNA manipulation is warranted, in order to advance progress with new treatments aimed at reversing pulmonary vascular remodelling.

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