The role of Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL) in Pulmonary Arterial Hypertension

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Research department and group members

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Contributions to experiments presented in thesis

I was involved in planning and performing experiments and data interpretation unless indicated otherwise. I am indebted to my departmental colleagues for their help and support and members of the pulmonary vascular research group;

Dr Allan Lawrie for performing the bulk of the rodent cardiac catheterisation presented in this thesis and along with Dr Claudia Paiva who undertook cell culture work presented in chapter 3 (Background data).

Ms Nadine Arnold for assistance with animal husbandry, harvesting of tissues after haemodynamic phenotyping, preparation of paraffin block sections and slides for immunohistochemistry.

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I am also grateful to Prof. Christopher Newman and Prof. David Crossman respectively for their support and mentoring during this and earlier periods of my career. Finally I would like to thank Prof. David Kiely (Director of the Sheffield pulmonary vascular disease Unit, Royal Hallamshire Hospital) for welcoming me into this fascinating field of medical endeavor and providing warming inspiration and support during my career.

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Personal

All praise and glory goes to the almighty for the countless blessings in my life. I would like to thank my family, particularly my parents for nurturing me with the importance of seeking knowledge, to my dear wife and three children (two were additions during the fellowship) for their support, love and utmost patience during this collectively eventful period in our lives. I hope I can now put to rest the endless number of times you have all enquired by asking “have you finished writing your book?” . I look forward to devoting more time for you all.

Brief notes about the student

I undertook my undergraduate training at the School of Medicine, University of Southampton between 1996 and 2002. During this period I also qualified with an intercalated BSc (Hons) degree in biomedical sciences studying a variety of topics spanning genetics, cardiovascular physiology, and immunology over the course a whole academic year. I also spent part of an additional year in a research lab exploring inflammatory mechanisms in atherosclerosis. This scientific exposure fueled me with an interest in mechanisms of disease.

As part of my general professional medical training (SHO level) I spent six months working in the Sheffield Pulmonary Vascular Disease unit, where I acquired experience in the management of a variety of patients evaluated for suspected or known pulmonary hypertension under the supervision of Dr David Kiely. During this period I was fascinated by the clinical aspects of this entity yet surprised at the limited bench research activity within the department. Thus it wasn’t a difficult decision for me to accept an offer to pursue pulmonary vascular research when the opportunity arose in 2007.

Subsequent to this research fellowship I rejoined the NHS and underwent higher specialist clinical training in cardiovascular medicine (2012-2018) with sub-specialisation in heart failure and pulmonary hypertension.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

LIST OF TABLES

LIST OF ABBREVIATIONS

ApoE	Apolipoprotein-E
BMDC	bone marrow derived cells
BMT	bone marrow transplantation
C.I	cardiac index
CSA	Cross Sectional Area
EC	endothelial cell
ePVRi	estimated pulmonary vascular resistance index
ERK1/2	Extracellular signal related Kinase 1/2
HFD	high fat diet
IHC	immunohistochemistry
LVEDP	left ventricular end-diastolic pressure
LVESP	left ventricular end-systolic pressure
MCT	Monocrotaline
NF-κB	Nuclear Factor Kappa Beta
NO	Nitric Oxide
OPG	Osteoprotegerin
PA AT	pulmonary artery acceleration time
PAEC	pulmonary artery endothelial cell
PAH	pulmonary arterial hypertension
PAP	pulmonary artery pressure
PASMC	pulmonary artery smooth muscle cell
PBS	Phosphate Buffered Saline
PCNA	proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet derived Growth Factor
PAH	pulmonary arterial hypertension
PAWP	pulmonary artery wedge pressure
PH	pulmonary hypertension
PVR	pulmonary vascular resistance
PVRi	Indexed PVR
qPCR	quantitative Polymerase Chain Reaction
RV	Right Ventricle
RVEDP	right ventricular end-diastolic pressure
RVSP	right ventricular end-systolic pressure
RVH	right ventricular hypertrophy
α SMA	alpha Smooth Muscle Actin
SEM	Standard Error of the Mean
SMC	smooth muscle cell
TRAIL	tumor necrosis factor related apoptosis-inducing ligand
VSMC	vascular smooth muscle cell
vWF	Von Willebrand factor

LIST OF PUBLISHED ARTICLES, ABSTRACTS & AWARDS FROM FELLOWSHIP

Original publications related to data presented in thesis

1. Hameed, A.G., N.D. Arnold, J. Chamberlain, J.A. Pickworth, C. Paiva, S. Dawson, S. Cross, L. Long, L. Zhao, N.W. Morrell, D.C. Crossman, C.M.H. Newman, D.G. Kiely, S.E. Francis, and A. Lawrie. **2012**. *Inhibition of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) reverses experimental pulmonary hypertension*. **The Journal of Experimental Medicine** 209:1919-1935.

2. Lawrie, A., Hameed A.G, J. Chamberlain, N. Arnold, A. Kennerley, K. Hopkinson, J. Pickworth, D.G. Kiely, D.C. Crossman, and S.E. Francis. **2011**. *Paigen Diet–Fed Apolipoprotein E Knockout Mice Develop Severe Pulmonary Hypertension in an Interleukin-1–Dependent Manner.* **The American Journal of Pathology** 179:1693-1705.

Additional Research papers arising from research activity during fellowship

3. Nadine D. Arnold, Sarah Dawson, Josephine A. Pickworth, Joana A. Carvalho, Adam T.Braithwaite, Hager Kowash, James Iremonger, Helen Casbolt, Lewis Renshall, Volker Germaschewski, Matthew McCourt**,** Abdul G. Hameed, Alexander M.K. Rothman, Maria G. Frid, A. A. Roger Thompson, Jennifer Louth, Beining Chen, Holly R. Evans, Mark Southwood, Christopher M. Newman, Nicholas W. Morrell, David C. Crossman, Moira K. B. Whyte, Kurt R. Stemark, Philip Bland-Ward, David G. Kiely, Sheila E. Francis, Allan Lawrie *A novel human anti-osteoprotegerin antibody attenuates severe experimental pulmonary arterial hypertension* **(paper under review)**

4. Thompson, A. A. R., R. S. Dickinson, F. Murphy, J. P. Thomson, H. M. Marriott, A. Tavares, J. Willson, L. Williams, A. Lewis, A. Mirchandani, P. Dos Santos Coelho, C. Doherty, E. Ryan, E. Watts, N. M. Morton, S. Forbes, R. H. Stimson, A. G. Hameed, N. Arnold, J. A. Preston, A. Lawrie, V. Finisguerra, M. Mazzone, P. Sadiku, J. Goveia, F. Taverna, P. Carmeliet, S. J. Foster, E. R. Chilvers, A. S. Cowburn, D. H. Dockrell, R. S. Johnson, R. R. Meehan, M. K. B. Whyte and S. R. Walmsley **(2017)**. *Hypoxia determines survival outcomes of bacterial infection through HIF-1α–dependent reprogramming of leukocyte metabolism*. **Science Immunology** 2(8)

5. Ellam, T., A. Hameed, R. ul Haque, M. Muthana, M. Wilkie, S. E. Francis and T. J. A. Chico **(2014)**. *Vitamin D Deficiency and Exogenous Vitamin D Excess Similarly Increase Diffuse Atherosclerotic Calcification in Apolipoprotein E Knockout Mice*. **PLoS ONE** 9(2): e88767

6. Hameed, A., E. Bennett, B. Ciani, L. P. C. Hoebers, R. Milner, A. Lawrie, S. E. Francis and A. J. Grierson **(2013)**. *No Evidence for Cardiac Dysfunction in Kif6 Mutant Mice*. **PloS ONE** 8(1): e54636

7. Condliffe, R., J. Pickworth, K. Hopkinson, S. Walker, A. Hameed, J. Suntharaligam, E. Soon, C. Treacy, J. Pepke-Zaba, S. Francis, D. Crossman, C. Newman, C. Elliot, A. Morton, N. Morrell, D. Kiely and A. Lawrie **(2012)**. *Serum osteoprotegerin is increased and predicts survival in idiopathic pulmonary arterial hypertension***. Pulmonary circulation** 2(1): 21-27

SCIENTIFIC ABSTRACTS

International scientific meetings

1. Hameed, A.G., J. Chamberlain, N. Arnold, C. Newman, S. Francis, D. Crossman, and A. Lawrie. **2011**. Beneficial Effects Of Inhibiting Trail In Monocrotaline-Induced Experimental Pulmonary Hypertension. ***Am. J. Respir. Crit. Care Med***. 183:A3407-(Poster abstract, presented at the American Thoracic Society, Denver, U.S May 2011)

2. Hameed, A.G., S.E. Francis, D.C. Crossman, and A. Lawrie. **2010**. TRAIL Deficiency Protects Fat-fed ApoE-/- Mice From Pulmonary Arterial Hypertension. ***Am. J. Respir. Crit. Care Med***. 181:A2283- (Oral Abstract, presented at the American Thoracic Society, New Orleans, U.S May 2010.)

National scientific meetings

1. Hameed, A.G, N. Arnold, J. Chamberlain, J. Pickworth, C. Newman, D. Crossman, S. Francis, and A. Lawrie. **2012**. 03 Tissue Trail Drives Pulmonary Vascular Remodeling and its Inhibition Reverses Experimental Pulmonary Arterial Hypertension. ***Heart*** 98:A1. **(Oral abstract presented for the young investigator award at the BAS/BSCR/BCS meeting, Manchester, May 2012)**
2. Hameed, A.G., N.D. Arnold, J. Pickworth, J.C. Chamberlain, C.M.H. Newman, D.C. Crossman, S.E. Francis, and A. Lawrie. **2011**. T6 TRAIL is a potential novel therapeutic target in pulmonary arterial hypertension. ***Thorax*** 66:A3 **(Oral abstract presented at the BTS for the Early career investigator awards, London, UK Dec. 2011)**
3. Hameed, A.G., J. Chamberlain, N.D. Arnold, S.E. Francis, C.M.H. Newman, D.C. Crossman, and A. Lawrie. **2010**. Abstract S151 TRAIL deficiency is protective in experimental pulmonary arterial hypertension. ***Thorax*** 65:A68 (oral abstract presented at BTS, London, Dec.2010)
4. Hameed A.G., J Chamberlain, N.D. Arnold, S.E Francis, C.M.H Newman, D.C Crossman and Lawrie A. **2009**. Abstract S8 TRAIL Expression is Increased in the rat moncrotaline model of Pulmonary arterial Hypertension ***Thorax*** 64:A7-A10.(oral abstract presented at BTS, London, Dec.2009)

PRIZES AND AWARDS

1. First prize in final year postgraduate research student oral presentations, faculty of medicine, University of Sheffield, **July 2012.**
2. British Heart Foundation Michael Davies Young Investigator Award for oral presentation at the spring meeting of the British Atherosclerosis Society/British Society of Cardiovascular Society held jointly with the British Cardiovascular Society, Manchester, UK, **May 2012.**
3. Runner up in the British Thoracic Society early career investigator award (main prize), London, UK, **Dec. 2011.**
4. First Prize for research abstract (poster) at the British Heart Foundation biennial national fellows day, Queen's college, University of Cambridge, Cambridge, UK, **April 2011**.
5. British Lung Foundation travel fellowship to attend and present data at the American Thoracic Society, New Orleans, U.S, **May 2010**.

ABSTRACT

Background Pulmonary arterial hypertension (PAH) is an obliterative vasculopathy characterized by endothelial and smooth muscle cell proliferation affecting small arterioles resulting in progressive elevation of pressure within the lungs. This poses significant load on the right heart which can lead to heart failure. It is a devastating and life threatening disease as patients are frequently diagnosed at an advanced stage.

Existing drug therapies augmenting pulmonary vasodilatation have significantly improved patient morbidity but insufficiently modify vascular remodeling and consequently have modestly improved survival. Halting or reversing vascular remodeling could revolutionise human therapy but has yet to come to fruition. To improve disease prognosis, identifying key disease pathways is a priority for developing newer therapies.

A role for Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) in regulating endothelial cell and smooth muscle cell physiology has been reported in the systemic vasculature but hitherto unexplored in the pulmonary circulation. I hypothesised that TRAIL is an essential mediator in the pathogenesis of PAH.

Methods: Expression of TRAIL in human and rodent PAH and the mitogenic effects of TRAIL on pulmonary artery smooth muscle cells (PASMCs) were characterised. The pathogenic role of TRAIL in three independent rodent models of disease was determined and the efficacy of inhibiting TRAIL in halting or regressing established disease was tested. Phenotyping included cardiac catheterisation, echocardiography, and pulmonary vascular immunohistochemistry.

Findings Gene and protein expression of TRAIL ligand and receptors were upregulated in PASMCs from patients with PAH. *In-vitro* TRAIL was a mitogen for PASMCs. TRAIL-deficient mice were protected from both hypoxia and diet-induced PAH. Antibody blockade prevented rats from developing MCT induced PAH. Bone marrow transplantation in chimeric mice supported a role for tissue derived TRAIL. In mice and rats with established disease, an anti-TRAIL antibody improved pulmonary haemodynamics, reversed pulmonary vascular remodeling, reduced proliferation and increased apoptosis in vascular lesions and significantly improved survival.

Conclusion: My preclinical studies are the first to determine the importance of TRAIL in the pathogenesis of experimental pulmonary arterial hypertension and highlight its potential as a novel therapeutic target for directing future therapies.

CHAPTER 1 INTRODUCTION

1.1 Overview of PAH

Early descriptions suggestive of a primary pulmonary vasculopathy (interestingly first described in a middle age man) were published in the mid-late 19th century (Klob 1865; Romberg 1891; Mönckeberg 1907) in case reports from single individuals. The bedside finding of a heart murmur related to high pulmonary pressure had also been appreciated (Steell 1888; Bramwell 1942). Subsequent case studies in the American (Sanders 1909; Brenner 1935; Seely 1938) and British (De Navasquez, Forbes et al. 1940; East 1940; Gilmour and Evans 1946) literature featured similar findings.

These reports described the clinical presentation, including features of right heart failure and subsequent postmortem findings. Histopathology revealed intimal and medial hyperplasia of small pulmonary arterioles (initially referred to as “arteriolar sclerosis”) and right ventricular hypertrophy in the absence of clinical or pathological evidence of disease of the lungs (Cor pulmonale), pericardium or left heart (typically rheumatic mitral valve disease). In the absence of recognized causes of PH (Steell 1888) these authors postulated a new, albeit rarer disease of primary pulmonary hypertension (Gilmour and Evans 1946). Indeed reading the accounts from these reports was fascinating as they elegantly highlighted the clinical features of the disease that remain evident today. Sadly however, these descriptions of hypertensive pulmonary vascular disease prior to the advent of currently available therapies also demonstrated the rather fatal and rapid natural history of disease, which remained largely the case until the early 1990s.

Following the introduction of cardiac catheterisation in man (Cournand and Ranges 1941) investigators were able to measure pulmonary artery pressure and correlate haemodynamics with the observed clinical and pathological findings (Dresdale, Schultz et al. 1951; Heath and Whitaker 1956; Brown, Heath et al. 1957; Wood 1958). This dawned a new age in the study of pulmonary hypertension leading to seminal works characterising and classifying the pathology of hypertensive pulmonary vascular diseases encountered in mitral stenosis, congenital heart disease and idiopathic PAH (what was then known as Primary pulmonary hypertension) (Harrison 1958; Wood 1958; Heath and Edwards 1960) which slowly progressed over following decades (Wagenvoort and Wagenvoort 1970; Smith and Heath 1979; Heath, Smith et al. 1987; Wagenvoort 1988; Palevsky, Schloo et al. 1989; Cool, Stewart et al. 1999).

In the late 1990s inflammation became an increasingly appreciated phenomenon of vascular lesions (Palevsky, Schloo et al. 1989; Tuder, Groves et al. 1994; Lee, Shroyer et al. 1998; Tuder and Voelkel 2002; Pietra, Capron et al. 2004). These features have been confirmed in contemporary histopathology studies from patients undergoing lung transplantation in the era of intravenous prostacyclin therapy , however surprisingly reveal persistent neointimal fibrosis and advanced plexiform lesions (Pogoriler, Rich et al. 2012; Stacher, Graham et al. 2012; Tuder, Archer et al. 2013) indicating progressive pathology and supporting the notion that these lesions are either partially or incompletely treatable.

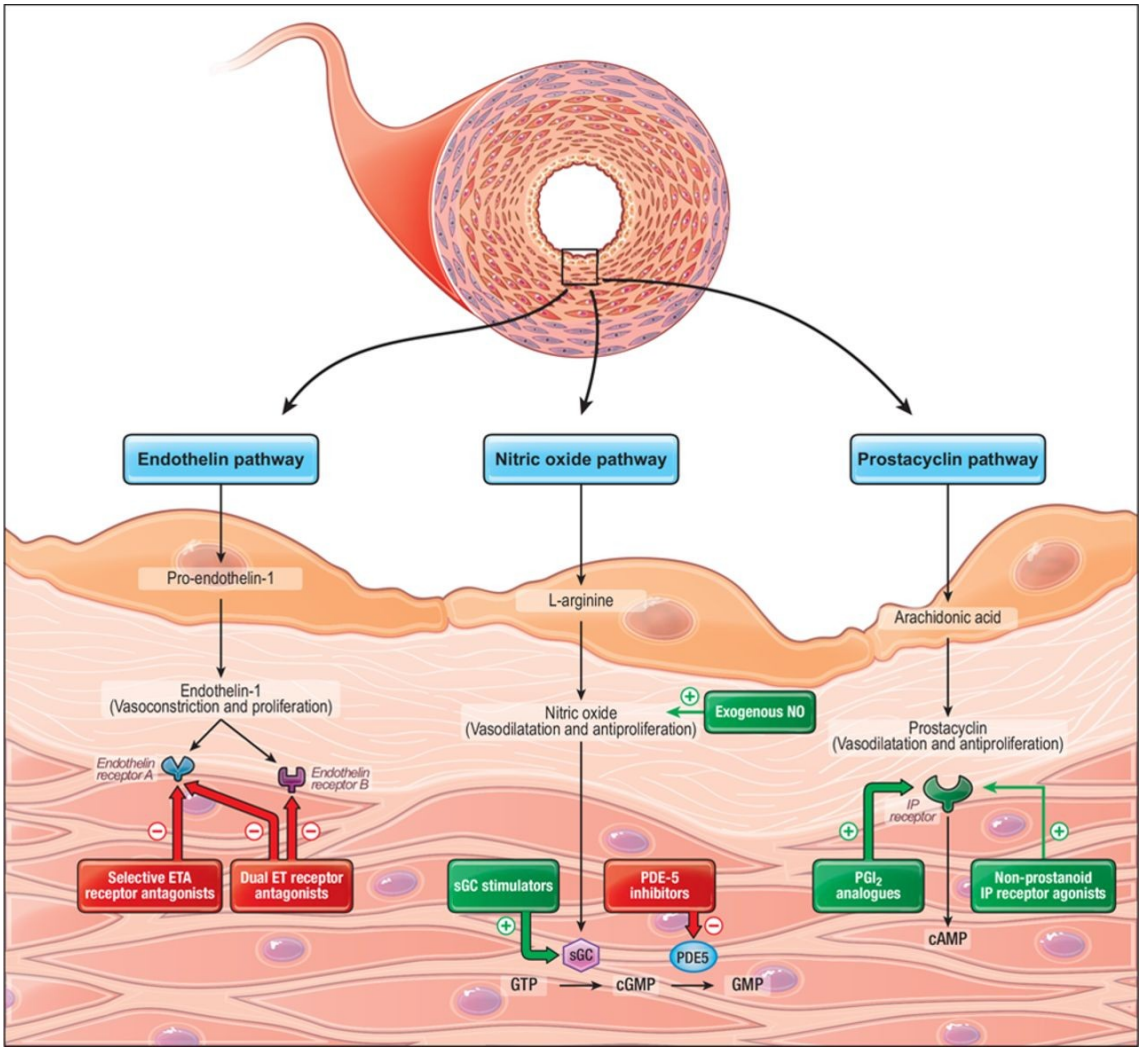


Figure 1.1 Currently approved pulmonary vasodilators in human PAH. Schematic illustration highlighting three principal pathways targeted by current drugs licensed for use in PAH. Redisplayed with permission from (Humbert, Lau et al. 2014).

During the 1980s and 1990s the vasoconstrictor hypothesis of PH burgeoned following the identification of the potent vasoconstrictor Endothelin, (Giaid , Yanagisawa et al. 1993) and vasodilators nitric oxide (Giaid and Saleh 1995) and prostacyclin (Higenbottam, Wheeldon et al. 1984; Barst, Rubin et al. 1994). These seminal works paved the way for several successes over next two decades leading to multiple clinical trials of drugs **(Fig. 1.1)** therapeutically exploiting this imbalance between pulmonary vasoconstriction and vasodilatation (Galiè, Humbert et al. 2016; Lau, Giannoulatou et al. 2017). Several members of the endothelin receptor antagonists (ERA), phosphodiesterase 5 inhibitors and prostacyclin analogues are in routine clinical use for patients with PAH (group 1 PH). These therapies have provided significant symptomatic benefit to patients and there is evidence, at least certainly for prostacyclin analogues that they can prolong survival (Galie, Manes et al. 2009).

At the turn of the millennium, the next chapter in the pulmonary hypertension saga opened following the identification of mutations within BMPR2 in PAH (Deng, Morse et al. 2000; Lane, Machado et al. 2000; Machado, Pauciulo et al. 2000; Thomson, Machado et al. 2000). This led to an evolution and refinement in the understanding of the molecular and cellular pathology of PAH (Archer, Weir et al. 2010; Rabinovitch 2012). Perturbations within genetic and epigenetic regulation (Austin, West et al. 2017; Chun, Bonnet et al. 2017; Ma and Chung 2017), mitochondrial physiology (Ryan, Dasgupta et al. 2015; Ryan and Archer 2015; Archer 2017), inflammation (Rabinovitch, Guignabert et al. 2014), growth factors (Hassoun, Mouthon et al. 2009), phenotypic heterogeneity of vascular cells (Stenmark, Frid et al. 2016; Stenmark, Frid et al. 2018) and right ventricular adaption (Ryan and Archer 2014) were increasingly recognized as novel contributors to the growing signature of PAH biology. The value and limitations of animal models have been well rehearsed (Stenmark, Meyrick et al. 2009; Gomez-Arroyo, Saleem et al. 2012) with greater emphasis on angioproliferative rodent models (Abe, Toba et al. 2010; Al Husseini, Bogaard et al. 2012; Voelkel and Gomez-Arroyo 2014).

Thus we have arrived at a crossroad where PAH is positioned as a complex and biologically heterogeneous disease, albeit with an established haemodynamic phenotype. However several disappointments in translating bench science is focusing the research community (Lythgoe, Rhodes et al. 2016). Whilst still in its infancy, systems biology and personalized medicine are now entering a new strategic phase in PAH research **(Fig. 1.2)** (Austin, West et al. 2017; Ghataorhe, Rhodes et al. 2017; Hemnes, Beck et al. 2017; Newman, Rich et al. 2017; Rhodes, Ghataorhe et al. 2017). The intended result of such endeavors is to ultimately improve patient care and convert PAH into a curable disease (Gurtu and Michelakis 2016; Bonnet,

Provencher et al. 2017; Michelakis 2017). However these endeavors will bring their own challenges as “big data” will require appropriate analysis and interpretation. It is likely many new molecular signatures and putative drug targets will come to light; however selecting the most appropriate targets for development may not be straightforward. The heterogeneous nature of PAH, cost of clinical trials and relatively limited number of PAH patients will require investigators to think carefully about drug development and design of clinical trials (Wilkins 2013; Ryan, Rich et al. 2015).

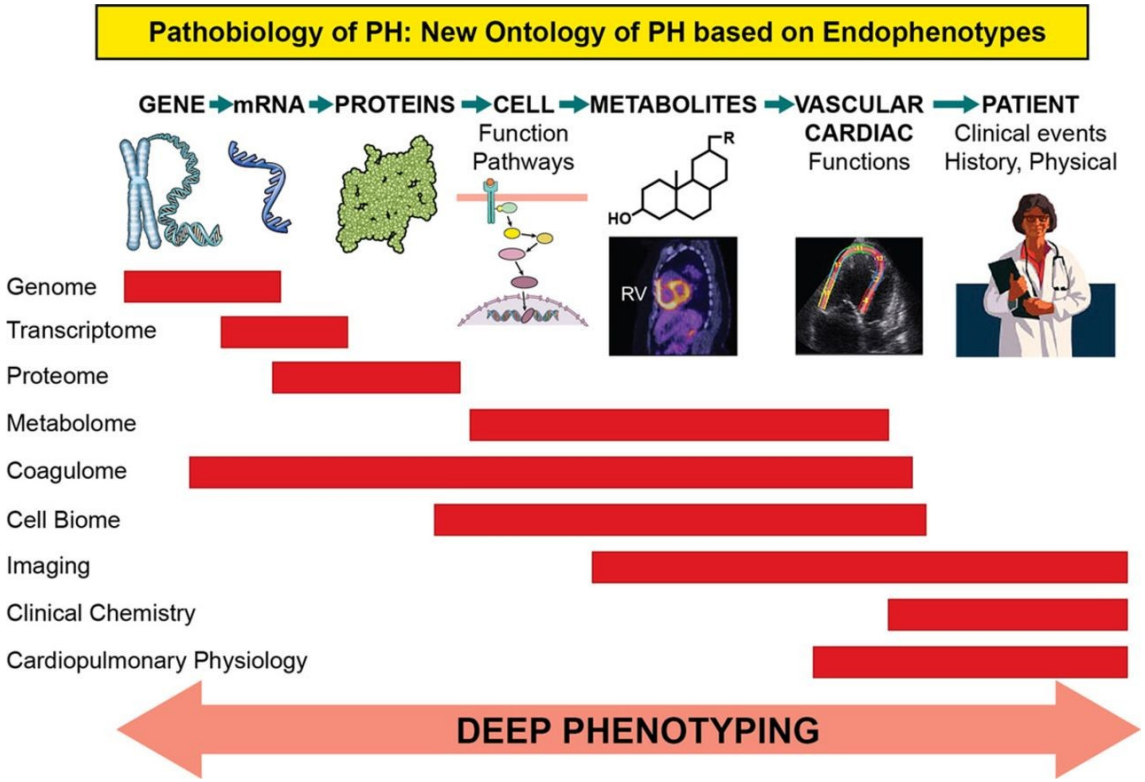


Figure 1.2: Era of Omics research in PH. Schematic highlighting the PVDOMICS program which aims to refine the molecular and clinical classification of pulmonary hypertension and provide a powerful platform for drug development. Redisplayed with permission from (Hemnes, Beck et al. 2017).

1.2 Physiology of the human pulmonary circulation

The human heart propels blood (approx. 5L/min at rest) in a pulsatile manner through two circulations (systemic and pulmonary) connected in series. The left ventricle pumps oxygenated blood (returning from the lungs) into the aorta and thence to the whole body to nourish every organ and tissue. Therein at the capillary-tissue interface, oxygen, metabolic and synthetic nutrients are extracted, whilst excretory (lactate, CO_2) and secretory (e.g. hormones, cytokines) products are released into the circulation. This blood is returned via a converging system of veins into the vena cava draining into the right atrium and finally filling the right ventricle. The latter propels this deoxygenated blood to the lungs through the pulmonary arterial tree which sequentially gets smaller as it branches into small arterioles (<100um) which closely follow alveolar sacs. Due to the very thin capillary alveolar membrane, the area for gas exchange area is immense (between 50-100m^2) and permits rapid replenishment of blood oxygen whilst also permitting removal of CO_2 through simple diffusion (West and Luks 2016). This oxygenated blood returns through a network of pulmonary venules which drain through at least 4 pulmonary veins into the left atrium to repeat the aforementioned cardiac cycle.

Remarkably the resting healthy adult heart will contract (and relax) approximately 100,000 times and propel over 7000L of blood in a single day. When put into context of a lifetime the numbers are truly astonishing and when combined with the large surface area of alveoli for gas exchange makes the cardiopulmonary unit a remarkable and elegant physiological system. Furthermore the heart and circulatory systems can regulate flow depending on physiological states such as exercise, pregnancy or hypoxia.

The lungs are the only organ exposed to full flow (cardiac output) at all times. The pulmonary circulation is a low pressure, low resistance circuit that can accommodate high flow rates due to its distensible properties. In health, for the same resting cardiac output, the pulmonary circulation operates at much lower arterial pressure (six times lower) compared to the systemic circulation (mean arterial pressure 15mmHg vs 90mmHg) with a corresponding tenfold lower vascular resistance. This is due to the highly distensible (compliant) nature of the pulmonary vasculature.

As a result pulmonary blood flow (cardiac output) can increase significantly (up to 20L/min) with little increases in pressure and resistance. Thereafter PA pressure rises slightly. This homeostatic regulation appears to be partly blunted with increasing age. For example, healthy older people (age>50 years) have been shown to have a exaggerated response to exercise in a one series (Kovacs, Olschewski et al. 2012) but not in a more recent study (Oliveira, Agarwal et al. 2016) The growing literature on normative ranges for pulmonary haemodynamics during exercise has recently led to some welcomed consensus on the subject (Kovacs, Herve et al. 2017).

1.3 Definition and clinical classification of pulmonary hypertension

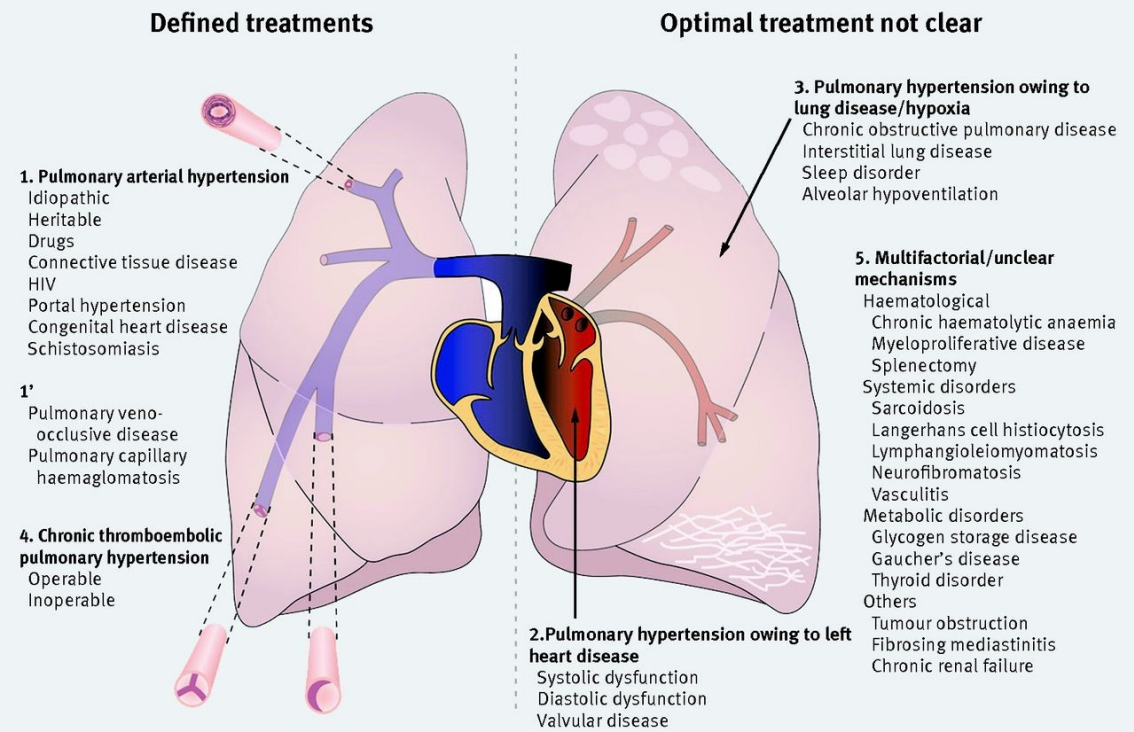


Figure 1.3 Clinical classification of pulmonary hypertension. Groups 1, 3 and 4 are primarily precapillary disease whilst Groups 2 is postcapillary and group 5 a mixture. Redisplayed with permission from (Kiely, Elliot et al. 2013).

Pulmonary hypertension (PH) refers to a pathophysiological state whereby the pressure within the pulmonary circulation is elevated. It is clinically heterogeneous with respect to clinical presentation, developmental origins, genetics, pathogenesis, histopathology, adaptation of the right ventricle and the potential for reversibility of the vasculopathy. It is suffice to say that contemporary view underscores it is a complex syndrome, even within the various clinical subtypes (Kulik and Austin 2017).

PH is specifically defined by a mean pulmonary arterial (PA) pressure that is ≥25mmHg. The elevated pressure can develop in one or more sites within the pulmonary circulation leading to arterial hypertension (pre capillary disease) or venous hypertension (post capillary disease). Less commonly it can arise due to disease within either the post capillary venules or capillary bed however this clinically manifests as a precapillary haemodynamic phenotype and can be

diagnostically difficult to distinguish from idiopathic PAH. It is not uncommon for systemic heart and vascular disease to affect both venous and arterial circuits and in fact PH related to left heart disease is the commonest form of PH. The clinical classification of PH, although imperfect has slowly evolved and the latest (5^{th}) classification (Simonneau, Gatzoulis et al. 2013) is highlighted in **Fig. 1.3.**

PAH is a subtype of PH. At the 5^{th} World symposium on Pulmonary hypertension in 2013, the haemodynamic criteria of PAH remained unchanged and were defined (by cardiac catheterisation) by a mean PA pressure ≥25mmHg, a normal venous pressure (PA wedge pressure <15mmHg) and increased pulmonary vascular resistance (>3 Wood Units) in the absence of diseases affecting the lung, left heart or caused by pulmonary thromboembolism (Hoeper, Bogaard et al. 2013). PAH is pathologically characterised by obliterative arteriolar disease with a range of histopathological lesions. Several subtypes exist relating to the underlying cause. Chiefly it can be idiopathic or heritable (due to one of several identified genetic mutations) that have almost identical pathological feature or associated to a number of well recognised conditions as listed in **Fig. 1.3**. The focus of my investigations presented in this thesis will focus on PAH and in particular the idiopathic form.

1.4 Epidemiology of PAH

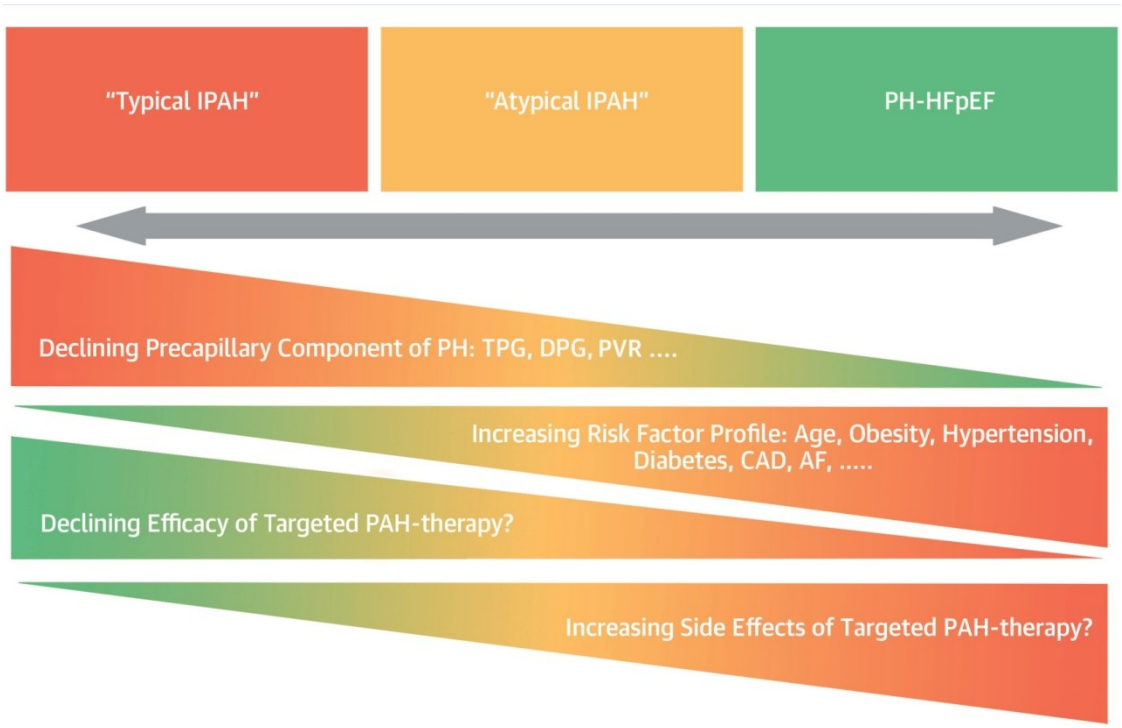


Figure 1.4: Evolving phenotype of PAH in the modern era: Spectrum of PAH ranging from "Typical" PAH towards "atypical" PAH and then to a subset of group 2 PH related to heart failure with preserved ejection fraction. Redisplayed from (Opitz, Hoeper et al. 2016) with no permissions required as open access article with a Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license. http://creativecommons.org/licenses/by-nc/4.0/

Idiopathic and heritable PAH are both uncommon diseases. Exact figures on incidence and prevalence are unknown but registries report them between 1- 10 cases/million and 5-50/million (Humbert, Sitbon et al. 2006; Peacock, Murphy et al. 2007; Frost, Badesch et al. 2011; Ling, Johnson et al. 2012; McGoon, Benza et al. 2013). Idiopathic and heritable PAH account for between 30-50%, PAH-CTD approx 30% (of which half are due to systemic sclerosis) and PH-CHD about 20% (Lau, Giannoulatou et al. 2017).

It has long been recognised that young females have accounted for over two-thirds of the cases of idiopathic PAH and females have higher (42%) disease penetrance of BMPR2 mutations compared to males (14%) (Aldred and Morrell 2012; Larkin, Newman et al. 2012). Whilst likely related to differences in sex hormones the precise mechanisms are still

incompletely understood (Mair, Johansen et al. 2014; Ventetuolo, Praestgaard et al. 2014; Singla and Machado 2015).

National registry data (**Table 1.1** p.29) highlight that the mean age at diagnosis of idiopathic PAH has increased, particularly in developed nations and consequently patients have a greater prevalence of cardiovascular co-morbidities (Hoeper, Huscher et al. 2012). Several reasons have been cited to account for this apparent change in PAH demographics including: a change in disease biology, heterogeneity in natural history, improved non-invasive detection and increased use of pulmonary vasodilators. It is likely that a combination of these factors accounts for the demographic changes (Lau, Giannoulatou et al. 2017). This increasing profile in contemporary western IPAH patients has led to the term “atypical IPAH” when compared to younger “typical IPAH” female patients. The former phenotypes overlaps with PH due to left heart disease **(Fig. 1.4)** which as a result of misclassification could partly account for the aforementioned “age drift” (Opitz, Hoeper et al. 2016). This recognition is an important consideration when planning and interpreting results from clinical studies.

1.5 Natural history of PAH

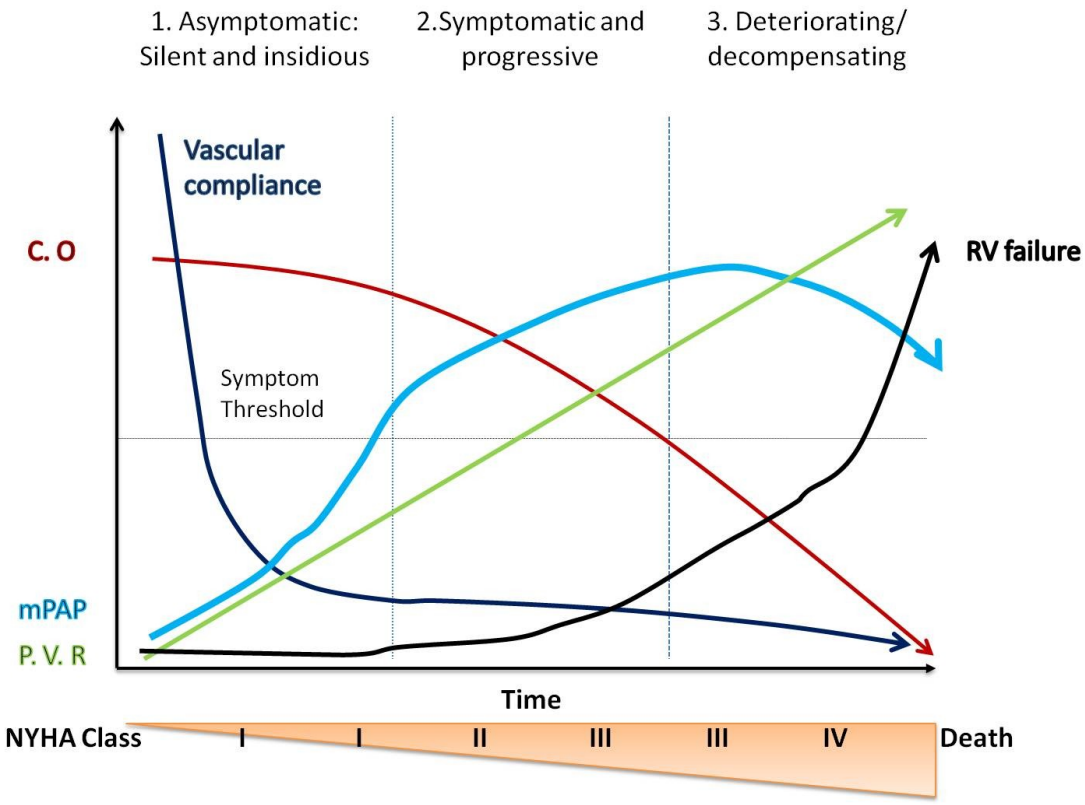


Figure 1.5: Schematic illustration of the natural history of PAH. Modified from figure 4 in (Haddad, Couture et al. 2009) with permission. C.O Cardiac output, mPAP; mean PA pressure PVR; pulmonary vascular resistance.

Unfortunately many patients with PAH are diagnosed at a late stage of disease due to low disease incidence, non specific symptoms and the insidious nature of PAH all compounded by delayed diagnosis. Indeed the original NIH registry (Rich, Dantzker et al. 1987) and modern registries such as REVEAL (Brown, Chen et al. 2011) highlight a mean of 2 years in delay for many patients. Symptoms of early PAH (breathlessness, fatigue) are nonspecific whilst in advanced stages (chest pain, dizziness and collapse) signs of right heart failure may also be present (ankle swelling, abdominal swelling, symptoms at rest) supporting why over 70% of patients are in NYHA class III-IV at diagnosis.

Given that the pulmonary circulation has a large physiological reserve, significant loss (estimated at >70%) in compliance can occur before PA pressure and vascular resistance rise **(Fig. 1.5)**. At this stage patients usually notice symptoms that can be quite alarming and progressive. Cardiac output is usually adequate at rest but is inadequate during exercise leading to breathlessness. As the disease progresses cardiac reserve falls further. Additionally

marked ventilatory inefficiency and reduced peripheral muscle oxygen uptake contribute to dyspnoea In PAH (Dumitrescu, Sitbon et al. 2017)). In advanced PAH aggressive medical therapy, atrial septostomy and lung transplantation remain the principal treatment options. Within the illustrated schema there is heterogeneity between and within PAH subtypes in disease progression and response to therapy as illustrated in the next section.

1.6 Survival in PAH

Registry	Time period	Mean age (years)	Women (%)	NYHA functional class III–IV (%)	6MWD (m)	Estimated survival at 1 year (%)	Estimated survival at 3 years (%)
NIH[12]	1981–1985	36 ± 15	63	75	NA	68	48
PHC[16]	1982–2004	46 ± 14	76	80	NA	91	75
Japanese[23]	1992–2012	33 ± 14	74	87	267 ± 154	98	92
Scottish[29]	2002–2009	49 ± 11	62	NA	NA	NA	NA
French[14]	2002–2003	52 ± 15	62	81	328 ± 112	83	58
UK and Ireland[10]	2001–2009	50 ± 17	70	84	292 ± 123	93	73
REVEAL[18]	2006–2009	53 ± 15	83	55	374 ± 129	91	74
COMPERA[22]	2007–2011	65 ± 15	60	91	293 ± 126	92	74
Spanish[19]	2007–2008	46 ± 18	73	70	382 ± 117	89	77
New Chinese[20]	2008–2011	33 ± 15	70	52	394 ± 114	92	75
Korean[24]	2008–2011	45 ± 16	73	63	398 ± 116	NA	NA

Table 1.1: Demographics and survival in idiopathic PAH from national registries. Adapted from (Lau, Giannoulatou et al. 2017) with permission. Hyperlinks to individual studies are preserved.

Natural history studies from the early and mid 20th century cited above highlighted the poor prognosis of patients with PAH but cases numbers were small. The first study to formally characterise the natural history of idiopathic PAH was led by the NIH in the 1980s (Rich, Dantzker et al. 1987). 187 patients from 32 centres were prospectively followed up, prior to the era of modern pulmonary vasodilator therapies. These were mostly young females with a typical idiopathic PAH phenotype. The rather poor outlook was subsequently confirmed by 1, 3 and 5 year survival rates of 68%, 48% and 34% respectively. Estimated median survival was 2.8 years (D'Alonzo, Barst et al. 1991).

Data from more recent French, American REVEAL and UK registries of PAH reported median survival is now at least 5 years with 1, 3 and 5 year survival approximately 83-93%, 58-74% and 57-60% respectively which coincide with increased availability of pulmonary vasodilator drugs.

These and other national registries (in PAH cohorts with both incident +/- prevalent cases) have shown improvements in symptoms, haemodynamics and improved overall survival. However they continue to highlight older age, advanced disease on presentation (NYHA class III-IV), greater preponderance of women, and a worse prognosis in older age groups and incident cases (summarised in **Table 1.1**) (McGoon, Benza et al. 2013; Lau, Giannoulatou et al. 2017). Latest audit figures (2017) from the United Kingdom's large national PH database reported a median survival of just under 4 years in 4100 patients with PAH from a cohort of 8500 PH patients during the period 2009-2017 **(Fig. 1.6)** and confirm prior similar trends with respect to age, sex and functional class on presentation (NHS-Digital 2017).

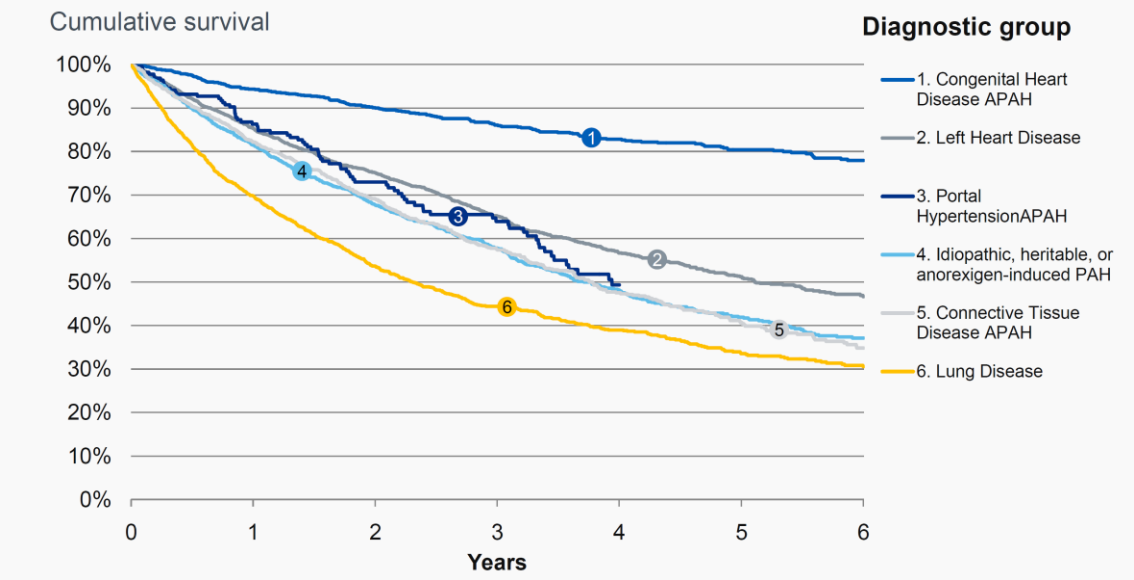


Figure 1.6: Survival of adult PH patients in the U.K from first diagnosis in the 8th National Audit of PH for Great Britain 2009-2017, published 31st Oct. 2017. Reproduced under the terms of the Open Government Licence v3.0 (http://www.nationalarchives.gov.uk/doc/open-government-licence/version/3/)

It is important to highlight that prognosis within the 5 groups of PH **(Fig. 1.3)** is heterogeneous and even with Group 1 (PAH), where it varies between subtypes; generally patients with Congenital HD-PAH do the best, whilst idiopathic PAH is much better compared with CTD-PAH. Group 3 PH (PH related to lung diseases) tend to fare the worst **(Fig. 1.6)**. Recent large PH registries such as the Sheffield ASPIRE study (2001-2010) have confirmed these observations. Specifically within the PAH group, 3 year survival in idiopathic PAH was 62%, 52% in PAH-CTD and 82% in Eisenmenger-PAH (Hurdman, Condliffe et al. 2012; Hurdman, Condliffe et al. 2013). Similar patterns were recently reported from a large German cohort (Gall, Felix et al. 2017).

1.7 Pathology of pulmonary arterial hypertension

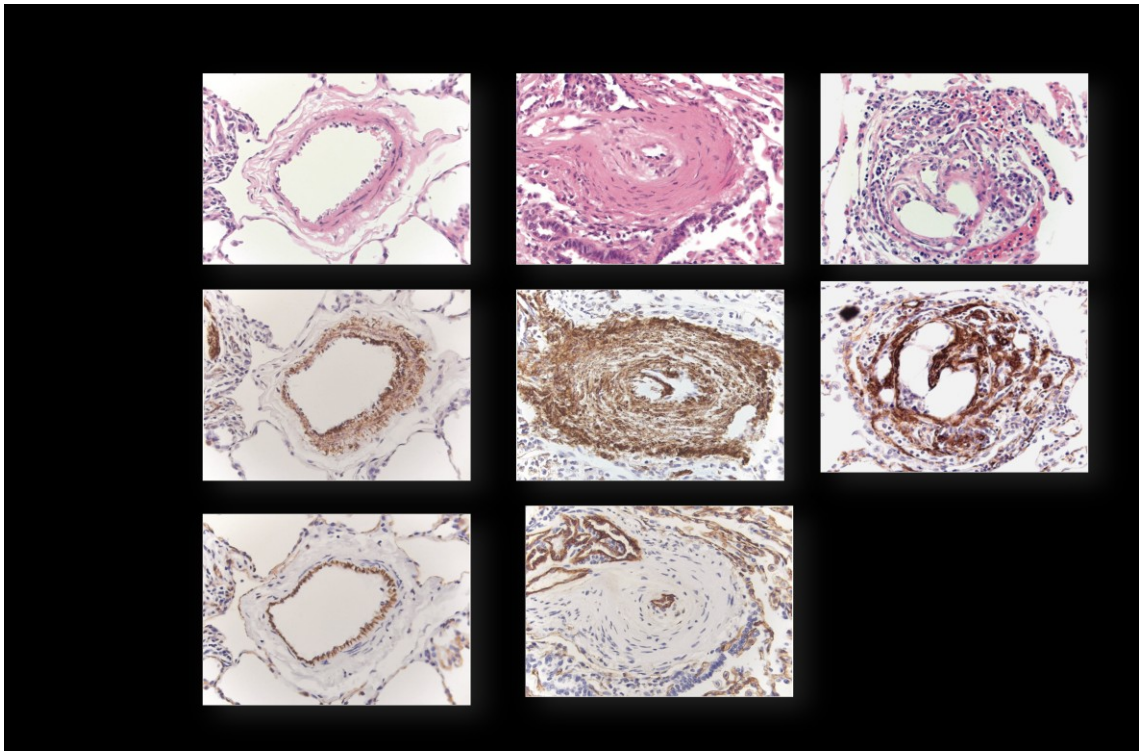


Figure 1.7 Histopathological lesions in PAH. Representative lung sections demonstrating the morphology of concentric and plexiform lesions in IPAH. SMA staining in brown reveals increased smooth muscle density within the medial vessel layer. Endothelial staining with CD31 demarcates a tiny residual lumen and with underlying neointimal proliferation. Plexiform lesion shows a more disorganized vascular architecture with multiple endothelial lined channels. H&E Haemotoxylin and Eosin, SMA alpha smooth muscle actin, CD31; endothelial marker. Images courtesy of Dr Allan Lawrie, University of Sheffield.

PAH is characterized by a range of histopathological changes that appear qualitatively quite similar irrespective of the underlying aetiology which likely represents a common response of the pulmonary vasculature in response to a variety of stimuli (Pietra, Capron et al. 2004). PAH has a predilection for the distal pulmonary vasculature, usually in small order arterioles (<150µm) that are usually devoid of smooth muscle but become characteristically muscularised and constrictive in PAH. This is due to one or more of medial hypertrophy, neointimal proliferation and adventitial thickening and associated vasoconstriction **(Fig.1.7)**.

This consequentially leads to significant reductions in the lumen diameter, altered vasomotor tone leading to increased pulmonary vascular resistance, raised PAP and invariably to death from right heart failure. In situ thrombosis is also a recognized phenomenon. Advanced lesions in PAH include concentric laminar fibrosis, eccentric fibrosis, plexiform lesions and occlusive

vasculopathy. It is still unclear whether plexiform lesions are the precursor for subsequent vessel occlusion (Tuder 2017).

The plexiform lesion has long intrigued pathologists and clinicians alike and is most often, although not exclusively associated with IPAH. It is classified as a complex lesion often seen in advanced disease and defined by focal proliferation of endothelial channels with disruption of the normal architecture of the surrounding vessel and often contains thrombi (Lee, Shroyer et al. 1998; Pietra, Capron et al. 2004) **(Fig.1.7)**.

A plethora of molecular and cellular mechanisms initiate, maintain and perpetuate the observed vascular remodelling. However there is a growing appreciation of cross talk between pathways (Sutendra and Michelakis 2014).

1.8 Genetic, cellular and molecular pathogenesis of PAH

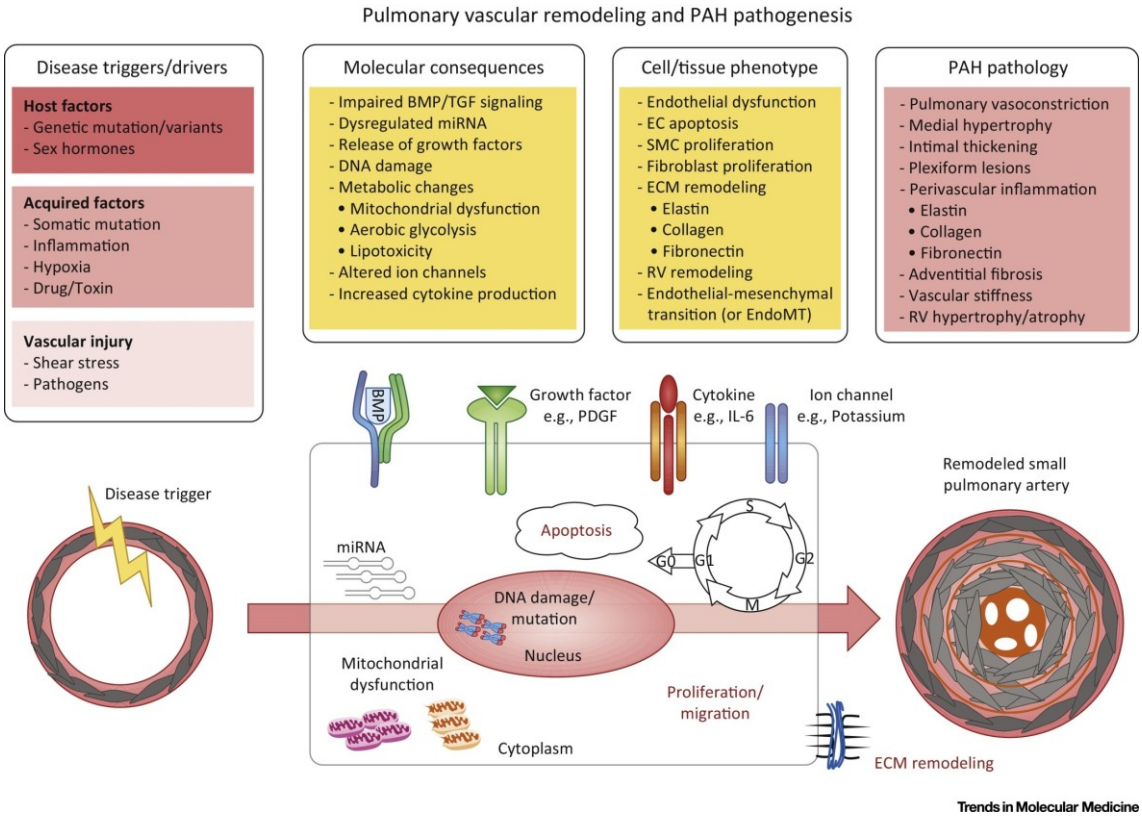


Figure 1.8: Key aspects in the cellular and molecular pathogenesis of PAH. Redisplayed with permission from (Thompson and Lawrie 2017).

It is reasonable to assert that the pathophysiology of PAH is increasingly viewed as complex and a complete story is lacking. Key molecular and cellular abnormalities are highlighted schematically in (**Fig. 1.8)**. Perturbation in genetics, inflammation, immunity, mitochondria, cell metabolism, ion channels, vasomotor tone, fluid mechanics and hypoxia are key features that lead to dysfunctional cell growth and repair in this pulmonary vasculopathy (Hemnes and Humbert 2017). The end result is obliterative arterioles as illustrated in **(Figs. 1.7-1.8).** Several unifying theories of PAH are emerging focusing on altered metabolism and inflammation linking seemingly diverse abnormalities. I shall begin by summarising the genetics of PAH as subsequent research has tried to integrate defects to upstream dysfunctional BMPR2 signaling.

1.9 Genetics of PAH

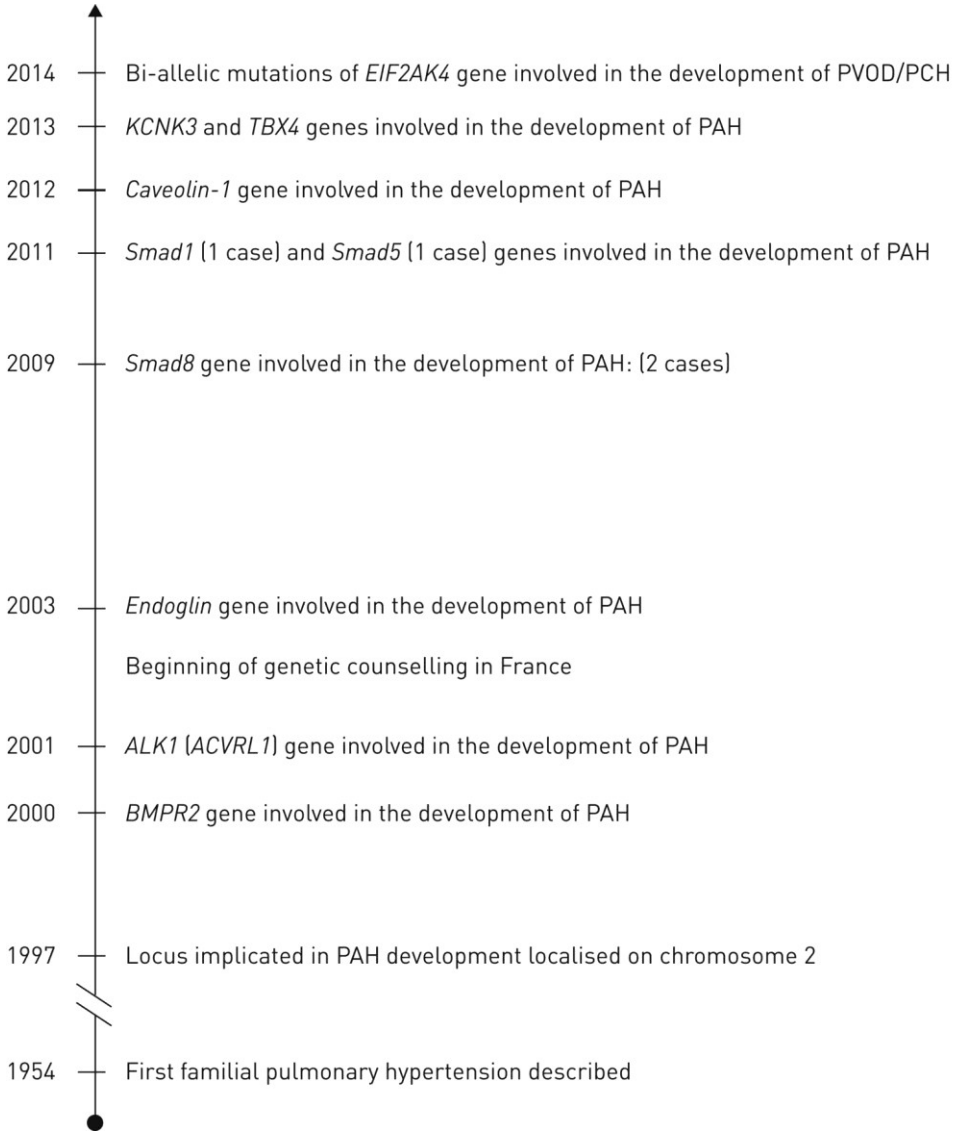


Figure 1.9: Historical overview of genetic discovery in PAH. Redisplayed with permission from (Girerd, Weatherald et al. 2017) Reproduced with permission of the © ERS 2018. *European Respiratory Review Sep 2017, 26 (145) 170037; DOI: 10.1183/16000617.0037-2017*

The observation that PAH could occur in first degree relatives in an autosomal dominant fashion had been recognised decades ago (Dresdale, Michtom et al. 1954; Kingdon, Cohen et al. 1966; Parry and Verel 1966; Thompson and McRae 1970). The phenomenon of incomplete gene penetrance was subsequently reported (Loyd, Primm et al. 1984). Linkage analysis led to localisation of a putative gene at locus on the long arm of chromosome 2 (Morse, Jones et al. 1997; Nichols, Koller et al. 1997) paving the way for subsequent identification of heterozygous mutations within the bone morphogenetic protein receptor II (BMPR2) gene (Deng, Morse et al. 2000; Lane, Machado et al. 2000). Mutations in BMPR2 were also detected in apparent

cases of sporadic idiopathic PAH (Thomson, Machado et al. 2000; Newman, Wheeler et al. 2001) and patients with drug induced (Fenfluramine) PAH (Humbert, Deng et al. 2002). Mutations in other TGF-β receptors (ALK1, ENG) and BMPR2 signalling related genes (SMAD 1,5,8) have also been identified further underscoring this pathway **(Figs. 1.9 and 1.10)**. More recently through whole exome sequencing, mutations in caveolin 1 (Austin, Ma et al. 2012) and a potassium channel KCNK3 (Ma, Roman-Campos et al. 2013) have been identified in small numbers of hereditary PAH. The precise pathophysiological mechanisms for these newer mutations are still work in progress.

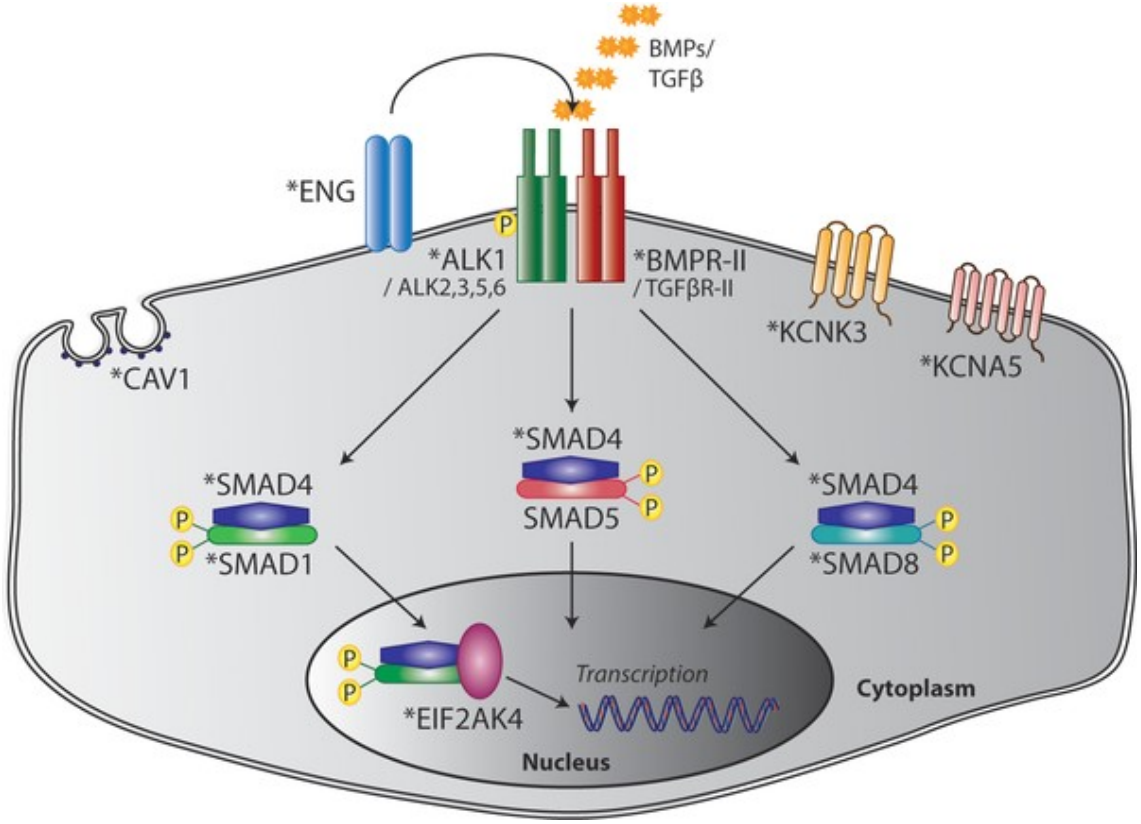


Figure 1.10 Mutations in BMPR2 related and non related genes in PAH. Schematic illustration highlighting where mutations* in PAH have been detected and in particular within the BMPR2 SMAD axis. Redisplayed with permission from (Machado, Southgate et al. 2015).

Heterozygous mutations in BMPR2 account for approximately 70-80% of familial PAH and 20% IPAH (Machado, Eickelberg et al. 2009; Morrell 2010) making it the most causally associated gene in PAH. Nearly 670 mutations have been identified in the BMPR2 gene of which the majority are pathogenic (Machado, Southgate et al. 2015). Pathogenic mutations lead to loss of function and approximately 70% of them are due to frame shift, missense or nonsense mutations (Machado, Southgate et al. 2015; Ma and Chung 2017). A recently published large individual patient meta-analysis reported patients with a BMPR2 mutation to present at a

younger age, have worse haemodynamics and survival compared to non-carriers (Evans, Girerd et al. 2016). Furthermore patients with BMPR2 mutations have worse right ventricular function (van der Bruggen, Happé et al. 2016) which could be related to defects in RV energy metabolism (Hemnes, Brittain et al. 2013; Talati, Brittain et al. 2016).

BMPR2 is one of three type II receptors belonging to the TGF-β receptor super family. It is a serine/threonine receptor kinase. Upstream of the receptors are at least 20 ligands and combined with 10 receptors that interact as hetromeric complexes to activate a number of canonical (SMAD dependant) and non-canonical (non SMAD e.g ERK, MAPK) downstream signalling cascades leading to nuclear events. In addition there are a number of co-receptors and decoy receptors. Unsurprisingly as a result of this diversity combined with spatiotemporal expression and context dependant signalling, a variety of functions for BMP ligands in mammalian physiology have been described (Yadin, Knaus et al. 2016). Mutations in these receptors can lead to a seemingly diverse array of conditions including cancer and range of cardiovascular diseases (including PAH).

BMPR2 deficiency primarily leads to endothelial cell dysfunction, apoptosis and loss of vascular integrity. Functional reduction (non genetic) in lung expression of BMPR2 has been identified in human PAH (Atkinson, Stewart et al. 2002; Dewachter, Adnot et al. 2009), monocrotaline rats (Morty, Nejman et al. 2007) and in mice exposed to hypoxia (Brock, Samillan et al. 2012) , Sugen-Hypoxia (Ciuclan, Bonneau et al. 2011) and high fat fed $ApoE^{-/-}$ mice (Lawrie, Hameed et al. 2011).

However mutations within BMPR2 do not fully account for the disease as evidenced by incomplete gene penetrance (approximately 20%) and thus the majority of individuals harboring mutations do not go on to develop disease. Additionally what has also been unclear and frustrating is why despite germ-line mutations in BMPR2 there is a predilection for the disease within the pulmonary vasculature (White and Morrell 2012). This has led to the notion that “multiple hits” are required and that BMPR2 mutations are required but alone are insufficient for disease (Yuan and Rubin 2005; Austin, West et al. 2017). This has led to a huge body of work trying to elucidate the mechanisms in dysfunctional BMPR2 signaling and integration into emerging mechanisms of disease pathogenesis as described below.

1.10 Endothelial and vascular smooth cells in PAH

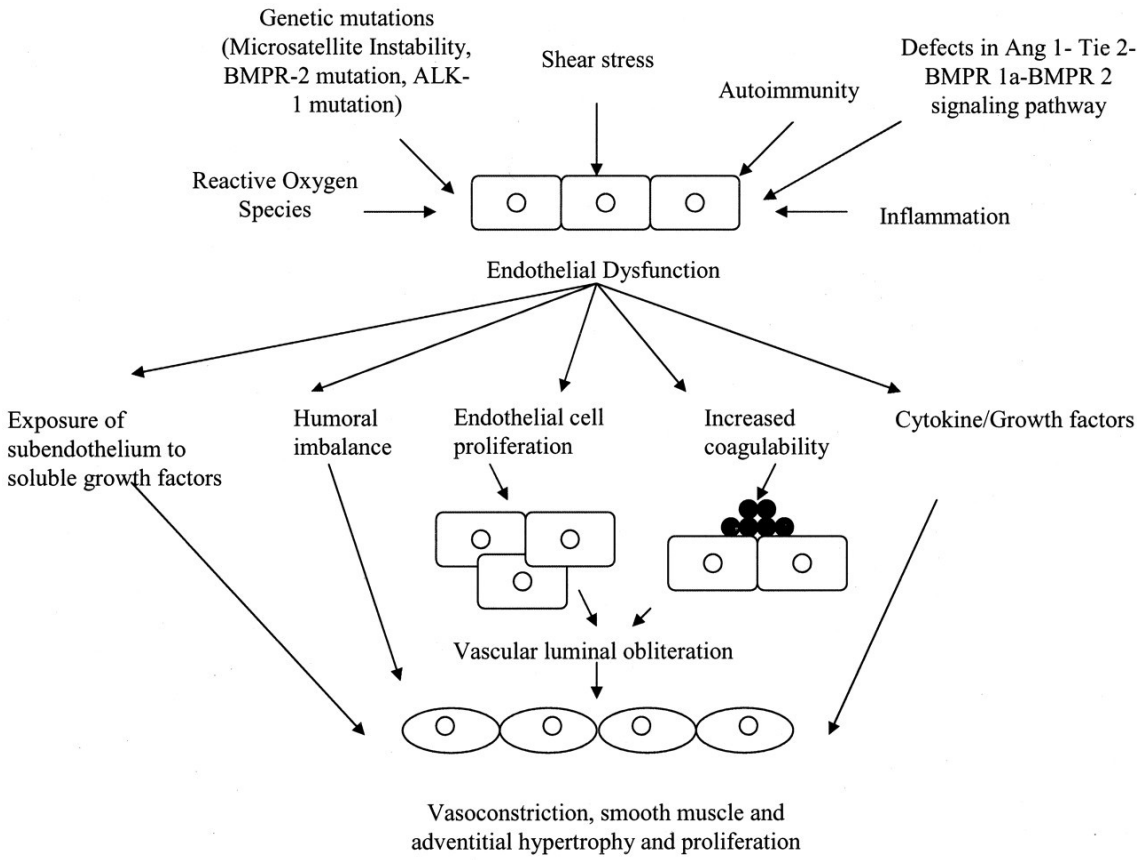


Figure 1.11: Endothelial dysfunction and PAH pathogenesis. Schematic illustration of upstream factors of that can induce endothelial dysfunction and the subsequent effects on endothelial and PASMC biology. Redisplayed with permission from (Budhiraja, Tuder et al. 2004).

Pulmonary vascular endothelium has an important role in maintaining vascular homeostasis by regulating vascular tone, vascular barrier, permitting active and passive transport of molecules into underlying tissue as well as maintaining flow and preventing thrombosis. Several lines of evidence demonstrating endothelial cell dysfunction, apoptosis and hyperproliferation induced by inflammation, oxidative and shear stress, genetics, epigenetics and mitochondrial dysfunction **(Fig. 1.11)**. This has been derived from human studies (serum biomarkers, patient derived cells, lung immunohistochemistry) and rodent models of disease (Budhiraja, Tuder et al. 2004; Hiress, Tu et al. 2015; Bonnet and Provencher 2016; Benoît, Lloyd et al. 2017).

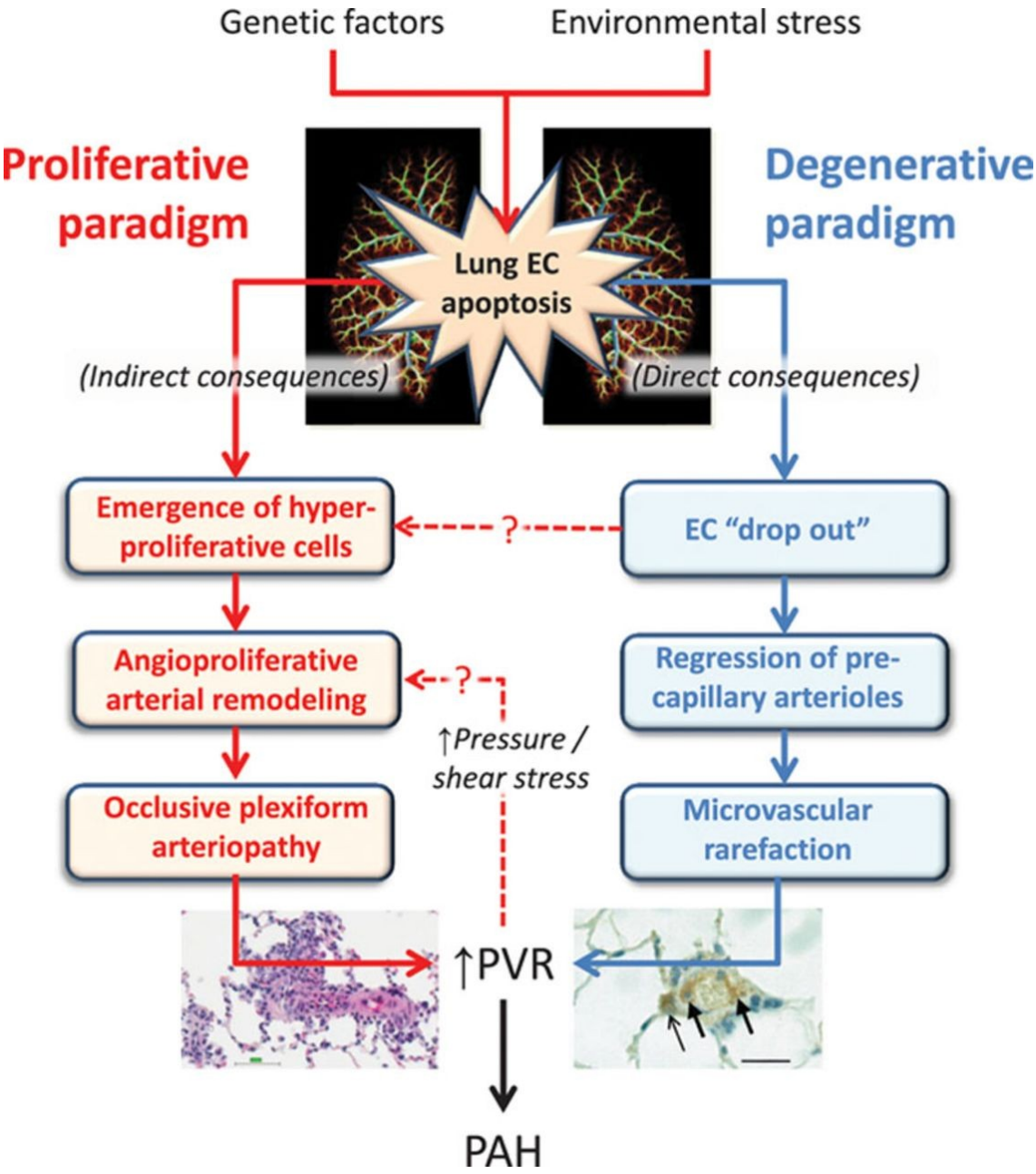


Figure 1.12: Proliferative and degenerative hypotheses of PAH. Redisplayed with permission from (Chaudhary, Taha et al. 2017).

Although the field remains complex, it is believed that endothelial cell injury induced by a number of factors leads to early endothelial cell dysfunction/apoptosis and loss of vascular integrity followed by activation of endothelial repair mechanisms and proinflammatory signalling stimulating medial and adventitial remodelling (Sakao, Taraseviciene-Stewart et al. 2005; Sakao, Tatsumi et al. 2009) . Normal BMPR2 signalling appears be a protective regulatory factor in this regard (Teichert-Kuliszewska, Kutryk et al. 2006; Song, Coleman et al. 2008;

Alastalo, Li et al. 2011; Tamosiuniene, Tian et al. 2011) and prevents PASMCs from switching to a proliferative and contractile phenotype (Nasim, Ogo et al. 2012; Maruyama, Dewachter et al. 2015).

However initial endothelial cell apoptosis is believed to lead to the selective and clonal expansion of hyperproliferative apoptotic resistant endothelial cells (Sakao, Tatsumi et al. 2010; Voelkel, Gomez-Arroyo et al. 2012; Goldthorpe, Jiang et al. 2015). These cells are regarded by some to represent an aberrant angiogenic response to injury but resulting in concentric intimal fibrosis and plexiform lesion formation. This "angioproliferative" hypothesis of PAH has principally been derived from studies in the Sugen-Hypoxia rat model of PAH (Taraseviciene-Stewart, Kasahara et al. 2001; Abe, Toba et al. 2010; Lavoie, Ormiston et al. 2014; Toba, Alzoubi et al. 2014).

An alternative hypothesis is the "degenerative" model **(Fig. 1.12)** which regards the primary defect within endothelial cell loss resulting in vascular degeneration and loss of arteriolar architecture. This hypothesis proposes that augmenting lung vascular regeneration would be a more suitable therapeutic strategy (Chaudhary, Taha et al. 2017). In this regard either endothelial progenitor cells (Jurasz, Courtman et al. 2010; Gurtu and Michelakis 2015) or restoring functional BMPR2 signalling (Spiekerkoetter, Tian et al. 2013; Long, Ormiston et al. 2015; Ormiston, Upton et al. 2015; Spiekerkoetter, Sung et al. 2015) are potential therapeutic strategies to try restore endothelial integrity and function.

PASMCs are regarded as the main cell driving medial hypertrophy in response to a number of growth factors and dysfunctional BMPR2 signalling. Aberrant growth properties of PASMCs have been noted in patients (Morrell, Yang et al. 2001; Yang, Long et al. 2005; Dewachter, Adnot et al. 2009; Zabini, Granton et al. 2018) and rodent models (Thomas, Docx et al. 2009). PASMCs display heterogeneity within different compartments of the lung vasculature and there is growing recognition that other cells (endothelial, pericytes, fibroblasts and myofibroblasts) may migrate and differentiate into SMC like cells through processes such as endothelial to mesenchymal transition (Stevens, Phan et al. 2008; Ranchoux, Antigny et al. 2015; Stenmark, Frid et al. 2018).

1.11 Inflammation and immunity in PAH

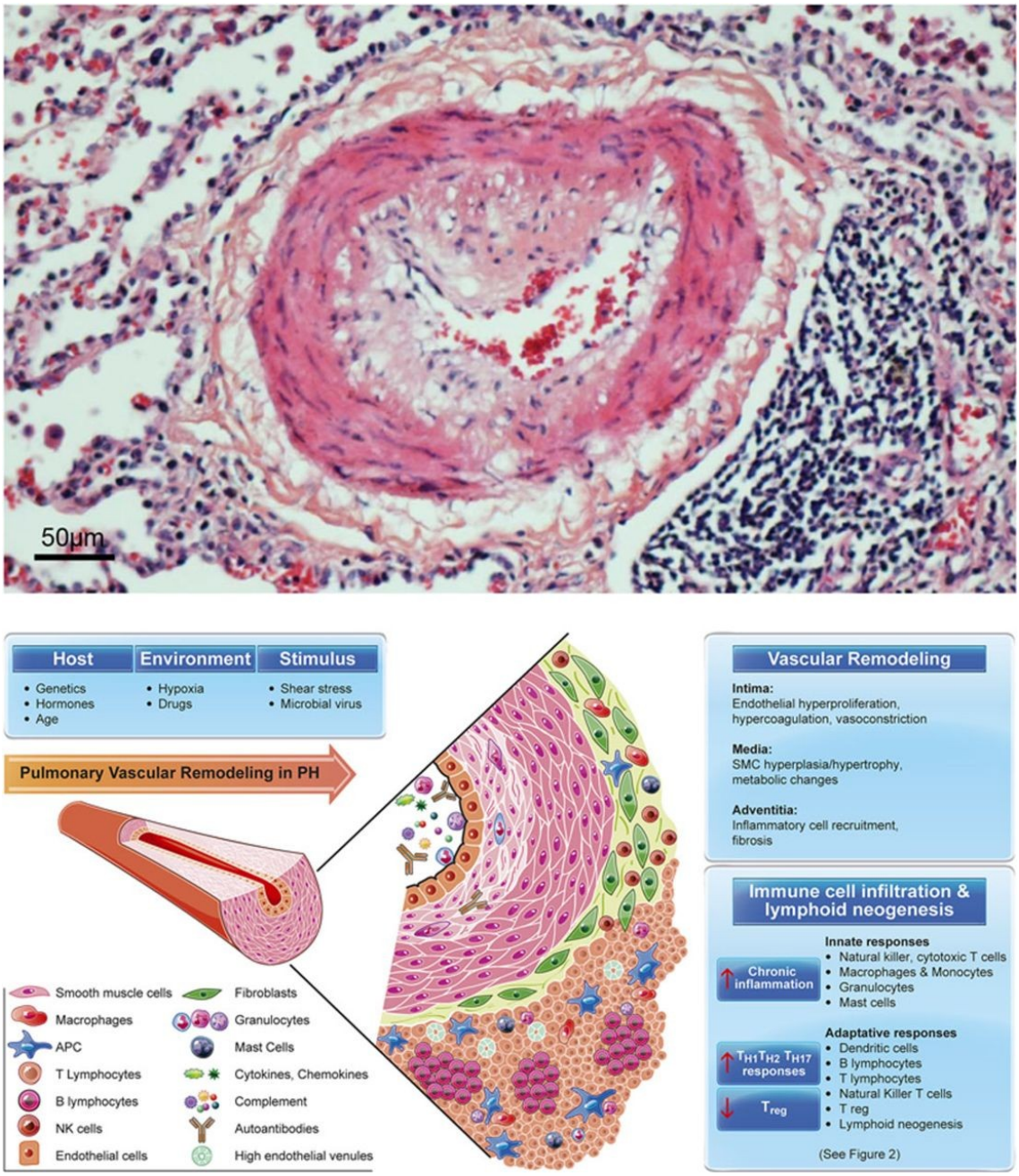


Figure 1.13 Immune and inflammatory involvement in PAH. Histopathology of a vascular lesion from human PAH demonstrating neointimal proliferation, medial hypertrophy and marked inflammatory cell accumulation in the periadventitia. The lower schematic illustrates the types of immune cells and inflammatory mediators reported in human and animal PAH. Redisplayed from (Rabinovitch, Guignabert et al. 2014) with permission.

There are several lines of evidence indicating a role for inflammation in human and experimental PAH (Hassoun, Mouthon et al. 2009; Price, Wort et al. 2012; Rabinovitch, Guignabert et al. 2014). Elevated blood levels of cytokines have been demonstrated from relatively small numbers of patients with PAH and rodent models. A range of inflammatory and immune cells have been observed in histopathological lesions from patients. Transgenic mice have highlighted a pathogenic role for some cytokines and anecdotal reports of improvement in PAH with the use of biological therapies.

Elevated serum levels of interleukins 1 and 6 (Dorfmuller, Perros et al. 2003; Cracowski, Chabot et al. 2014) and TNFα have been detected in patients with PAH and predict survival (Soon, Holmes et al. 2010; Heresi, Aytekin et al. 2014). Several chemokines (Dorfmuller, Zarka et al. 2002; Perros, Dorfmuller et al. 2007) have also been reported in IPAH.

In animal models IL-6 over-expression in PASMCs drives pulmonary hypertension in hypoxic mice (Savale, Tu et al. 2009; Steiner, Syrkina et al. 2009). IL-6 and FGF2 have been shown to stimulate pericyte growth in human and experimental PAH (Ricard, Tu et al. 2014). IL-1 signaling is also dysregulated in human and experimental PAH (Lawrie, Hameed et al. 2011; Parpaleix, Amsellem et al. 2016; Pickworth, Rothman et al. 2017). These are exciting observations because biological drugs targeting IL-1 and IL-6 are licensed for clinical use in certain inflammatory diseases and thus provide potential for repurposing in PAH (Gomez-Arroyo, Abbate et al. 2016). Interestingly case reports have reported marked clinical benefit with the anti-IL6 antibody Tocilizumab in patients with PAH in the context of adult onset Still's disease (Kadavath, Zapantis et al. 2014) or an uncommon lymphoproliferative disorder, Castleman's disease (Arita, Sakata et al. 2010; Furuya, Satoh et al. 2010).

Histopathological studies have noted inflammatory cells around vascular lesions in PAH prompting the significance of this observation (Savai, Pullamsetti et al. 2012; Stacher, Graham et al. 2012; Tuder, Archer et al. 2013). Regulatory T cells limit experimental PH (Tamosiuniene, Tian et al. 2011) whilst altered natural killer and dendritic cell responses have been observed in human and rodent PH models (Perros, Dorfmuller et al. 2007; Ormiston, Chang et al. 2012). Macrophage recruitment and release of leukotriene B4 has been therapeutically explored in PAH (Tian, Jiang et al. 2013).

BMPR2 mutant cells from patients exhibit proinflammatory responses (Helbing, Rothweiler et al. 2011; Davies, Holmes et al. 2012) whilst cytokines and growth factors can repress BMPR2 signaling *in-vitro* and in rodent models (Hagen, Fagan et al. 2007; Kim, Song et al. 2013;

Sawada, Saito et al. 2014; Vengethasamy, Hautefort et al. 2016; Pickworth, Rothman et al. 2017). The archetypal proinflammatory cytokine TNFα (Sutendra, Dromparis et al. 2011; Sawada, Saito et al. 2014) via NFκB signaling (Wang, Prakash et al. 2012; Hosokawa, Haraguchi et al. 2013; Li, Kim et al. 2017) has been linked altered metabolism and miRNA dysfunction in PAH.

Although still not definitive, there is a persuasive body of evidence supporting a role for deranged immune and inflammatory responses being causal in initiating and perpetuating disease pathogenesis **(Fig. 1.13)**. However apart from mechanistic/therapeutic studies in experimental rodents, the clinical evidence base is small and restricted to patients with a immune/inflammatory diseases linked to PAH such as mixed connective tissue disease or SLE (Hassoun 2014). Given the growing evidence for a pathogenic role for IL-1 and IL-6 in PAH and the availability of licensed drugs for their targeting there is the possibility of a quick "win". A small phase II proof of concept trial (TRANSFORM-UK) is underway testing the safety and haemodynamic efficacy of blocking IL-6 with Tocilizumab in idiopathic PAH (Hernández-Sánchez, Harlow et al. 2018). Trials therapeutically targeting other aspects of immunity and inflammation in early phase are summarized in (**Fig. 1.14**).

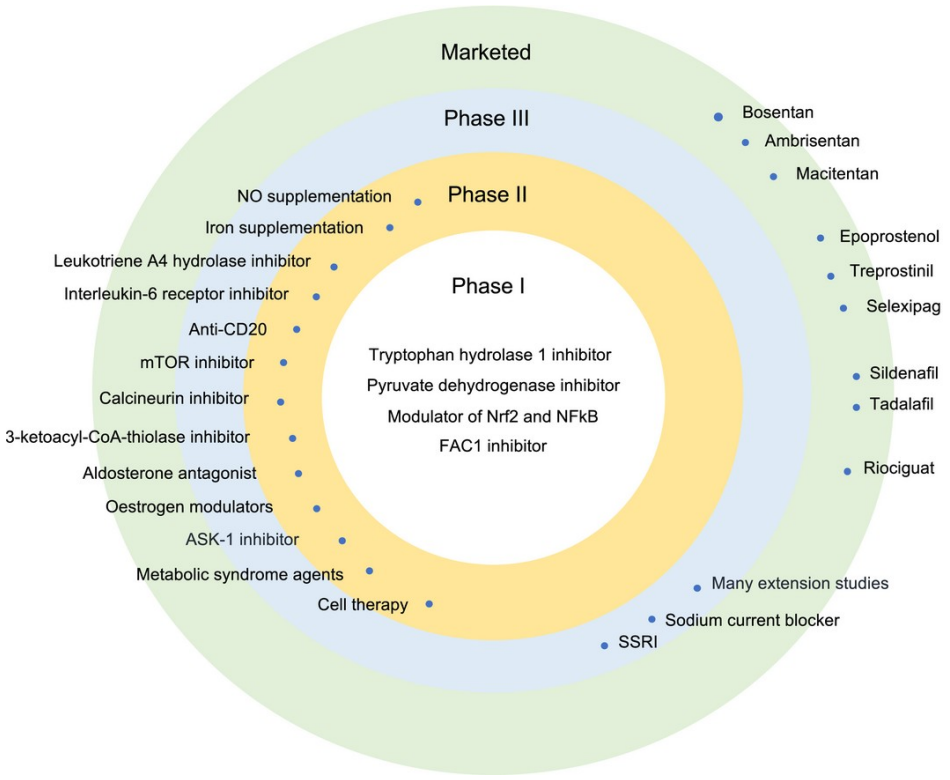


Figure 1.14. ongoing early phase clinical trials in PAH. Redisplayed with permission from (Ghataorhe, Rhodes et al. 2017).

1.12 Metabolic Hypothesis of PAH

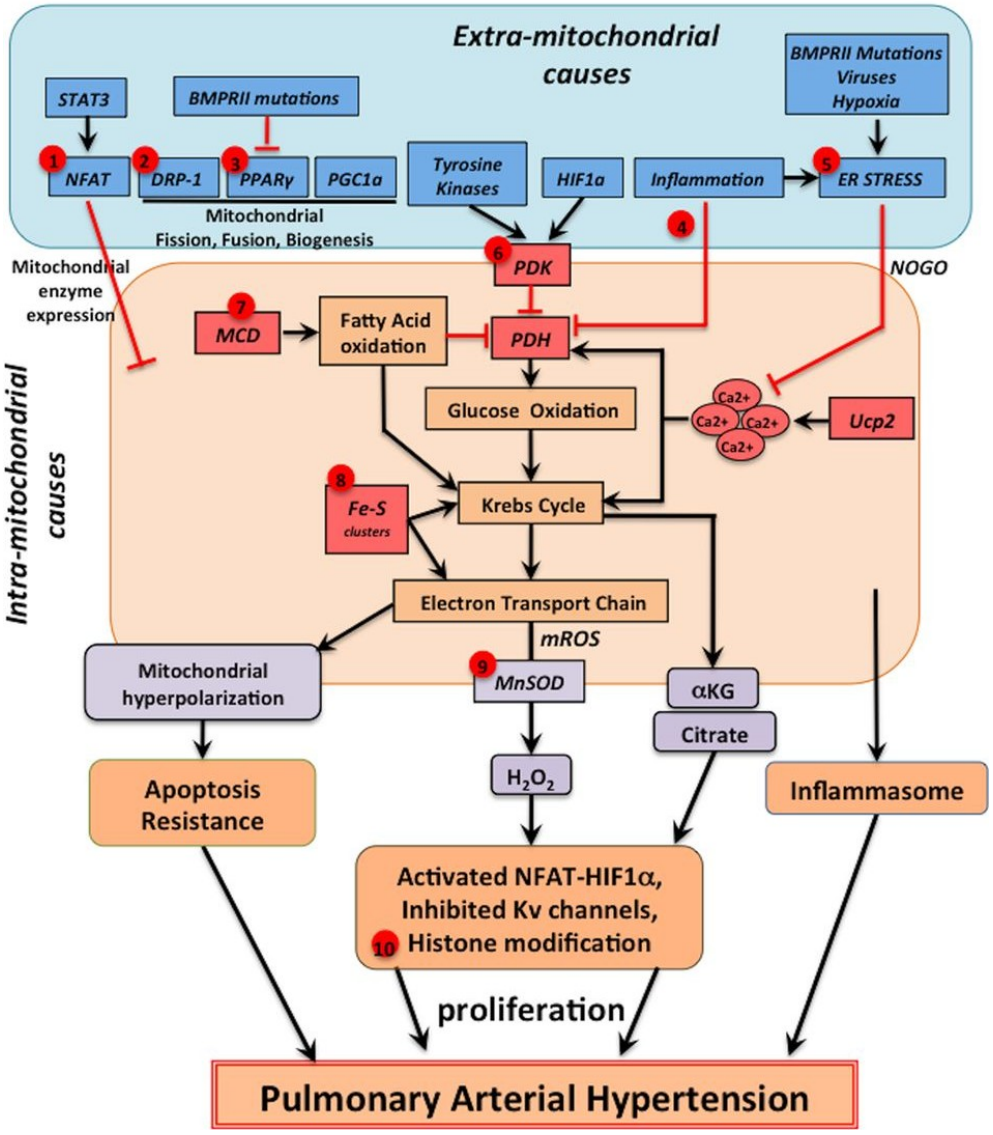


Figure 1.15 Mitochondrial dysfunction in PAH. Schematic illustration summarising how mitochondrial suppression (manifested primarily as reduced calcium levels and membrane potential) in a pulmonary endothelial and smooth muscle cells may lead a hyperproliferative, apoptosis resistant and pro-inflammatory phenotype. Upstream a variety of triggers can inhibit the enzyme PDH and two principal downstream transcription factors (NFAT and HIF1 α) can drive the three features of this phenotype. Potential sites for therapeutic exploitation are numbered. Redisplayed with permission from (Paulin and Michelakis 2014).

There has been a growing body of literature in recent years highlighting a role for mitochondrial dysfunction as a central feature in the pathogenesis of PAH (Dromparis, Paulin et al. 2013; Paulin and Michelakis 2014; Sutendra and Michelakis 2014). These authors argue that mitochondrial dysfunction is a potentially unifying theory of PAH to amalgamate many of

the diverse defects described in the pathogenesis. Mitochondria are the “power houses” of a cell and maintain life through generation of ATP. In addition they are important in mediating apoptosis through the extrinsic pathway (described in a subsequent section on TRAIL). There are two key features of mitochondrial dysfunction; hyperpolarized membranes and decreased mitochondrial calcium levels. Mitochondrial membrane hyperpolarisation has been observed in pulmonary vascular endothelial, smooth muscle and fibroblast cells by several groups (Bonnet, Michelakis et al. 2006; Archer, Gomberg-Maitland et al. 2008; Archer, Marsboom et al. 2010; Xu and Erzurum 2010; Hemnes, Wittmann et al. 2012; Goveia, Stapor et al. 2014).

In essence mitochondrial suppression due to a variety of upstream triggers already appreciated in PAH **(Fig. 1.15)**, leads to hyperpolarized mitochondrial cell membranes in PASMC and PAECs with a resultant metabolic fuel switch towards glycolysis (and shut down of the more energetically efficient process of generating ATP by oxidative phosphorylation). This leads to a “Warburg” effect akin to that first described and widely appreciated in tumour cells (Archer 2017) and explains the basis for via FDG-PET imaging is used to detect rapidly dividing cells in cancer (and lung and RV in human PAH). This leads to inhibition of cell apoptosis, activation of two key transcription factors NFAT (Nuclear Factor of Activated T cells) and HIF1α (hypoxia inducible factor 1 α) which stimulate cell proliferation, further inhibit apoptotic mechanisms and activate inflammatory pathways that feedback to drive the aforementioned cell phenotype.

A key upstream mitochondrial enzyme inactivated in PAH is PDH (Pyruvate dehydrogenase) which is inhibited by PDK (PDH kinase- labeled 6 in Fig.1.15 above). PDH promotes oxidative phosphorylation and maintains normal mitochondrial metabolism and function. A body of preclinical and translational science over a decade has tried to therapeutically exploit PDK inhibition using a clinically available drug called DCA (Dichlorate Acetate). DCA inhibits PDK and releases PDH to restore oxidative phosphorylation and normal membrane potential. Recently this endeavor resulted in the first multicentre human trial of DCA in 20 patients with IPAH (Michelakis, Gurtu et al. 2017). Significant clinically meaningful improvements in haemodynamics and exercise capacity were observed in well treated (mostly on dual oral therapy) IPAH patients. However a group of non responders to DCA were observed in whom it was subsequently determined that they harbored a genetic modifier in two proteins permitting mitochondrial suppression independent of PDK.

1.13 MicroRNAs in PAH

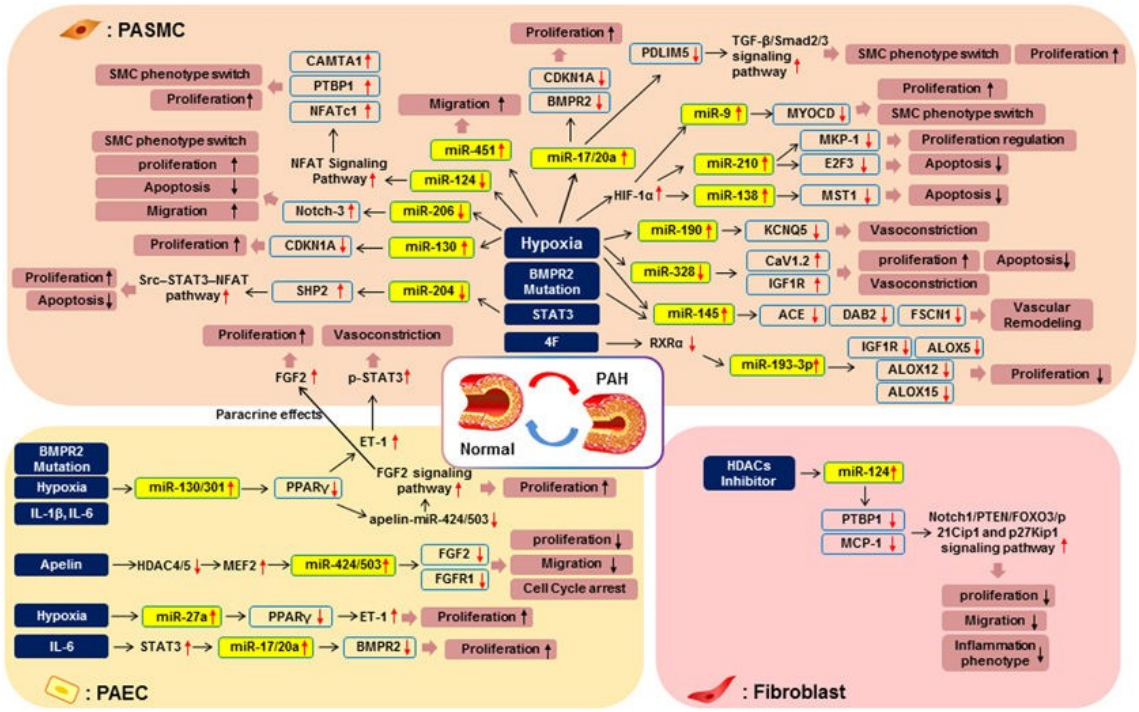


Figure 1.16: MicroRNA networks in Pulmonary Hypertension. Schematic illustration summarising how existing pathophysiological mechanisms interact with novel and emerging miRNA networks in key pulmonary vascular cells. Redisplayed from (Kim, Lee et al. 2015). No permission was required as this is an open access publication.

Micro RNAs (miRNA) belong to a group of at least 10 different classes of non-coding RNA. They are short (19-24 nucleotides long) non coding RNA molecules that repress the translation of multiple genes (up to 60% of all protein encoding genes) and thus afford a physiologically important epigenetic mechanism for diversity and regulation of several cellular and molecular processes. They have been well described in cancer biology (Esteller 2011). At least 5000 miRNAs have been reported some of which can regulate hundreds of genes.

There is a growing list of miRNAs **(Fig. 1.16)** implicated in PAH, modulating inflammatory and metabolic pathways to drive a hyperproliferative anti-apoptotic phenotype in PAECs and PASMCs (Zhou, Chen et al. 2015; Gamen, Seeger et al. 2016; Chun, Bonnet et al. 2017). For example IL-6 activates the miR 17/92 cluster to repress BMPR2 protein expression in PAECs (Brock, Trenkmann et al. 2009) activate HIF1α (Chen, Zhou et al. 2016) and induce experimental disease *in vivo* (Chen, Zhou et al. 2015). Our group recently identified another

novel mechanism of reduced BMPR2 signaling through ubuiquination, controlled by miR 140 (Rothman, Arnold et al. 2016). Collectively these findings could account for the functionally reduced BMPR2 signaling described earlier and could be permissive factors for the development of PAH in non genetic disease.

The use of a systems biology approach has begun elucidating a hierarchy of key miRNAs in PAH (Parikh, Jin et al. 2012; Bertero, Lu et al. 2014; Chun, Bonnet et al. 2017) and permitted integration with defects such as altered cell metabolism (Caruso, Dunmore et al. 2017; Zhang, Wang et al. 2017). By adopting this approach key miRNAs can be identified that drive multiple disease pathways, however the therapeutic safety and clinical efficacy of this approach remains unproven (Negi and Chan 2017).

Another potentially valuable role for miRNAs relevant to disease is their utility as biomarkers in human PAH. There is a significant unmet clinical and research need for good biomarkers. Recent data could fill this gap as they supported a role for miRNA 150 as independent predictor of survival in PAH (Rhodes, Wharton et al. 2013). Further studies employing this approach are emerging, and data from the PVDOMICs program will be eagerly awaited (Hemnes, Beck et al. 2017).

1.14 Summary of potential translational targets for drug therapy in PAH

From the discussion so far it can be appreciated that a complex array of molecular and cellular defects is expanding our current understanding of the pathophysiology of PAH. Most have been studied mechanistically in rodent and cell culture models supported by some evidence in human PAH. A few are being therapeutically exploited in early phase clinical trials but most remain experimental **(Fig. 1.17).** The progressive nature of pulmonary vascular disease results in an unmet clinical need for developing more effective therapies to improve prognosis.

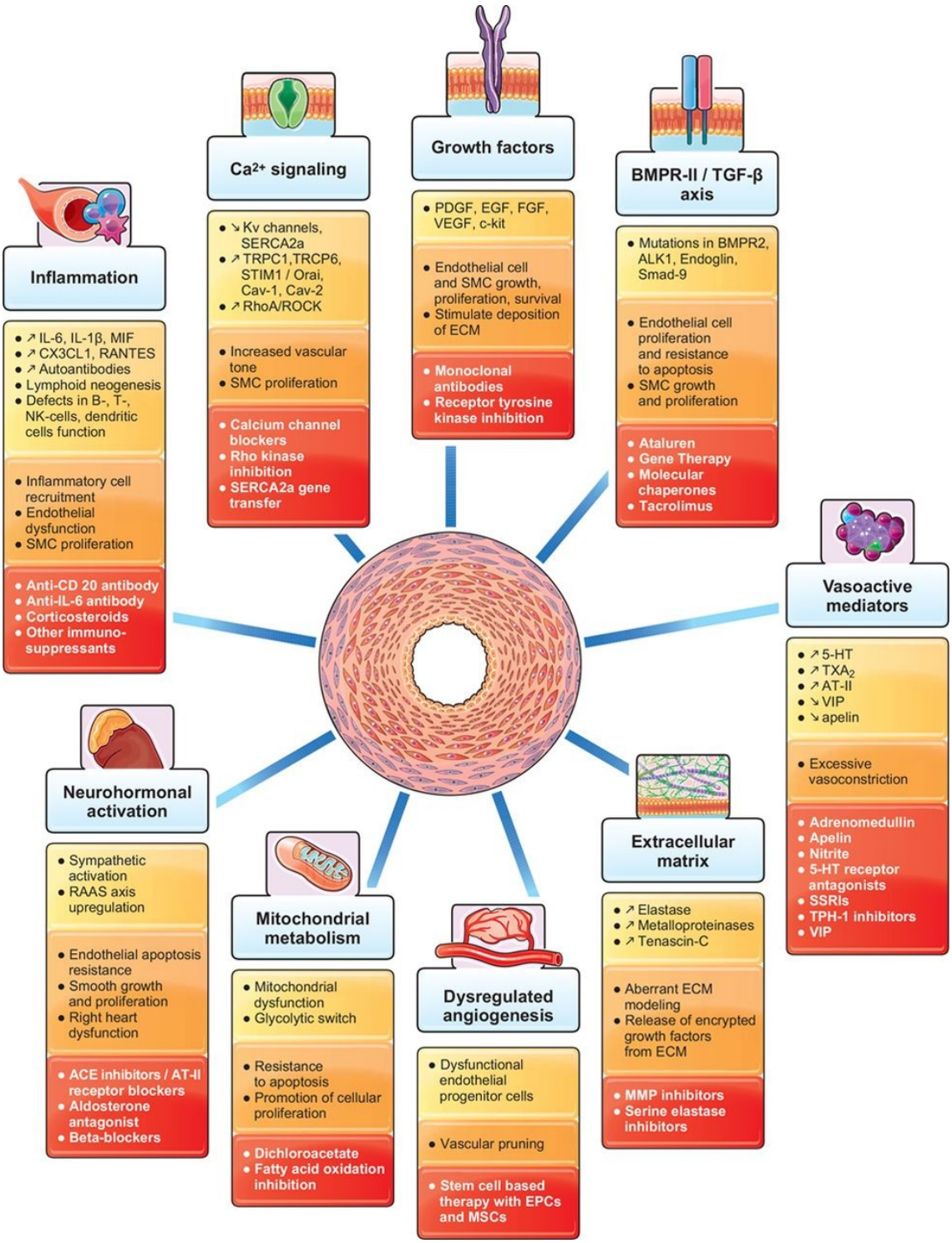


Figure 1.17. Emerging pathophysiological defects and potential targets for therapy in PAH. Specific mediators or defects (yellow), pathophysiological consequences (orange) and potential therapies studied or underway (red). Redisplayed with permission from (Humbert, Lau et al. 2014).

1.15 Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL)

1.15.1 Brief overview of TRAIL

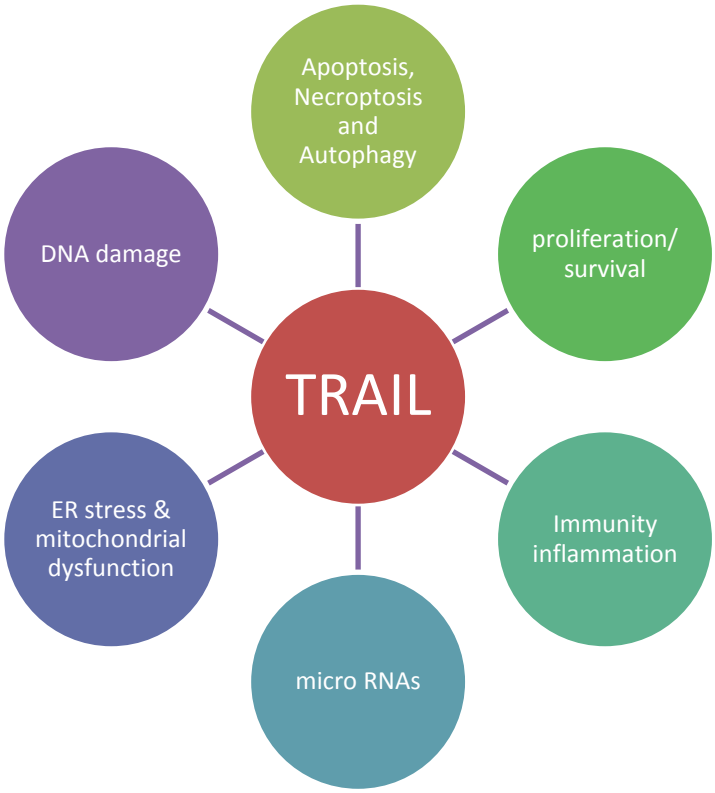


Figure 1.18 Diversity in TRAIL physiology. TRAIL can be induced in response to DNA damage, ER stress and by miRNAs whilst also regulating cell growth, survival, apoptosis with links to inflammation and mitochondrial function, that have been noted in PAH.

TRAIL is a multi functional cytokine **(Fig. 1.18)** part of the TNF superfamily. Following its discovery over two decades ago, huge hopes were placed on it after inducing apoptosis of xenograft tumors in mice (Ashkenazi, Pai et al. 1999; Walczak, Miller et al. 1999). Studies in TRAIL deficient mice revealed a propensity for greater tumor growth and metastases. TRAIL mediated apoptosis through two "death" receptors (TRAIL R1 and R2) led to the clinical development of recombinant forms of TRAIL and agonistic antibodies. Over the past decade these have demonstrated safety but no significant efficacy in several human cancers (Ashkenazi 2015; von Karstedt, Montinaro et al. 2017). As with many disappointments in

cancer drug discovery and not surprisingly, most human cancers were found to be TRAIL resistant. Work from other areas started elucidating a role for TRAIL in regulating inflammation, cell survival, immune cell function in the context of systemic vascular biology, erythropoiesis, infection resolution and auto-immune and inflammatory diseases. These studies also suggested roles for the non apoptotic TRAIL receptors and a body of data has emerged supporting non-canonical TRAIL signaling to explain the diverse effects of TRAIL which appear context and disease dependent (Azijli, Weyhenmeyer et al. 2013). In particular a role for TRAIL in systemic vascular biology emerged (Zauli and Secchiero 2006; Kavurma and Bennett 2008) but the effects of TRAIL had not been explored in pulmonary vascular disease.

1.15.2 TRAIL structure and tissue expression

TRAIL was identified by two independent teams over two decades ago (Wiley, Schooley et al. 1995; Pitti, Marsters et al. 1996) after noting that it was able to induce apoptosis in transformed cell lines but not normal cells. It displayed sequence homology (20-30% in the C-terminal domain) to other members of the TNF superfamily (Ware 2008), particularly FAS Ligand. TRAIL is a type II transmembrane protein consisting of 281 amino acids (291 in mice) with a molecular weight of approximately 35 kDa. TRAIL can be cleaved by cysteine proteases (the precise protease(s) has not been identified) within the cell membrane to release a soluble form that is reported to retain biological activity (Zauli and Secchiero 2006). TRAIL is widely expressed in several tissues including lung, thymus, spleen, and prostate (Daniels, Turley et al. 2005; Zauli and Secchiero 2006). Furthermore TRAIL is expressed by vascular endothelial and smooth muscle cells (discussed later) as well as cells from the innate and adaptive immune system; T lymphocytes (Ehrlich, Infante-Duarte et al. 2003), mast cells (Berent-Maoz, Salemi et al. 2010), platelets (Crist, Elzey et al. 2004) neutrophils (Cassatella, Huber et al. 2006) , monocytes (Griffith, Wiley et al. 1999) macrophages (Ho, Chen et al. 2011) dendritic cells (Fanger, Maliszewski et al. 1999) and NK cells.

1.15.3 Function of TRAIL

The initial reports of TRAIL having the ability to induce apoptosis in tumor cell lines and regress tumors in mice with little effect on normal cells in-vitro was an exciting discovery (Ashkenazi, Pai et al. 1999). It stimulated an intense effort into studying TRAIL signaling as a pathway for targeting cancer (Johnstone, Frew et al. 2008; Corazza, Kassahn et al. 2009; Newsom-Davis, Prieske et al. 2009). However after over a decade of multiple clinical trials using either recombinant TRAIL or agonistic death receptor it became apparent that most human tumors were resistant to the apoptotic effects of TRAIL. Several mechanisms of acquired resistance have been described (Trivedi and Mishra 2015). Furthermore the fact that TRAIL receptors are ubiquitously expressed on cells throughout the body (Daniels, Turley et al. 2005) supports the role for non-apoptotic functions of TRAIL. TRAIL has been shown to regulate processes linked to haematopoesis (Zauli and Secchiero 2006; Secchiero and Zauli 2008), inflammation (Adam, Paul et al. 2009; Tang, Wang et al. 2009), immune surveillance (Falschlehner, Schaefer et al. 2009) and vascular biology (Secchiero, Candido et al. 2006; Zauli and Secchiero 2006; Vaccarezza, Delbello et al. 2007). These diverse functions of TRAIL that have become apparent appear to be related to the complexity within the receptor signaling and subcellular assembly of scaffolding proteins.

1.15.4 Regulation of the human TRAIL gene

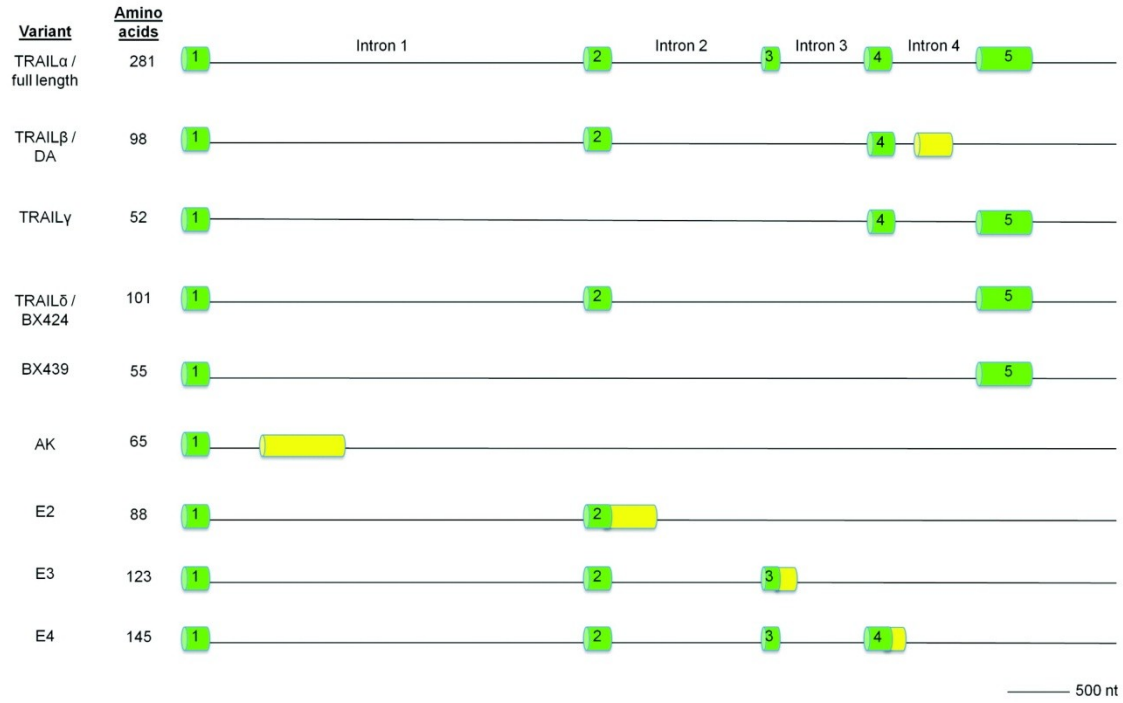


Figure 1.19 Structure of human TRAIL gene and splice variants. Green indicates exons and Yellow novel sequences in splice variants. Exon 1 codes for transmembrane and cytoplasmic domains, Exons 4 and 5 code for the extracellular domain and receptor binding whilst Exon 5 also codes for the C-terminal domain. Redisplayed from (Allen and El-Deiry 2012) with no permissions required as open access publication.

The gene for TRAIL is on chromosome 3q26 in humans and mice (Ware 2008). It consists of 5 exons and 4 introns (Gong and Almasan 2000). 8 splice variants **(Fig. 1.19)** have been reported. TRAIL β, TRAILγ and TRAILδ were shown to not induce apoptosis but hypothesised to neutralize full length TRAIL. Additional variants described in 2011 were shown to induce NFκB signaling and stimulate inflammatory cytokine and chemokine release but not apoptosis (Krieg, Krieg et al. 2003; Wang, Lu et al. 2011). Patterns of expression and effects of splice variants have not been characterized in diseased tissues.

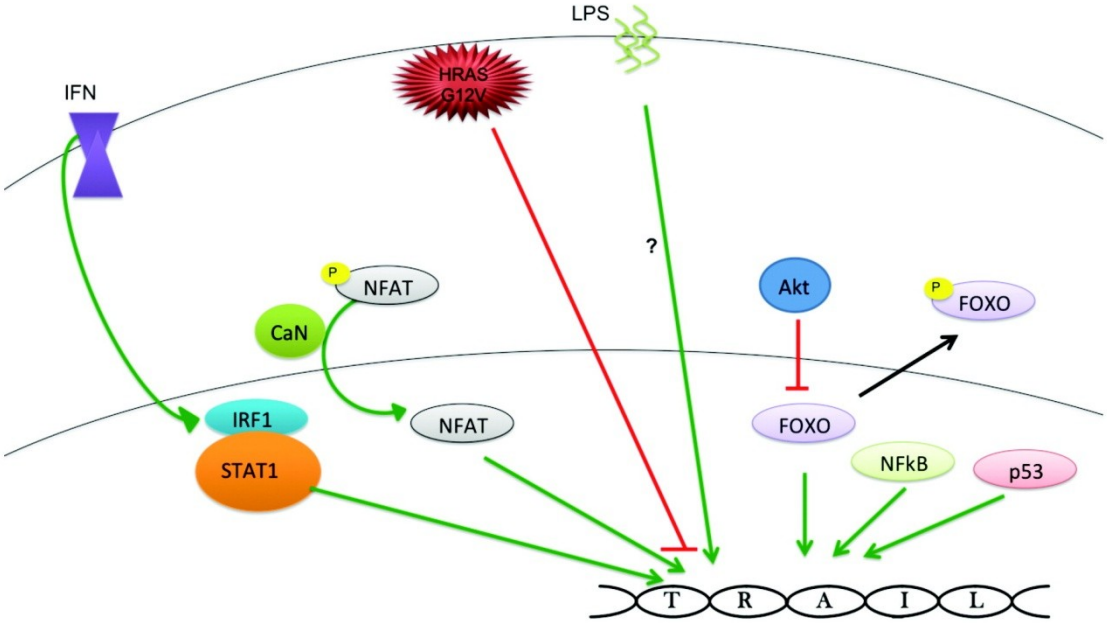


Figure 1.20: Transcription regulation of TRAIL gene. Schematic illustration of key transcription factors regulating TRAIL gene expression, most of which have already been implicated in PAH (Pullamsetti, Perros et al. 2016). Redisplayed from (Allen and El-Deiry 2012) with no permission required (open access article).

Several growth and transcription factors can regulate TRAIL **(Fig. 1.20)** (Allen and El-Deiry 2012; Azahri and Kavurma 2013). Several of these have already been implicated in PAH and examples of positive regulation (many from vascular biology) include FGF2 (Chan, Prado-Lourenco et al. 2010), PDGF (Azahri, Di Bartolo et al. 2012) NFκB (Kavurma, Schoppet et al. 2008; Chan, Prado-Lourenco et al. 2010) PPARγ (Ho, Chen et al. 2011) and TNFα (Secchiero, Zerbinati et al. 2004; Nguyen, Olesen et al. 2007; Corallini, Secchiero et al. 2010). Further discussion on putative links between existing pathways in PAH and TRAIL are covered in chapter 3.

In the context of neoplasia, TRAIL expression/function has been reported to be regulated by; microRNAs (Ovcharenko, Kelnar et al. 2007; Jeon, Middleton et al. 2015; Joshi, Jeon et al. 2015), altered mitochondrial metabolism (MacFarlane, Robinson et al. 2012; Robinson, Dinsdale et al. 2012) and endoplasmic reticulum stress (Akita, Suzuki-Karasaki et al. 2014; Huang, Wang et al. 2015; Iurlaro and Muñoz-Pinedo 2016; Jiang, Chen et al. 2017) concepts which as discussed earlier have emerged in PAH research and share similarity with some of the hallmarks described in cancer (Hanahan and Weinberg 2011).

1.15.5 TRAIL receptors

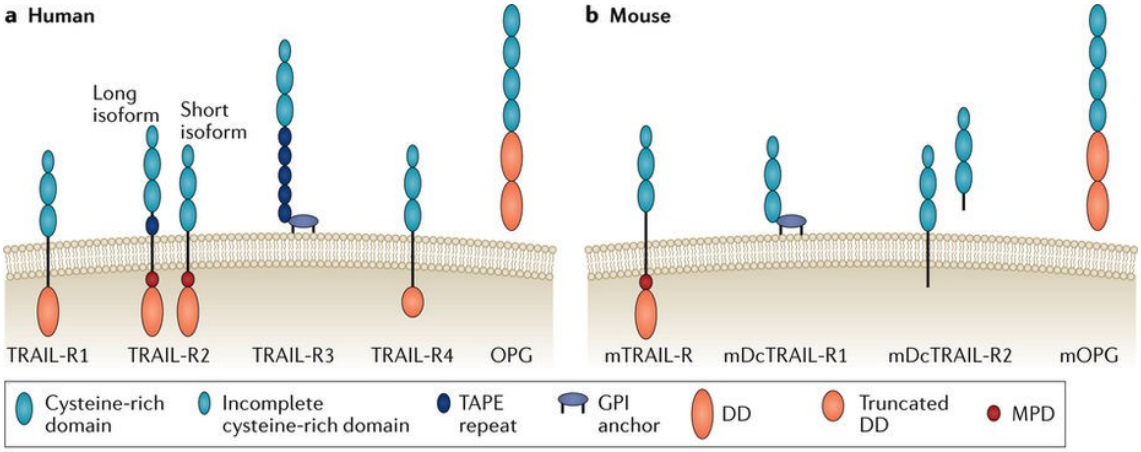


Figure 1.21 Structure of TRAIL receptors in humans and mice. TAPE (threonine, alanine, proline and glutamine) domain. DD death domain. GPI glycosylphosphatidylinositol , MPD; membrane-proximal domain. Redisplayed with permission from (von Karstedt, Montinaro et al. 2017).

Like other members of the TNF superfamily, TRAIL forms a homotrimer before binding to its cognate receptors. Five receptors have been described for TRAIL **(Fig. 1.21).** TRAIL R1 and R2 contain intracellular death domains and were initially shown to trigger apoptosis by the extrinsic and intrinsic pathways (Kimberley and Screaton 2004; Falschlehner, Emmerich et al. 2007; Guicciardi and Gores 2009)). In addition two further cell surface receptors (R3 and R4) that lack functional death domains have been described, and were proposed to merely act as decoy receptors (Marsters, Sheridan et al. 1997; Sheridan, Marsters et al. 1997). However precise functional details on R3 and R4 remain to be fully defined. Osteoprotegrin (OPG) was identified as the fifth receptor for TRAIL and has been shown to prevent TRAIL induced apoptosis *in vitro* (Emery, McDonnell et al. 1998; Vitovski, Phillips et al. 2007). OPG has an important role in bone remodeling and also is a soluble receptor for RANK Ligand.

A major driver for focusing on TRAIL apoptotic signaling was due to the potential for commercial and therapeutic exploitation in oncology. As a result most of our mechanistic understanding about TRAIL receptor signaling relates to TRAIL death receptors (R1 and R2). Data on the mechanisms of signaling via TRAIL R3 and R4 are still relatively limited.

1.15.6 Canonical (apoptotic) TRAIL signaling

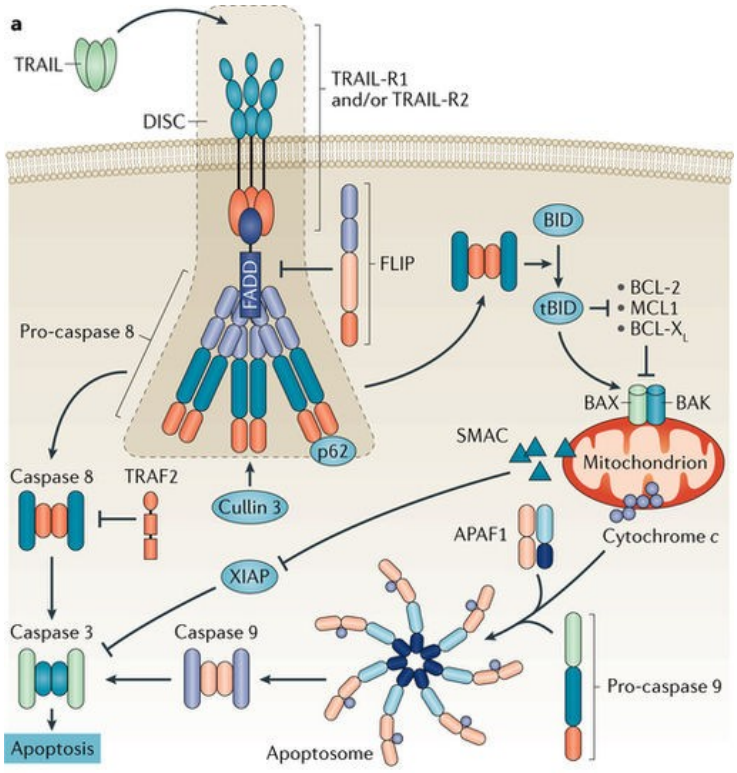


Figure 1.22. Apoptotic TRAIL receptor signaling via caspases. Schematic illustration of canonical TRAIL signaling. After ligand-receptor hetrotrimerisation of DISC (death inducing signal complex) is assembled which sequentially activates caspase 3. This initiates apoptosis via the intrinsic pathway. In type II cells apoptosis is triggered via the extrinsic (mitochondrial) pathway. Inhibitors and regulators of this pathway are marked and highlight mechanisms for how both transformed and non transformed cells can be resistant to TRAIL induced apoptosis. Redisplayed with permission from (von Karstedt, Montinaro et al. 2017).

The initial apoptotic effects of TRAIL on tumor cells led to studies focusing on the mechanisms of apoptosis signaling. Upon binding of TRAIL to a death receptor, the intracellular death domains of the receptors bind to a FAS associated death domain (FADD) adaptor protein to form a DISC assembly. This leads to sequential activation of caspases triggering apoptosis directly or via mitochondria **(Fig. 1.22).** Although many normal tissues and cell types express TRAIL and its receptors, due to inbuilt mechanisms most cells do not undergo apoptosis after TRAIL ligation. It is also apparent however that many cancer cells have acquired resistance to the apoptotic effects of TRAIL (von Karstedt, Montinaro et al. 2017).

1.15.7 Non-canonical TRAIL signaling

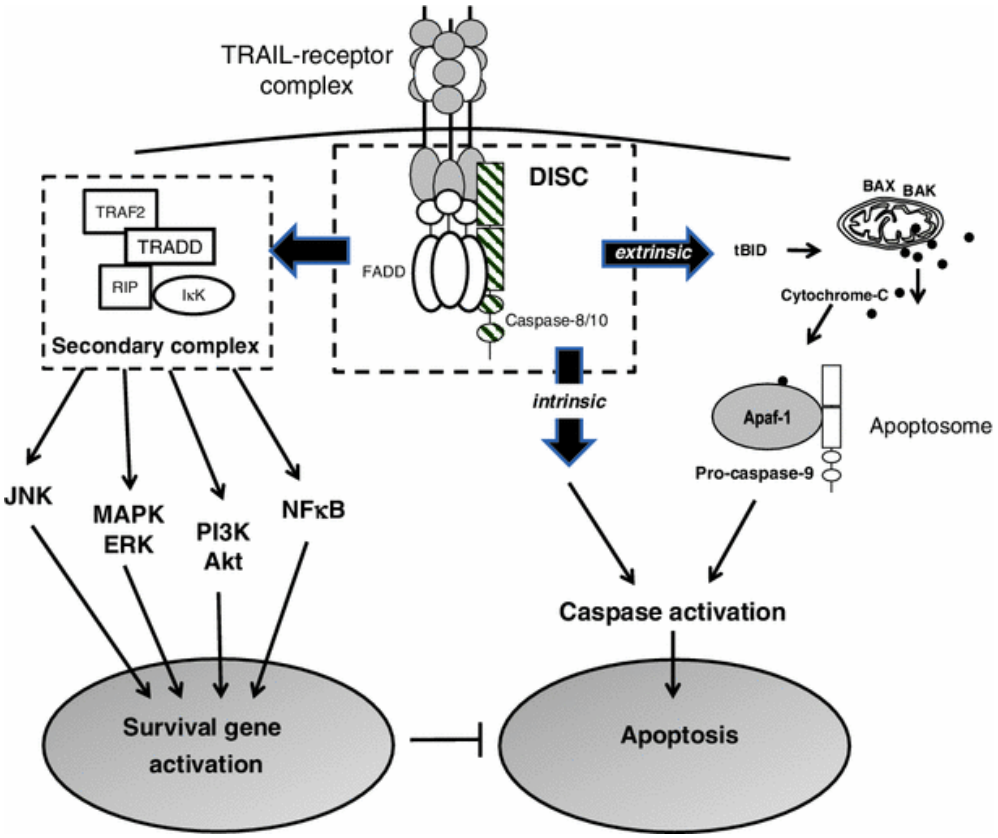


Figure 1.23 Noncanonical TRAIL signaling. TRAIL signaling via death receptors can form a secondary cytosolic complex consisting of additional adaptor proteins that subsequently activates several kinases known to induce survival and inflammatory gene activation. Redisplayed with permission from (Azahri and Kavurma 2013).

Given the diverse functions for TRAIL **(Fig. 1.18)** in non transformed cells such as promoting survival and inflammation as well as the observation that paradoxically TRAIL can promote survival and invasion in some cancers has fuelled intense research that has started elucidating additional TRAIL signaling pathways **(Fig. 1.23)** (Azijli, Weyhenmeyer et al. 2013; Fulda 2013; Siegmund, Lang et al. 2017; von Karstedt, Montinaro et al. 2017) explaining prior work highlighting a role for NFκB induced inflammation with TRAIL (Plantivaux, Szegezdi et al. 2009). Recent studies are shedding further light on the dynamics and complexities of TRAIL signaling with respect to TRAIL R1 and R2 (Jiang, Chen et al. 2017; Kearney and Martin 2017; Lafont, Kantari-Mimoun et al. 2017; Lafont, Hartwig et al. 2018).

1.16 A role for TRAIL in systemic vascular biology and cardiovascular disease

TRAIL was first detected in the aorta and pulmonary artery of rodents (Gochuico, Zhang et al. 2000) and culture of VSMC from these vascular beds expressed TRAIL. TRAIL was also detected in atherosclerotic plaques (Schoppet, Al-Fakhri et al. 2004; Michowitz, Goldstein et al. 2005; Kavurma and Bennett 2008; Kavurma, Schoppet et al. 2008) where it was shown to increase both VSMC migration (Secchiero, Candido et al. 2006; Kavurma, Schoppet et al. 2008) and VSMC apoptosis (Sato, Niessner et al. 2006) thus promoting both plaque stabilization and instability.

In-vitro TRAIL has been consistently shown to stimulate the migration, proliferation and survival of VSMCs (Secchiero, Zerbinati et al. 2004; Kavurma, Schoppet et al. 2008; Chan, Prado-Lourenco et al. 2010). TRAIL has been detected in stenosed vein grafts in areas of increased VSMC proliferation (as detected by PCNA-proliferating cell nuclear antigen staining). Interestingly no changes in apoptosis levels were observed supporting a pro-proliferative role for TRAIL in vascular remodeling. Further evidence to support this phenomenon was confirmed *in-vivo.* TRAIL was demonstrated to be an essential mediator in the neointimal proliferation observed after arterial injury in a murine model. Specifically $TRAIL^{-/-}$ mice had a 2-3 fold reduction in VSMC proliferation, with no differences in detectable apoptosis (Chan, Prado-Lourenco et al. 2010).

Data suggest that non transformed cells such as VSMC have several redundant mechanisms for being intrinsically resistant to apoptosis beyond the expression profile of TRAIL receptors (van Dijk, Halpin-McCormick et al. 2013). Further discussion on TRAIL signaling in systemic VSMCs is covered in chapter 3.

There has been a slowly growing body of literature on the role of TRAIL in endothelial cell biology (Zauli and Secchiero 2006) that is derived predominantly from *in-vitro* cell culture work. TRAIL has been shown to promote survival and proliferation of ECs although some studies have also observed apoptosis. This could reflect differences in the type of EC studied (dermal, umbilical vein, aortic and microvascular origin) and contextual biology of TRAIL signaling.

For example TRAIL has been shown to induce some apoptosis (30%) in HUVEC and human dermal ECs whereas in surviving cells it induced inflammatory gene expression (NFκB), and

upregulation of adhesion molecules promoting leucocyte adhesion (Li, Kirkiles-Smith et al. 2003). Additionally OPG was shown to bind TRAIL to prevent endothelial cell apoptosis (Pritzker, Scatena et al. 2004). The proliferative effects of TRAIL in VSMC are mediated via AKT and ERK signaling (Secchiero, Zerbinati et al. 2004) and a similar effect has been shown in human umbilical vein and aortic ECs (Secchiero, Gonelli et al. 2003).

In addition to effects on endothelial cell growth, there are links between TRAIL and other important vascular endothelial responses such as oxidative stress (D'Auria, Centurione et al. 2015), inflammation (Viemann, Goebeler et al. 2006) microparticle release (Mackman 2009; Simoncini, Njock et al. 2009), nitric oxide production (Zauli, Pandolfi et al. 2003) and cell permeability (Stagg, Bowen et al. 2013). Studies in $TRAIL^{-/-}$ mice reveal a role for TRAIL in promoting angiogenesis (Hubert, Davies et al. 2009; Di Bartolo, Cartland et al. 2015) supporting prior in-vitro findings (Secchiero, Gonelli et al. 2004).

Collectively these data support a role for TRAIL in vascular endothelial cell homeostasis. Given that endothelial dysfunction and EC apoptosis are regarded as early features of PAH with a hyperproliferative EC phenotype later in disease; these data suggest that TRAIL could mediate pathologically relevant effects in PAH. Although not studied in this thesis, the role of TRAIL in pulmonary endothelial cell biology merits further attention and further discussion on this provided in chapter 8.

Several clinical studies have consistently highlighted inverse relationships between serum TRAIL and adverse outcome (including mortality) in atherosclerotic vascular disease (Michowitz, Goldstein et al. 2005; Schoppet, Sattler et al. 2006; S, S et al. 2010; Shaker, El-Shehaby et al. 2010), instent restenosis (Deftereos, Giannopoulos et al. 2011; Deftereos, Giannopoulos et al. 2012) acute myocardial infarction (Secchiero, Corallini et al. 2009; Secchiero, Corallini et al. 2010; Secchiero, Gonelli et al. 2010) and ventricular dysfunction (Schoppet, Ruppert et al. 2005; Lula, Rocha et al. 2009; Niessner, Hohensinner et al. 2009; Hage, Michaelsson et al. 2017). In a large population study low serum TRAIL levels independently predicted worse survival in older aged individuals with cardiovascular risk factors (Volpato, Ferrucci et al. 2011).

Finally several studies in mice investigating TRAIL have consistently supported a protective role for TRAIL in atherosclerosis (Secchiero, Candido et al. 2006; Di Bartolo, Chan et al. 2011; Watt, Chamberlain et al. 2011; Di Bartolo, Cartland et al. 2013; Liu, Xiang et al. 2014; Cartland,

Murphy et al. 2016) whilst there is also a growing literature on the protective role of TRAIL in diabetes and obesity (Harith, Morris et al. 2013) collectively suggesting TRAIL may be protective in systemic cardiovascular diseases. Further discussion on this subject is covered in chapter 4.

1.17 Evidence for a possible role of TRAIL in Pulmonary Arterial Hypertension

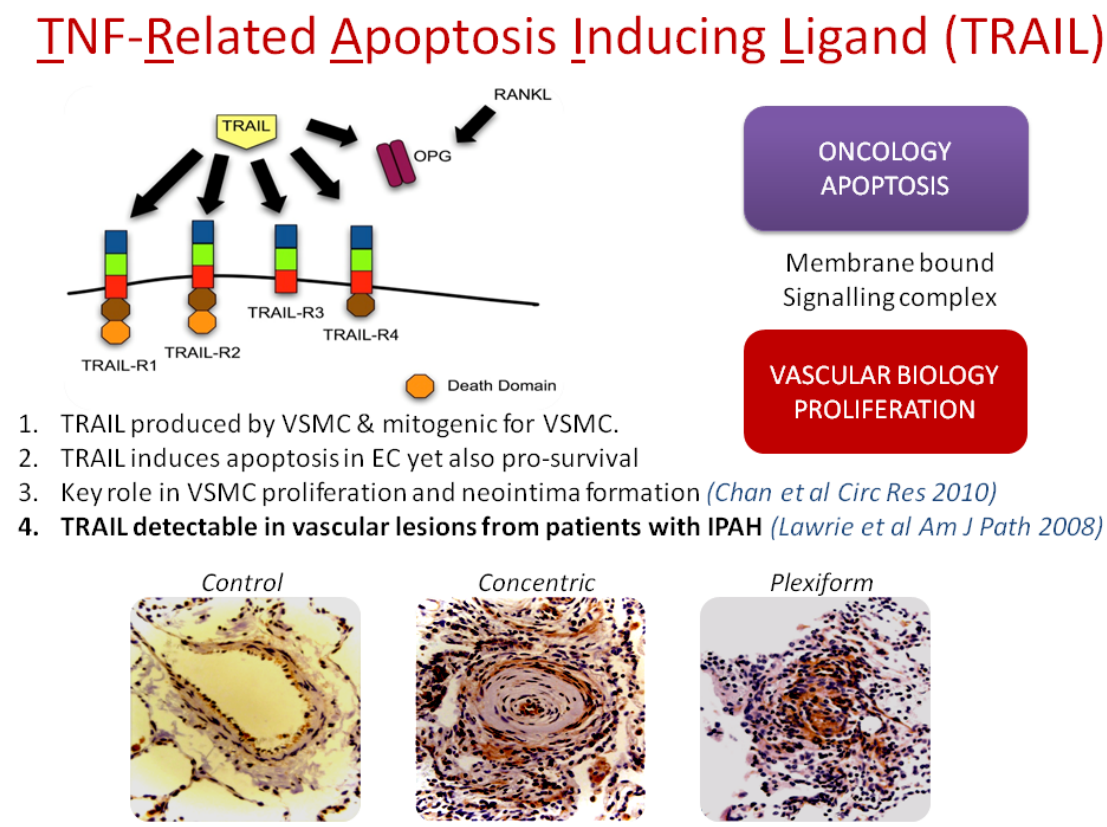


Figure 1.24: Evidence supporting a role for TRAIL in PAH.

The function of TRAIL has evolved from one predominantly describing apoptosis (in oncology) towards proliferation in systemic vascular biology. TRAIL is a proliferative and migratory stimulus for VSMC (vascular smooth muscle cells) and EC (endothelial cells) *in-vitro* and *in-vivo*. TRAIL has been detected using immunohistochemistry in advanced pulmonary lesions from patients with idiopathic PAH (lower histology slides).

Given the body of emerging literature supporting a role for TRAIL in vascular biology, our group observed expression of TRAIL in pulmonary vascular lesions of patients with IPAH (Lawrie, Waterman et al. 2008). TRAIL was found to be diffusely present throughout areas of medial hypertrophy as well as luminal and peri-adventitial sites in both concentric and plexiform lesions **(Fig.1.24).** Excess VSMC proliferation leading to medial hypertrophy of previously unmuscularised pulmonary arterioles represents a key component of the abnormal vascular remodeling observed in PAH. Whether this is in response to endothelial injury or dysfunction induced by inflammation, toxins or shear stress is still not clear.

Although not studied in the context of PH, some of the BMP ligands BMP2 and BMP7 decreased, whereas TNFα increased gene expression of TRAIL in aortic vascular smooth muscle cells (Nguyen, Olesen et al. 2007) further implicating a possible role for TRAIL in vascular pathology such as PAH. Collectively these observations lend support to the hypothesis that TRAIL could play an important role in the pulmonary vasculopathy encountered in PAH.

1.18 THESIS HYPOTHESIS AND OBJECTIVES

Hypothesis

TRAIL is an important mediator in the pathogenesis of PAH and could potentially provide a novel and rationale target for future drug development.

Study objectives

To determine

- The pattern of TRAIL ligand and receptor expression on patient cells (PASMCs).
- The mitogenic effects of TRAIL on PASMCs *in-vitro.*
- The pathogenic role of TRAIL in three preclinical rodent models of disease (hypoxia, high fat diet and monocrotaline).
- The contribution of bone marrow versus tissue derived TRAIL in experimental disease.
- Whether targeting TRAIL could reverse or halt disease in rodent models of experimental PAH.

CHAPTER 2: MATERIALS AND METHODS

2.1 Rodents

2.1.1 Rats and Monocrotaline

Outbred male, albino Sprague Dawley rats (Charles River or Harlan, U.K) (starting weight approx. 200g) were used in the described experiments. A single subcutaneous injection of monocrotaline (MCT) (see below) into the left thigh was used to induce pulmonary arterial hypertension. We used an established dose of 60mg/kg which leads to the development severe PAH and which is invariably fatal within 4-6 weeks.

200mg of Monocrotaline (MCT) (#C2041-500, Sigma Aldrich, UK) was first fully dissolved in 0.6ml of 1M Hydrochloric Acid and vortexed for 40min. Sterile water was added to make the volume to 5ml and the pH adjusted to 7.0 with sterile NaOH. Sterile water was added to achieve a final volume of 10ml (concentration 20mg/ml).

2.1.2 Mice

All inbred mice were on a C57BL/6 background and were homoyzygous deficient for either the Apolipoprotein-E ($ApoE^{-/-}$), TRAIL ($TRAIL^{-/-}$) or both genes ($ApoE^{-/-}/TRAIL^{-/-}$). Mice were available from in house colonies at the University of Sheffield. $ApoE^{-/-}$ (Jax 2052) mice were originally sourced from Jackson laboratories (Bar Habor, ME, USA). $TRAIL^{-/-}$ deficient mice were supplied by way of a prior material transfer agreement from Amgen **(**having been originally developed at the Peter Mcallum Cancer Institute, Melbourne, Australia (Cretney, Takeda et al. 2002). They were crossed with $ApoE^{-/-}$ mice to generate $ApoE^{-/-}/TRAIL^{-/-}$ double deficient colonies here in Sheffield. Male mice aged between 8-14 weeks were used for all *in vivo* experiments.

2.1.3 Diets and husbandry

All rodents were fed standard laboratory chow (4.3% fat, 0.02% cholesterol, and 0.28% sodium, Harlan, UK). Where indicated experimental mice were fed a high fat atherogenic diet (referred to as the Paigen diet from here on) which consisted of 18.5% fat, 0.9% cholesterol, 0.5% cholate, and 0.259% sodium for either 8 or 12 weeks (#829110, Special Diet services, UK). The Paigen diet was supplied as a solid block. It was prepared for use by first breaking it into powder form and then sterile water was added to create a thick paste consistency. It was then placed on baking trays, lined with greaseproof paper, loosely covered with aluminium baking foil before being placed in a drying chamber set to 40^0C for 48 hours. Following dehydration it

became firm yet retained a chewy texture. It was then frozen at -20^{0}C until use, prior to which it was thawed at room temperature.

All animals had access to drinking water and fed *ad-libitum.* They were housed in dedicated laboratories with controlled temperature, humidity and a 12h day-night cycle. Animal care and investigation conformed to the Universitys ethical policy statement and the UK Home office guidance in the operation of Animal Scientific Procedures Act 1986. I was in a receipt of a U.K home office personal license (PIL 40/9332) and worked with procedures detailed in a H.O project license held by my principal supervisor Dr A. Lawrie (PPL 40/2952).

2.2 Antibodies for Intervention studies

Where stated recombinant mouse TRAIL-rmTRAIL (Peprotech, Cambridge, UK), polyclonal goat anti-mouse TRAIL (Anti-TRAIL) or goat anti-mouse (as control) IgG isotype antibodies (R&D systems, UK) where delivered to rodents through subcutaneously implanted osmotic pumps (Durect Corp., CA, USA). Interventions were delivered via an Alzet® 1004 micro pump (100µl reservoir, 0.1µl/hour for 4 weeks) in mice and via an Alzet® 2002 mini-pump (200µl reservoir, 0.5µl/hr for 2 weeks) in rats. Cost primarily governed the maximum concentration of antibody we could reconstitute in the osmotic pumps.

2.2.1 Pump Implantation protocol

Manufacturer's recommendations were followed for filling pumps with solutions. Each pump was filled with the appropriate intervention under sterile conditions in a class II laminar flow hood and placed in sterile 0.9% saline at 37^{o}C for 24 hours prior to implantation. Under isoflurane gas anaesthesia (2-3%, IsoFlo® 100% w/w inhalation vapour liquid, Abbot laboratories Ltd, Kent, UK) through 100% oxygen (flow rate 1.5L/min) overlying fur was clipped, the skin cleaned and sterilised prior to making a 1-1.5cm cutaneous incision over the left posterolateral thoracic wall, inferior to the lower costal margin in rats. Under sterile surgical conditions, pre-filled pumps were implanted into a subcutaneous pocket created with blunt dissection. The wound was subsequently cleaned and closed using interrupted 2-0 Vicryl absorbable sutures (B-Braun, Sheffield, UK). Implantation for mice was identical except pumps were primed for 48 hours, implanted posterior to the cervical spine (scruff line) and wounds closed with interrupted non absorbable silk sutures (Silkam®, B-Braun Sheffield, UK).

2.3 Experimental Protocol

2.3.1 Mice

ApoE$^{-/-}$, TRAIL$^{-/-}$ and ApoE$^{-/-}$/TRAIL$^{-/-}$ knockout mice (aged between 8-16 weeks old, n as per relevant chapter) where fed either chow or Paigen for 8 weeks before disease phenotyping (see below).

In separate experiments ApoE$^{-/-}$/TRAIL$^{-/-}$ mice (8-12 weeks of age, n=4-6/group) were treated with either rmTRAIL (10ng/hr) or placebo (PBS) by osmotic micro-pump for 4 weeks that coincided with the onset of feeding Paigen diet.

To determine the efficacy of inhibiting TRAIL in mice with established disease, ApoE$^{-/-}$ mice (8-10 weeks of age, n=6-7/group) were fed a Paigen diet for 8 weeks and then received an anti-TRAIL antibody (20ng/hr) or isotype control with phenotyping performed at week 12.

2.3.2 Rats

In time course experiments rats (200-260g, n=7/group/time-point) underwent haemodynamic study and sacrifice either 2,7,14,21 or 28 days after injection with MCT (60mg/kg) or saline control.

To investigate if TRAIL was required for the development of disease (Prevention study) rats (200-240g, n=4/group) were treated with an anti-TRAIL antibody (84ng/hr) or isotype control delivered for 2 weeks by osmotic pumps, commencing at baseline with MCT injection. Disease phenotyping was undertaken one week later, i.e.: 21 days after MCT injection.

To determine the efficacy of inhibiting TRAIL in established disease (Survival study), rats (200-240g, n=6/group) with MCT induced PAH (day 21 after MCT) received an anti-TRAIL antibody (84ng/hr) or isotype control for 2 weeks. We compared survival in the two groups and rats were sacrificed at day 35 post MCT (1-14 days following intervention) or sooner if they displayed morbidity and evidence of right heart failure. The latter were defined by outward illness (breathlessness, lethargy, ruffled fur) and significant weight loss (defined as >5% weight loss in 24 hours or a total of 10% over 48 hours). At all times echocardiographic and haemodynamic studies were performed immediately prior to sacrifice.

2.4 Disease phenotyping

Each rodent underwent echocardiography (where indicated) before cardiac catherterisation and was then euthanized whilst still under anaesthesia. Blood was collected by cardiac puncture for blood (for subsequent isolation of serum and RNA where indicated). The abdominal aorta was cut and lungs were perfused with PBS, using a 5ml syringe and orange needle in the right ventricle to expel blood until such that the lungs became visibly white. The heart and lungs were removed en-bloc. The right lung was quickly separated by tying sutures at the hilum, before immediately being snap frozen in liquid nitrogen for subsequent biochemical analyses. The left lung was perfusion fixed, via the trachea with 10% (v/v) formalin at an inflation pressure of 20cm H_20 and then placed with the heart in 10% formalin overnight at 4^0C. The left lung was used for all subsequent histological and immunohistochemical analyses.

2.5 Rodent Echocardiography

Transthoracic echocardiography was performed with a preclinical high frequency ultrasound imaging system (Vevo 770®, Visual Sonics, Toronto, Canada) using either a RMV707B (mouse central frequency 35MHz) or RMV710B (rat, central frequency 25MHz) scan heads. Standard frame rates were 100FPS but this could be increased by reducing sector width. Rodents were anaesthetised with isoflurane via oxygen before being placed supine on a heated platform and covered to minimise heat loss. Maintenance Isoflurane (0.5-1.5%) with oxygen was delivered via a nose cone and adjusted to achieve maximal heart rate (approx. 500bpm for mice and 350bpm for rats) that was continuously recorded along with respiration rate and rectal temperature. The chest of the mouse was depilated and preheated ultrasound gel was applied (Aquasonics 100 Gel, Parker Labs Inc. New Jersey, U.S) for subsequent image acquisition.

2.5.1 Left Ventricle Study echocardiography Protocol

Standard parameters of the left ventricle were measured in the short axis view at the mid-papillary muscle level. Manual tracing of the LV end diastolic and systolic areas were made to derive the fractional area change (FAC) as the primary index of contractility. M-Mode measurements were made for the LV wall and cavity dimensions (LVIDd), from which the ejection fraction (EF%), fractional shortening (FS%) and corrected LV mass were determined by standard automated analysis. Pulse wave tissue Doppler (TDI) velocities were manually

recorded from the endocardial aspect of the posterior wall of the left ventricle and represented another independent index of contractility. Stroke volume was derived from measuring the Velocity Timed integral (VTi) of flow and diameter at the level of aortic valve annulus and multiplied by heart rate to obtain the cardiac output.

2.5.2 Right Ventricle/Pulmonary Artery Echocardiography study protocol

From the right parasternal long axis view, right ventricle free wall measurements were recorded with M-Mode function. From the short axis view, Doppler flow was recorded from the proximal pulmonary artery (just after the pulmonary valve). From the spectral Doppler tracing the time from onset of flow to peak velocity (PA acceleration time; PAAT), the duration of ejection (PA ejection time; PAET) and stroke distance (PA VTI) were measured.

Analysis was performed offline using the accompanying software (Vevo 770, V3.0). Measurements were taken during the relevant phase of the cardiac cycle that did not coincide with inspiration artefact. To minimise inter-observer variability all image acquisition and analyses were performed by a single, experienced operator (AGH) blind to the status experimental subjects.

2.6 Cardiac Catheterisation and invasive haemodynamics

Following echocardiography, right and left (where indicated) ventricular catheterisation was performed using a closed chest method via the right external jugular vein and right internal carotid artery. Data were acquired with high fidelity micromanometer catheters (Millar instruments, Houston, Texas, USA). In mice, a 1Fr SPR-1000 for pressure or a 1Fr PVR-1045 pressure volume catheter was used. Corresponding catheters used in rats were a 2Fr SPR-320 pressure or a SPR-838 pressure volume catheter. All catheters were presoaked in normal saline at room temperature for 30 minutes and volumes calibrated as per the manufacturer's instructions. The catheters were connected to a Millar MPVS 300 and a PowerLab 8/30 data acquisition system (AD Instruments, Oxfordshire, UK) and recorded using Chart v7 software (AD Instruments).

Pressure tracings were recorded when tracings had stabilised and reached a steady state. An average of between 15-20 heartbeats (pressure waveforms) was used to record the pressure from the relevant cardiac chamber. Right Ventricular End Systolic Pressure (RVSP) was used to define pulmonary hypertension as it is equivalent to the Pulmonary Artery Systolic Pressure (PASP) in the absence of an obstruction between the right ventricle and pulmonary artery. The latter was routinely evaluated with echocardiography as described above. Where indicated pressure volume analysis was performed using PVAN v2.3 to determine the Cardiac output (µl/min) which was indexed to body weight to derive the Cardiac index (µl/min/g).

As mean PA pressure (mPAP) could not be measured directly with the closed chest method it was estimated; (EmPAP) from the RVSP (which is equivalent to the PASP) by adapting the equation published by (Chemla, Castelain et al. 2004) [eMPAP=(0.61xRVSP) +2mmHg]. This has been validated more recently (Chemla, Humbert et al. 2015). EmPAP was then substituted for mPAP in the standard textbook equation used to determine Pulmonary vascular resistance (PVRi)=[80*(mPAP-Left Ventricular End Diastolic Pressure/cardiac index) to give an estimated PVRI (ePVRi). The ePVRi in this thesis has been expressed using the units mmHg/ml/min/g however is more often expressed as dynes-sec-cm^{-5}.

2.6.1 Protocol for vascular access surgery

Animals were anaesthetised with 3-5% Isoflurane using an anaesthetic vaporiser and induction chamber (Harvard Apparatus, UK) through 100% medical oxygen (flow rate 2l/min). Mouse and rat surgery protocols followed a published protocol (Pacher, Nagayama et al. 2008).
The first step in the haemodynamic phenotyping required isolation of the right external jugular vein (for antegrade catheterisation of right heart chambers) and right internal carotid artery (for retrograde aortic and left ventricular catheterisation) where necessary and both were isolated, before insertion of catheters as described below.

2.6.2 Measurement of right heart pressures

Animals were placed on a heated pad (#TR200 Fine Science Tools Inc). Once the pedal reflex had been abolished a small incision in the neck was made to the right of the midline. With the use of a dissecting microscope and lateral blunt dissection the right external jugular vein (RJV) was identified. Curved forceps were used to free the vein with blunt dissection. The distal RJV was tightly tied off with 5-0 non-absorbable silk suture (Silkam®, B-Braun, Sheffield, UK) to halt venous return to the heart. Proximal to the insertion point of right subclavian vein a loose silk suture attached to a mosquito clip with traction was applied to the RJV. This left approx a 1cm length of vein in which to insert our catheter. Under direct microscopic visualisation the vein was cleaned of any fatty tissues to enable successful cannulation of the true lumen of the vein. Using a 25G 5/8" orange needle with tip bent at 90^0 and the bevel pointing downwards the vein was punctured and the superior wall of the vein was immediately but gently lifted upwards to allow simultaneous insertion and advancement of the catheter. Catheters had been pre-soaked in saline for at least 30min.

The appropriate catheter was advanced forwards and the proximal sling was tightened to prevent bleeding. Real time visualisation of pressure recording helped us to identify characteristic tracings for the right atrial and ventricular chambers. Once the catheter was stable within each chamber recordings were taken for subsequent analysis. A recording of at least 15-20 heart beats was used to average the pressure measurements.

Right ventricular systolic pressure (RVSP), maximum and minimum derivatives of pressure (max and min dp/dt) were specifically recorded. RVSP served as a surrogate of pulmonary artery pressure as recordings of the latter were not possible with these straight tipped catheters. RVSP is identical to PASP in the absence of any obstruction at the level of the pulmonary valve. Once the catheter was removed the proximal RJV was tightly secured with a suture.

2.6.3 Measurement of left heart pressures

Following completion of RVSP measurements the right internal carotid artery was identified, deep and lateral to the trachea. The artery had been isolated and prepared in tandem with the jugular vein as described. It was freed similar to the vein using curved forceps. A tight distal, loose mid and proximal segment ties (5-0 silk Suture) were applied. The latter was attached to a mosquito clip and traction applied. Using a similar technique for the vein, an arteriotomy was created and the catheter advanced into the aorta and left ventricle. Once pressure and volume tracings were stable and clear, the recordings were taken. An aortic pressure tracing by catheter pullback from the LV was recorded prior to removal of catheter. A recording of at least 15-20 heart beats was used to average the pressure measurements.

2.7 Harvesting and processing of tissue

2.7.1 Blood

Blood collected in clinical specimen tubes was allowed to coagulate on the bench and subsequently centrifuged at 1200rpm for 15min. The serum was collected, aliquoted, labeled and frozen at -80^{o}C until subsequent analyses. Tubes containing whole blood for RNA (PAXgene®, Qiagen/BD U.K or Tempus®, Applied Biosystems, UK) were frozen at -20^{o}C until subsequent isolation of RNA.

2.7.2 Lung tissue protocol

After cardiac puncture the rodent was overdosed with gas anaesthetic followed by cervical dislocation. An incision in the upper abdominal wall was made to expose the liver. Whilst applying upward traction on the xiphoid process of the sternum, the diaphragm was carefully cut with fine scissors. The sternum and chest wall were resected away. The abdominal aorta was identified and cut to permit exsanguination. Using a 25G orange needle and 5ml syringe, the right ventricle was identified and flushed with PBS to drain out as much blood from the lungs (until they became visibly pale). The trachea was identified and freed between the medial clavicular borders. Whilst applying firm upward traction on the trachea, the heart and lungs were removed en-bloc from the posterior wall of thoracic cavity. Great care was taken to avoid inadvertent lung puncture.

The right lung was secured tightly at the hilum using 5-0 silk sutures, was separated and cut into three sections before being snap frozen in liquid nitrogen for subsequent isolation and determination of whole lung protein and RNA expression.

Polyethylene tubing was inserted into the trachea and secured tightly with a suture, to prevent leaks. The left lung was gently inflated manually with a syringe containing 10% phosphate buffered formalin (0.4% w/v $NaH_2PO_4{\cdot}2(H_2O)$, 0.65% w/v $Na_2HPO_4{\cdot}2(H_2O)$ and 4% v/v formaldehyde in water) and then both heart and left lung were fixed in formalin for 24hours before transfer into PBS. From the rat prevention study onwards lungs were inflated using 20cm H_20 clamp set up to standardise inflation. The left lung was separated from the heart for subsequent histology.

2.7.3 Estimation of right ventricle hypertrophy (RVH)

RVH was simply defined as the weight of the Right ventricle (RV) divided by the weight of the left ventricle/septum (RV/LV+S) as first described (Fulton, Hutchinson et al. 1952)

Right ventricle dissection: Using a small pair of fine scissors surrounding fat, tissue and great vessels were removed from around the heart. The atria were excised, cleared of any thrombus and weighed. The right ventricle was separated from the left ventricle and septum by the use of anatomical landmarks.

Starting from the right ventricular outflow tract (RVOT) the septal margin of the RV was dissected away to ensure no ridges of tissue were left. An incision was also made from the RVOT adjacent to and encircling the aortic root towards the medial tricuspid valve annulus to separate the base of the RV. From the lateral tricuspid annulus the RV free wall was cut away ensuring again no ridges of RV tissue remained. The incision continued towards the apex and back up towards the RVOT. Thus the RV was dissected off whole so as to obtain a structure that resembled a crescent.

Finally the left ventricle was cut, longitudinally and any clot removed from it before all chambers were padded dry and weighed.

2.8 Lung histology

2.8.1 Tissue processing and histology

The left lung was divided into 2-3 segments in the longitudinal (sagital) plane in rats or transverse plane in mice. Lungs were processed by first dehydrating them in graded alcohols (50% up to 100%). They were then placed in Xylene before being embedded in molten paraffin wax. 5µm thick paraffin embedded sections were cut with a microtome (Leica RM 2135) and mounted onto slides for subsequent histology, immunohistochemical staining and morphometric analyses.

All slides were initially dewaxed by placing in Xylene for 10mins and then repeating for 2mins. Slides were then rehydrated in graded alcohols (1min in each of 100%, 100%, 90%, 70%, 50% and then finally water). Following any staining as a final common step, all slides were dehydrated in an identical but reverse order and mounted in DPX (Dibutyl Phthalate Xylene) and allowed to dry overnight. Slides were then coded, placed in designated storage racks and stored at room temperature.

2.8.2 Alcian Blue Elastic Van Gieson (ABEVG)

Dewaxed and rehydrated slides were oxidised in 0.25% potassium permanganate for 3min and then rinsed in distilled water before being bleached with 1% Oxalic acid for 3min. Following rinsing, slides were stained with Carazzi's Haematoxylin for 2min and differentiated in acid alcohol (1% v/v HCl in 70% IMS) for a few seconds prior to being submerged in hot running tap water for 5min. Slides were then stained with Alcian Blue (1% w/v in 3% aqueous acetic acid, pH2.5) for 5 min. Slides were rinsed again with water and soaked rapidly in 95% IMS before being dipped into Millers elastin stain for 30min. Slides were then rinsed, placed in 95% IMS for a few seconds and rinsed in water again. They were then stained with Curtis modified Van Gieson reagent for 6min. Slides were then dehydrated in identical but reverse order to those for rehydration above before mounting in DPX.

2.9 Lung immunohistochemistry

Paraffin embedded 5µm lung sections underwent immunohistochemical staining with Smooth Muscle Actin alpha (α-SMA) to identify vascular smooth muscle cells, von Willebrand Factor (vWF) for endothelial cells and PCNA (proliferating cell nuclear antigen) for cells undergoing proliferation. Immunostaining for TRAIL was performed to identify any expression within pulmonary vascular lesions. Levels of apoptosis were determined with a colorimetric assay to detect DNA fragmentation (FRAGEL®, Calbiochem, UK) as specified by the manufacturer's instructions. A positive control was generated with DNAse treatment of a control slide.

2.9.1 Protocol for immunostaining for SMA,vWF and TRAIL

Following dewaxing and rehydration of slides, endogenous tissue peroxidases were blocked by incubating slides in 3% (v/v) hydrogen peroxide for 10mins before being rinsed in tap water. Antigen retrieval (Slide permeabilisation) was done by incubating slides in either:

a) citrate buffer, pH 6.0 preheated to 95^{o}C for 20min. before cooling for 20min at RT. Tissue was then permeabilised by incubation in 0.5% (v/v) tritonX100 for 10mins at RT (IHC for TRAIL).
b) 0.1% (w/v) Trypsin/TBS, pH7.8, preheated to 37C for 10minutes before stopping reaction by immersing in water (IHC for vWF).
c) For SMA staining an antigen retrieval step was not performed.

Slides were then blocked (to prevent non specific binding of secondary antibody) in 1% (w/v) skimmed milk/PBS for 30mins at RT. Milk was tipped off and excess blotted away. The relevant primary antibody diluted in PBS was added and incubated as follows:

a) Monoclonal mouse anti-human aSMA 1:150, (#m081, Dako) for 1 hour at RT
b) Polyclonal rabbit anti-human vWF 1:300 (#A0082, Dako) for 1hour at RT.
c) Polyclonal rabbit anti-human TRAIL 1:100 (#ab2435, Abcam) overnight at 4C

Slides were washed in PBS three times for 5 mins before adding a species specific biotinylated secondary antibody (1:200 dilution in PBS) for 30minutes at RT. Slides were washed again in PBS three times for 5mins and an avidin biotinylated enzyme complex added (Vectastain ABC Kit, Vector laboratories Inc. CA, US). Following a further PBS washing step, diaminobenzidine

(DAB) substrate was added for 5-10min. After optimal development the colour reaction was stopped by washing slides in tap water. Slides were then counterstained with Carazzi's haematoxylin for 1 minute before a final wash in water. Slides were dehydrated as described and mounted with DPX mountant. Slides were allowed to dry overnight before being examined under light microscopy.

2.10 Morphometric lung tissue analysis

The degree of pulmonary vascular remodeling was quantified in arterioles by two methods and categorised according to vessel size (20-50µm, 50-100µm and >100µm) (Schermuly, Dony et al. 2005). Vessels were scored blinded to the experimental status of rodents.

2.10.1 Media to Cross Sectional Area (Media/CSA) ratio

Cross sectional area was the total area defined by the outer vessel circumference with the media defined as the area between the internal and external elastic lamina of the vessel. Medial area/CSA represented the proportion of the total vessel area that was taken up by the medial layer, as determined from α-SMA stained slides. The ratio was directly proportional to the degree of muscularisation (medial hypertrophy) of the vessel. Six vessels of each size group were analysed at a 40X objective (18 vessels/section and 1 section/rodent).

2.10.2 Percentage of vessels thickened

Percentage of vessels thickened was determined using slides stained with ABEVG. For each slide 3-4 random fields of view were sampled using a 10x Objective (100x mag). The number of vessels that were fully occluded, partly occluded and non-thickened per size group were counted and expressed as a percentage of the total number of vessels in each view.
Slides were viewed with a light microscope (Nikon eclipse E600) connected to a digital camera (Nikon digital site DSRi1) and NIS basic elements software (Nikon Inc.).

2.10.3 Quantification of vascular proliferation and apoptosis levels

To determine the levels of proliferation within remodeled vessels, the number of PCNA positive stained nuclei were counted and expressed as a percentage of total nuclei counted within the vessel. Nuclei within the adventitia and adjacent perivascular area were also counted if they were in direct continuation from the vessel of interest. Six vessels of each size were scored from each section (one section/animal) at a 40X objective.

In an identical manner the percentage of apoptosis positive nuclei (as determined from a colorimetric assay for levels of DNA fragmentation) were quantified for six vessels of each size per lung section (one section/animal).

2.11 Studying the expression of gene and protein from tissues

2.11.1 Isolation and purification from Protein and RNA from lung tissue

Lung segments frozen in liquid nitrogen were ground using a pestle and mortar containing liquid nitrogen to a fine powder and weighed. Precautions were taken to minimise contamination by RNAase. Total protein and RNA were isolated using a commercial RNA/Protein purification Kit (Qiagen) according to the protocol supplied by the manufacturer. The purification kit employed a spin column chromatography technique and allowed elution of proteins and RNA from the same sample within 30 minutes.

Protocol.

Briefly lysis solution was added to the lung tissue and then ethanol added. This was loaded on to a spin-column. After centrifugation at 14000rpm, all nucleic acids within the solution were bound by a resin whilst the proteins were removed in the flow through. The bound RNA was washed, spun again and then purified RNA was eluted. The concentration of RNA in the elution was determined with a spectrophotometer (NanaDrop®, Thermo Scientific) before freezing at -80C. Following pH adjustment the protein flow through was reloaded on to the original spin column, centrifuged, washed and eluted.

Finally protein concentrations were determined using a commercial assay (DC™ protein assay #500-0116, BioRAD Life Sciences, UK) according to the protocol provided by the manufacturer. In principle it is a colorimetric assay that utilises a reaction between the protein and an alkaline copper tartrate solution. This is followed by a reduction step using Folin reagent. Absorbance was read at 750nm. The quantity of protein was determined from absorbance data generated from a protein standard curve with Albumin (BSA #23209, Pierce, Thermo Scientific Fisher, UK.) Protein samples were stored at -80C.

2.12 Western Immunoblotting for lung protein levels.

Proteins were separated by SDS-Polyacrylamide gel electrophoresis using a commercial electrophoresis kit (NuPAGE® Kit, Invitrogen). All buffers and reagents were part of the NuPAGE range unless otherwise stated. A volume containing 35 µg of protein purified from whole lung lysates in sample buffer and a reducing agent made to a final volume of 30 µl (in deionised water) was heated to 70^{o}C for 10 min. Samples and a pre-stained marker ladder were then loaded onto 10 well pre-cast SDS polyacrylamide gels (NuPAGE® 4-12% Bis-Tris Mini gels, Invitrogen). In addition a sample of mixed experimental lung tissue was also loaded onto every gel as an additional control to allow for subsequent quantitative analysis.

Immediately prior to placing the loaded gels into an electrophoresis cell (XCell SureLock® Mini cell, Invitrogen) that already contained SDS running buffer, 500 µl of antioxidant was added. The Gel was run at 200V for 35 min.

Gels were transferred onto a nitrocellulose membrane (membrane and blotting pads had been pre-soaked in the transfer buffer and air bubbles removed) in transfer buffer (containing antioxidant and 10% methanol v/v) and ran at 30V for 60min. Ponceau S staining was used to confirm adequate transfer.

The membranes were then blocked for 1h in 10ml of PBS with 5% milk (w/v) and 0.1% Tween-20 (v/v) on a shaking platform. Blots were rinsed in PBS/0.1% tween-20 three times before adding the relevant primary antibody in 5% milk/PBS/0.1% Tween-20 on a shaking platform overnight at 4^{o}C. (Mouse anti-human TRAIL 1:50, Novo Castro Laboratories, Co Durham, UK and anti Mouse Beta Actin 1:2000, #c56 Santa Cruz, CA, USA).

Blots were rinsed three times for 10min before adding an appropriate, species specific peroxidise labeled secondary antibody diluted in PBS (polyclonal goat anti-mouse immunoglobulin/HRP 1:2000, #p0447, Dako, Ely, UK).

Following a further rinse step as described enhanced chemoluminescence was performed by adding 1ml of a commercial assay on to the blots for 5min. in the dark (#34075 West Dura Super Signal, Thermo scientific Fisher). Blots were developed in a dark room using autoradiography film (#28906836, HyperFilm^{TM} GE Amersham, UK) and developer/fixer solutions.

Blots were stripped (#2502, Reblot Plus Mild Chemicon solution, Millipore) and reprobed for actin as described above.

The developed blots were dried and the ladder marked. The quantity of TRAIL in the bands was determined by normalising to actin and control samples using the densitometry function on commercial software (Syngene SNAP software, Chemigenius2 bioimaging system, SynGene).

2.13 Quantitative real time Polymerase Chain Reaction

This step was performed using components provided in a SuperScript™ III first strand synthesis system (#18080-051 and #18080-044, Invitrogen™ Life technologies, UK). A volume containing 3ug of total RNA isolated from the lungs (and whole blood using PAX-gene tubes) of experimental rodents was made to 10µl using molecular grade water. 1µl of random hexamer primers (50ng) and 1µl of a 10mM dNTP were added to this and heated to 65^{o}C for 5 minutes as a denature step. Samples were put on ice until 10µl of a cDNA synthesis mix [containing 10xRT buffer (2µl), 25mM $MgCl_2$ (4µl), 0.1M DTT (2µl), RNaseOUT™ (1µl) and SuperScript™ III reverse transcriptase (1µl)] was added to this solution and mixed. Samples were heated in a thermal cycler (G Storm GS1, GRI Ltd, Essex, UK) with parameters set as follows i) 25^{o}C for 10min. (annealing step), ii) 50^{o}C for 50min. (cDNA synthesis) and finally iii) 85^{o}C for 5min. before being held at 4^{o}C- (to terminate the reaction). 1µl of RNaseH was added to each tube before a final incubation step at 37^{0}C for 20min.

Alternatively (for all mouse and rat interventions) 2µg of RNA was reverse transcribed using a commercial high capacity RNA to cDNA kit (Applied Biosystems) according to enclosed protocol. Briefly RNA was added to PCR tubes containing 10µl of 2x RT buffer and 1µl of an RT enzyme mix. Samples were heated in a thermal cycler (G Storm GS1, GRI Ltd, Essex, UK) with parameters set as follows; i) 37^{o}C for 60min ii) 95^{o}C for 5min and then held at 4^{o}C to terminate the reaction.

Real Time quantitative PCR

Amplification of the target lung cDNA derived from the RT step above was then next performed. A volume containing 50ng of each cDNA was diluted to a volume of 4.5µl using sterile water. 5µl of a TaqMan® gene expression master mix-2X (#4369016, Applied Biosystems™ Life Technologies, UK) along with 0.5µl of the relevant target gene primers (10X) were added to the cDNA into the relevant well of a 384-well plate. The following target genes were tested (all from Applied Biosystems™): OPG (#Rn00563499-m1), TRAIL (#Rn00595556-m1 or (mouse: #Mm01283606-m1), CCL5 (#Rn00579590-m1), BMPR2 (#Rn01437210-m1), IL1R1 (#Rn00565482-m1), IL1R2 (#Rn00588589_m1). 18s (#Hs03003631-g1) was selected as an endogenous control and had been determined with prior testing. Samples (in duplicate) for each gene were loaded on the same plate. The plate was centrifuged at 1000rpm for 1 min and the reaction was run on a 7900HT fast real time PCR system (Applied Biosystems™). Relative expression for each gene was quantified by comparing the test gene with the housekeeping control gene and comparing this ratio between an experimental and control subject (delta, delta CT method) for each gene using SDS software (v2.2.1, Applied Biosystems™).

2.14 Statistical analysis

Data were plotted and analysed using Prism® v6.0 software (Graphpad, USA) software. Data are expressed as Mean [standard error of the mean] unless indicated otherwise. Either a Mann- Whitney test when comparing two groups, or a Kruskal-Wallis followed by the Dunns post-hoc test with a 95% confidence level where used unless indicated otherwise. Statistical significance was defined by a p value of ≤0.05.

CHAPTER 3 BACKGROUND DATA: TRAIL EXPRESSION AND FUNCTION ON HUMAN PASMCS

3. 1 INTRODUCTION

The pathogenesis of PAH remains incompletely understood, yet historical and contemporary pathological studies have reported a consistent pattern of changes in the distal pulmonary vascular bed that include intimal thickening, fibrosis, medial hypertrophy and plexiform lesion formation (Heath and Edwards 1958; Pietra, Capron et al. 2004; Stacher, Graham et al. 2012). These findings provide strong support for perturbed cell turnover as a significant feature of the disease process. PASMCs are major contributors to pulmonary vascular remodeling. PASMCs switch from a quiescent (contractile) to a synthetic (proliferative) phenotype and display aberrant growth characteristics (Morrell, Yang et al. 2001; Eddahibi, Guignabert et al. 2006; Marsboom and Archer 2008). This phenotypic switch is manifested in PASMCs through excessive proliferation and migration which constitutes a central theme in pulmonary vascular remodelling. Pulmonary vascular cells harboring a mutation in BMPR2 display aberrant growth responses: PASMCs are hyperproliferative and resistant to apoptosis.

Several cytokines and growth factors (Eddahibi, Humbert et al. 2001; Perros, Montani et al. 2008; Hassoun, Mouthon et al. 2009; Izikki, Guignabert et al. 2009) have been demonstrated to induce PASMC hyperplasia however the interplay between these is unclear, whilst none of the current therapies licensed for use in PAH is able to directly target them. Therefore a continued search for additional pathogenic factors is necessary to improve our comprehension of disease pathogenesis and to enable the identification of more efficacious therapies that can regress the underlying pathological vascular remodeling.

Over the past decade a role for TRAIL in vascular biology has emerged (Zauli and Secchiero 2006) however hitherto, this has been limited to the study of cells from the systemic (non-pulmonary) circulation (mainly aortic and internal mammary artery SMCs or human umbilical vein endothelial cells). TRAIL and its receptors are expressed on VSMCs and are detectable in both human (normal and diseased) and rodent arteries. TRAIL induces a pro-proliferative and migratory effect on systemic aortic VSMCs that is mediated via ERK signaling (Gochuico, Zhang et al. 2000; Kavurma and Khachigian 2003; Secchiero, Zerbinati et al. 2004; Kavurma, Schoppet et al. 2008; Chan, Prado-Lourenco et al. 2010; Azahri, Di Bartolo et al. 2012).

Although TRAIL has previously been localized to the medial layer of normal mouse pulmonary artery (Gochuico, Zhang et al. 2000) our group were the first to detect TRAIL within the media and adventitia of remodeled pulmonary arterioles from patients with advanced PAH (Lawrie, Waterman et al. 2008).

It was appropriate that the relevance of TRAIL to human PAH was established before I commenced my series of investigations on TRAIL in experimental PAH. Thus, this chapter describes initial data from our group on the expression and function of TRAIL, its receptors and downstream signaling in patients with PAH and in human PASMCs *in-vitro.* These data will highlight that the TRAIL pathway is present within PASMCs, a major cell type in PAH and produces effects on PASMCs that are relevant to the pathophysiology of this disease.

3.2 AIMS

The specific aims of this work were to determine the:

1. Expression of TRAIL and its receptors on PA-SMCs from patients with PAH.
2. Effects of TRAIL on the proliferation and migration of normal human PA-SMC *in-vitro.*
3. Role of ERK1/2 signaling in mediating the effects of TRAIL.
4. TRAIL receptor(s) important in pulmonary vascular signaling.

3.3 METHODS

The cell culture work reported in this chapter was performed by Dr Allan Lawrie and Dr Clauida Paiva. I specifically contributed to performing PASMC proliferation assays and only contributed data to Figure 3B.

3.3.1 Isolation and culture of pulmonary artery smooth muscle cells (PA-SMCs) from human lung tissue

Ribonucleic acid (RNA) from PASMCs isolated from human lung tissue (with prior ethical approval) was provided by Prof. Nick Morrell, University of Cambridge, Cambridge U.K. The isolation and culture of PASMCs as well as the subsequent extraction of RNA from these cells had been performed by the Morrell group, as previously described (Morrell, Upton et al. 1999; Morrell, Yang et al. 2001).

Briefly PASMCs grown in culture had been isolated from the proximal and peripheral segmental pulmonary arteries (<1-2mm) of explanted lungs from female patients who had undergone lung or heart/lung transplantation for advanced PAH (n=3 with 1 case harboring a mutation in BMPR-2) and control patients with emphysema or cancer undergoing lung surgery (n=3). The PASMC phenotype was confirmed by immunofluorescence with antibodies to alpha-smooth muscle actin and smooth muscle myosin (hsm-v). Total RNA was extracted from growth arrested PASMCs using Trizol reagent (Invitrogen).

3.3.2 Quantification of TRAIL gene expression in PA-SMCs

Real time PCR was used to determine the gene expression of TRAIL in PASMCs. Briefly, total RNA isolated (as above) from PASMCs was reverse transcribed using Superscript III reverse transcriptase as per manufacturer's instructions (Invitrogen, Paisley, UK). Gene expression was performed using Taqman PCR with commercial gene master mix and gene expression assays (Applied Biosystems U.K) with primers for TRAIL (Hs00234356_m1), TRAIL-R1 (Hs00269492_m1), TRAIL-R2 (Hs00366278_m1), TRAIL-R3 (Hs00182570_m1), and TRAIL-R4 (Hs04187520_m1). Relative gene expression was normalised to the control housekeeping gene ribosomal 18s RNA using the comparative delta/delta CT quantification method.

3.3.3 Culture of primary human PA-SMCs *in-vitro*.

Commercially available human pulmonary arterial smooth muscle cells (hPASMCs) (Cascade biologics, Invitrogen, UK) were used in proliferation and migration experiments. Each supplied vial contained at least $5x10^5$ viable cells that had been cryopreserved after the tertiary stage of culture in a medium containing 10% DMSO by the manufacturer. The subsequent storage, initiation of culture from cryopreserved cells and subculture were performed according to the manufacturer's instruction*).* A haemocytometer was used to determine the concentration of cells and viability was assessed with Tryptan blue.

Unless indicated otherwise, PASMCs were grown in Media 231 containing smooth muscle growth supplement (SMGS) with added gentamicin/amphotericin (all from Cascade biologics™, Invitrogen™, UK). hPASMCs were allowed to grow to 70-80% confluence in a monolayer before use in all experiments below. Cells were not used beyond passage 10. All tissue culture work was performed in class II laminar flow hoods using aseptic precautions. Sterile (autoclaved) pipettes, tips and flasks were used. Sterile cell culture media and reagents were pre-warmed at 37°C in a water bath that was regularly inspected and cleaned. Cells were cultured at 37°C in 5% CO_2/95% air in a humidified cell culture incubator that underwent regular inspection and cleaning. Unless stated otherwise, wells were washed with sterile PBS three times (500µl/well) with aspiration between each wash step.

3.3.4 *In vitro* assay of human PA-SMC proliferation.

PASMC proliferation experiments were performed in 24 well polystyrene plates (Costar3524, Corning, N.Y, U.S). To facilitate cell adhesion, all wells were pre coated with 500µl of 0.2% (w/v) gelatin, for 15mins at room temperature. Wells were washed with PBS and hPASMCs were seeded at a concentration of $2.5x10^4$/ml (500µl/well) and incubated overnight at 37°C in 5% CO_2/95% air.

Subsequently, cells were washed in PBS and then growth arrested for 48 hours by exchange of media to Dulbecco's Modified Eagle medium (500µl/well; DMEM, Biowhittaker®, Lonza, UK) supplemented with 0.2% (v/v) fetal calf serum (FCS) 100IU/ml penicillin, 100µg/ml Streptomycin and 0.25µg/ml Actinomycin B (all Gibco, Invitrogen, UK). After quiescence, cells

were washed in PBS and were stimulated for 72 hours, before being counted as described below.

All recombinant proteins and antibodies (purchased from R&D systems, Abingdon, UK unless otherwise indicated; # indicates catalogue number) were reconstituted and stored as indicated by the manufacturer. Antibodies, recombinant proteins, inhibitors and controls (unstimulated) were all diluted in supplemented DMEM with 0.2% (v/v) FCS as described (250µl/well).

PASMCs were stimulated with recombinant human TRAIL (rhTRAIL, #375-TL) at doses between of 1-100ng/ml. This preparation of rhTRAIL is an extracellular domain of TRAIL (Thr 95 to Gly 281) and contains a 6x histidine tag at the amino terminal end. In separate experiments TRAIL was cross linked using a monoclonal anti-polyhistidine antibody (#MAB050) and used to assess cell proliferation. Recombinant human PDGF (BB isoform, #220-BB) was used as a positive control and DMEM containing 0.2% (v/v) supplemented FCS (as above) served as control. Where indicated, inhibition of TRAIL receptors by antibodies (all goat IgG) to the extracellular domain of human TRAIL-R1 (#AF347), TRAIL-R3 (#AF630) TRAIL-R4 (#AF633) and the MEK inhibitor PD98059 (MEK inhibits ERK1/2) were all added 30mins prior to the addition of recombinant TRAIL or PDGF-BB.

After 72 hours of stimulation, cell proliferation was assessed by the Coulter^{TM} counting method. Wells were washed with PBS before the addition of Trypsin/EDTA (500µl/well) for 5min. The contents of each well were collected and added to individual small plastic containers containing 9.5ml of a diluent (#8448011; Coulter Isoton II, Beckman Coulter, Bromley UK). Each container was placed consecutively on a loading tray and cells were counted automatically by a Coulter Z1 machine. A numerical result was displayed on the screen and was noted. This number was multiplied by twenty to derive the actual cell count given the prior 1:20 dilution step.

3.3.5 *In-vitro* assay of human PA-SMC migration.

The method used to assess cell migration in response to TRAIL was performed using the well established transwell chamber assay, first described by Boyden when investigating leucocyte chemotaxis (Boyden 1962). The principle of this assay is as follows; a chamber divided into two compartments is separated by a micro-porous membrane (of varying pore size). Culture media containing the cell type to be studied is placed in the top chamber and the chemotactic stimulus, is added to the lower chamber. After a period of incubation the membrane separating the two chambers is fixed and removed and the numbers of cells on the undersurface of the membrane are counted to determine the number of cells that have migrated.

As described in section 3.3.3 above, hPASMCs were serum starved for 48 hours in DMEM (0.2%FCS). 24 well cell culture inserts with a special membrane (PET track etched, 8µm pore size, #353097, BD Falcon^{TM}) were presoaked in human fibronectin (#F0895, Sigma Aldrich, Poole, UK) for 60min at room temperature so as to coat both inner and outer aspects of the well insert. The cell inserts were washed to remove fibronectin and placed in a 24 well plate with media containing the relevant stimuli. The latter step was done after a 24 well plate had been prepared by adding 750µl/well of starvation media containing, recombinant TRAIL (1-100ng/ml, #375-TL), PDGF-BB (10ng/ml) or control (DMEM with 0.2%FCS). hPASMCS were seeded at a concentration of $3x10^{4}$ cells/ml, (250µl/well).

3.3.6 Time course for ERK1/2 expression and western Immunoblotting

To determine a time course for the expression of ERK1/2 in PASMC, recombinant human TRAIL (30ng/ml) was used to stimulate hPASMC for between 0-60minutes in 12-24 well cell culture plates. PASMC lysates were then prepared by washing cells in ice cold PBS. Cells were scraped and collected in ice cold PBS and centrifuged at 200g for 5min at 4^0C. The supernatant was discarded and the remaining cell pellet was re-suspended in an ice cold lysis buffer and left for 30min on ice. After lysis the tube was spun in a microcentrifuge for 10min at maximum speed. The supernatant containing the lysed cells was collected. The protein concentration was quantified using a DC protein assay as described in the methods chapter. Cell lysates were frozen at -80^0C.

Western immunoblotting was used to determine the amount of total and phosphorylated levels of p44 and p42 MAP Kinases (ERK1 and 2) in lysates of human PASMC stimulated with TRAIL. The principles and details of western blotting are described in the methods chapter. Briefly a 20µg sample of each PASMC lysates was loaded on to 4-12% Bis-Tris-Nupage Gel and run under reducing conditions in MES running buffer (Invitrogen) before transfer to nitrocellulose membrane (Invitrogen). The membranes were blocked for 1h in 5% non-fat milk at room temperature. The blots were incubated with a phospo-p44/42 MAPK (ERK1/2; Thr202/Tyr204), rabbit monoclonal antibody (1:1000 dilution, #9101, Cell Signalling technology®) or p44/42 MAPK (ERK1/2) mouse monoclonal antibody (1:1000 dilution, #9107, Cell signaling technology®) for 1h at room temperature. A HRP conjugated and species specific antibody was added for 1 hour before performing an enhanced chemoluminescence (ECL, GE healthcare) reaction and exposure to autoradiographic film. The autoradiography, densitometry and normalization processes were performed as described in the methods chapter.

3.4 RESULTS

3.4.1 TRAIL expression is upregulated on PA-SMCs from patients with PAH.

Prior work from our laboratory has observed TRAIL immunostaining in concentric and plexiform lesions from patients with idiopathic PAH (Lawrie, Waterman et al. 2008). Given that a major role for PASMCs in the vascular remodeling of PAH is well established and TRAIL expression has been demonstrated on systemic VSMCs we first sought to confirm if TRAIL was also expressed on PASMCs from patients with PAH.

Using Taqman PCR, there was an approx. three-fold upregulation in TRAIL gene expression in PASMCs from patients with PAH was observed compared to controls **(Fig. 3A).** The PASMCs had previously been isolated from explanted lungs of patients, with advanced idiopathic PAH who had undergone lung transplantation and grown ex-vivo. Patients undergoing lung surgery for either emphysema (COPD) or operable lung cancer served as disease controls. Clinical details of the patients from whom PASMCs had been isolated are shown in **Table 3.1**.

Table 3.1 Clinical details for human cells used in experiments outlined in Fig. 3

Sample	Diagnosis	Gender	Age (yr)	mPAP (mm Hg)	CO (l/min)
1	IPAH	Female	38	56	2.8
2	IPAH	Female	23	70	2.0
3	hPAH (R491M BMPR2)	Female	45	50	2.4
4	Emphysema	Female	58	n/a	n/a
5	Centrilobular Emphysema	Male	62	n/a	n/a
6	Squamous cell carcinoma	Female	60	n/a	n/a

IPAH – Idiopathic pulmonary arterial hypertension, hPAH – hereditary pulmonary arterial hypertension, mPAP – mean pulmonary artery pressure, CO – cardiac output, n/a – not available.

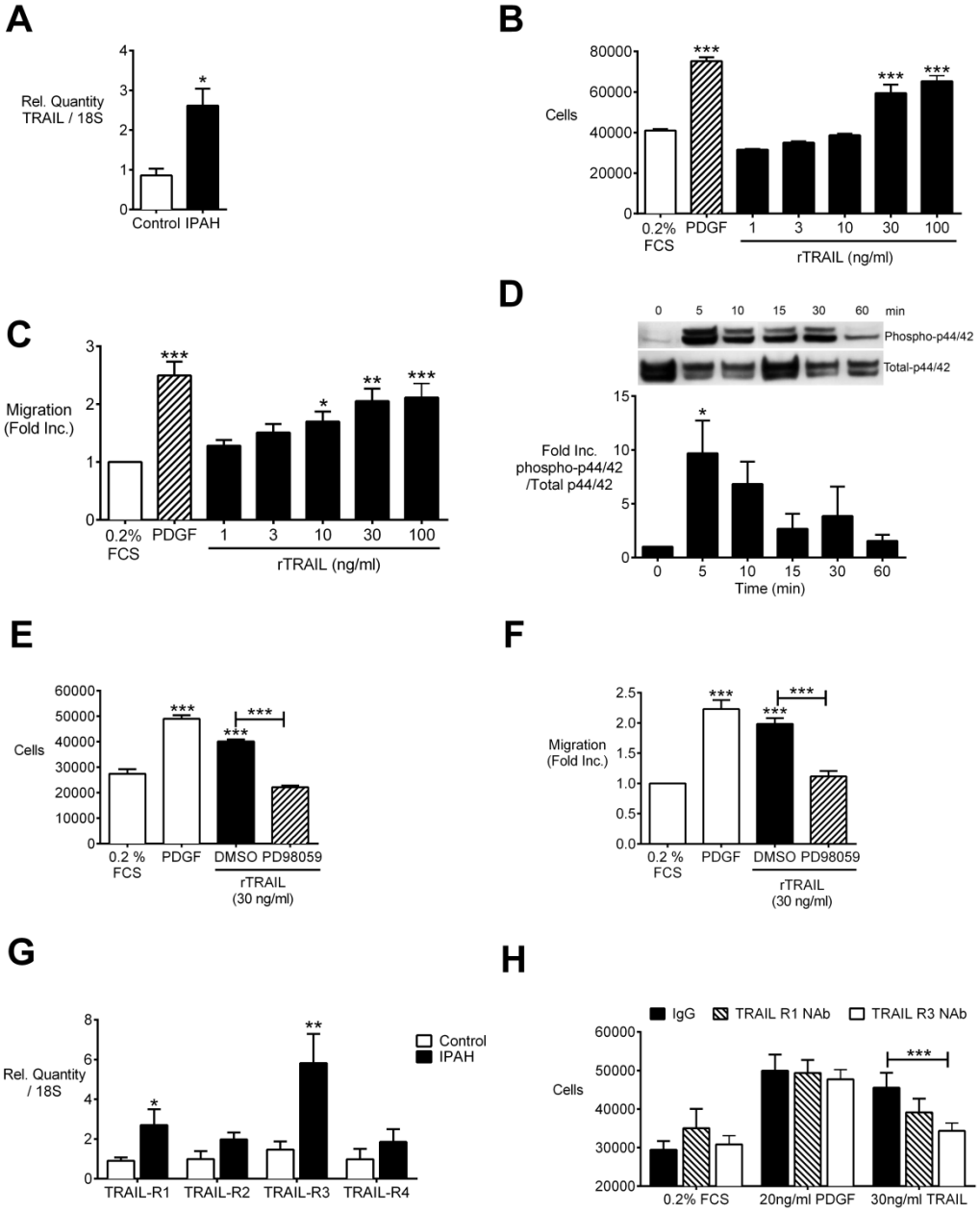


Figure 3. TRAIL induces proliferation and migration of PA-SMCs. (A) TaqMan expression of TRAIL in explanted PASMC from patients with IPAH normalised using ΔΔCT with 18S rRNA as the endogenous control gene. Human PASMCs were serum starved for 48 hours before stimulation with recombinant TRAIL (1-100 ng/ml) or 10 ng/ml PDGF-BB. **(B)** Proliferation was assessed by cell counting at 72 hours; **(C)** migration was measured at 6 hours using a Boyden Chamber assay and normalized relative to unstimulated cells (0.2% FCS). **(D)** Time course of p42/44 / ERK1/2 phosphorylation in PASMC using 30 ng/ml of TRAIL. Treatment of PASMC with the ERK1/2 inhibitor PD98059 inhibited TRAIL (30 ng/ml) induced proliferation **(E)** and migration **(F).** TaqMan expression of TRAIL receptors in explanted PASMC from patients with IPAH normalised using ΔΔCT with 18S rRNA as the endogenous control gene **(G).** Human PASMCs stimulated with recombinant TRAIL (30 ng/ml) or 20 ng/ml PDGF-BB with blocking antibodies for TRAIL-R1, TRAIL-R3 or an IgG control. Proliferation was assessed by cell counting at 72 hours **(H)** ***Bars represent mean +/- SEM, all experiments were performed in triplicate. For data using patient material (A & G) n=3. All remaining figures n=5. *=p<0.05, **=p<0.01, ***=p<0.001 compared to 0.2% FCS, control or 0h samples. These experiments were done by staff listed on p82.***

3.4.2 Receptors for TRAIL are upregulated on PA-SMCs from patients with PAH

All four membrane TRAIL receptors are expressed and upregulated on both human and rat aortic VSMCs (Secchiero, Zerbinati et al. 2004; Kavurma, Schoppet et al. 2008; Song, Choi et al. 2011). Using TaqMan PCR, PASMCs from both disease controls and IPAH expressed all five TRAIL receptors, although only the surface receptors TRAIL-R1 (two-fold) and in particular TRAIL R3 (six-fold) were both significantly upregulated **(Fig. 3G).** The soluble decoy receptor Osteoprotegerin (OPG:TRAIL-R5, was also upregulated three fold (data not shown).

3.4.3 TRAIL stimulates proliferation & migration of human PA-SMCs *in-vitro*.

As TRAIL induces the proliferation of aortic vascular smooth cells, we next determined if a similar response would occur with pulmonary arterial SMCs. Growth starved hPASMCS were stimulated with recombinant human TRAIL (rhTRAIL) for 72 hours and cell counts were determined using the Coulter^{TM} cell count method. Recombinant human TRAIL (rhTRAIL) induced the proliferation of normal human PASMCs in cell culture with a significant response observed with a concentration of 30ng/ml (performed in duplicate with n=5 experiments) **(Fig. 3B).**

TRAIL naturally forms a homo-trimer and experimentally, the effects (apoptotic) of rTRAIL have been be shown to be enhanced by oligimerisation with a cross linking antibody. Thus In separate experiments we crosslinked TRAIL (using an anti-histidine antibody) and stimulated hPASMCs *in-vitro* for 72 hours (Performed in duplicate, n=6 individual experiments). However no significant difference in human PASMC was observed when compared to non linked rhTRAIL (data not shown).

To determine if TRAIL displayed chemotactic properties towards PASMCs, a Boyden chamber migration assay was performed. rhTRAIL induced the migration of human PASMCs in a dose dependant manner, when compared to control (0.2% FCS treated) cells (performed in duplicate with n=5 experiments) **(Fig. 3C).**

3.4.4 TRAIL receptors and PA-SMC proliferation *in-vitro*

There are 4 cell surface receptors for TRAIL and previous work has identified that the proliferative effects of TRAIL on aortic VSMCs were mediated via receptors TRAIL-R1 and R3 (Kavurma, Schoppet et al. 2008). Our data demonstrate a similar upregulation of TRAIL R1 and R3 on PA-SMCs in PAH **(Fig. 3G)**. In cultured human PASMCs, antibody blockade of TRAIL-R3, but not R1 or R4 significantly, although modestly attenuated the proliferative response to TRAIL **(Fig. 3H).** We were unable to evaluate the contribution of TRAIL R2 to cell proliferation as no commercially available blocking antibody to TRAIL R2 was available at the time of this work.

3.4.5 TRAIL induced proliferation of PA-SMCs is mediated through ERK signaling.

MAPK (Mitogen Activate Protein Kinase) signaling via ERK (Extracellular signal Regulated Kinase, ERK1/2) is an established downstream signaling pathway in PASMCs. Furthermore the pro-survival effects of TRAIL on VSMCs have previously been demonstrated to activate ERK signaling (Secchiero, Gonelli et al. 2003; Secchiero, Zerbinati et al. 2004). A dose of 30ng/ml of rhTRAIL induced a rapid and significant upregulation of ERK1/2 signaling as evidence by an increase in the ratio of phosporylated P44/42 / total 44/42 levels in hPASMC lysates (**Fig. 3D**). Moreover the pro-proliferative and pro-migratory effects of rhTRAIL were inhibited by the MAPKK inhibitor- PD98059 when compared to vehicle (DMSO) treated cells **(Figs. 3E and 3F).** PD98059 inhibits MEK1/2 which sits immediately upstream of ERK1/2 and activates it through phosphorylation (Cargnello and Roux 2011).

3.5 DISCUSSION

The major findings from the investigations detailed in this chapter are:

1. TRAIL Ligand and its membrane receptors, TRAIL R1 and R3 were significantly overexpressed on PA-SMCs isolated from the lungs of patients with advanced IPAH.
2. Recombinant TRAIL stimulated proliferation and migration of human PA-SMC *in-vitro*.
3. The mitogenic effects of recombinant TRAIL *in-vitro* were mediated through the TRAIL-R3 receptor and required ERK signaling.

3.5.1 The role of the PASMC in PAH

PAH is a "proliferative vasculopathy" and although the precise sequence of molecular or cellular events that trigger PAH in susceptible individuals remain incomplete, aberrant PASMC growth with an imbalance towards excessive proliferation and migration underlies the vascular remodeling encountered in PAH (Archer, Weir et al. 2010; Schermuly, Ghofrani et al. 2011; Rabinovitch 2012). Several growth factors have been implicated to drive PASMC hyperplasia, including FGF-2 (Izikki, Guignabert et al. 2009), Platelet derived growth factor (PDGF) (Barst 2005; Schermuly, Dony et al. 2005) , Serotonin (Marcos, Fadel et al. 2004), Epidermal growth factor (EGF) (Merklinger, Jones et al. 2005) and TGF-B (Morrell, Yang et al. 2001). However the precise interplay of these factors are still not fully appreciated, although an adaptor protein, p130cas has been shown to be a common downstream factor in mediating the proliferative responses of several of the aforementioned mitogens (Tu, De Man et al. 2012). Therefore an antiproliferative therapeutic strategy is appealing for the treatment of PAH. Currently available therapies principally act through promoting vasodilatation and are unable to significantly reduce vascular remodeling, whereas recent efforts to clinically target some of the aforementioned growth factors have not been as promising as hoped (Dahal, Cornitescu et al. 2010; Hoeper, Barst et al. 2013). As a result there remains a need to continue identifying key pathways in disease pathogenesis that should permit the development of targeted and efficacious therapies.

3.5.2 TRAIL and VSMC growth

TRAIL is a member of the TNF ligand superfamily and was originally identified as a cytokine that induced apoptosis in malignant cells (Wiley, Schooley et al. 1995; Pitti, Marsters et al. 1996). It subsequently became apparent that TRAIL displayed non-apoptotic properties such as; cell growth, survival and differentiation (Falschlehner, Emmerich et al. 2007; Guicciardi and Gores 2009). These divergent, yet relevant effects of TRAIL theoretically suggested a role for TRAIL in proliferative vascular disease states such as atherosclerosis, in-stent restenosis and pulmonary hypertension, in which the balance between growth, apoptosis and overall cell turnover is critical.

A role for TRAIL in vascular biology was first reported over a decade ago, when TRAIL expression was detected in normal mouse pulmonary artery and aortic VSMCs from rat and human (Gochuico, Zhang et al. 2000). Subsequent studies reported TRAIL induced proliferation and migration of aortic VSMCs in-vitro (Secchiero, Zerbinati et al. 2004; Kavurma, Schoppet et al. 2008; Bumdelger, Kokubo et al. 2016). Moreover TRAIL expression was associated with markers of proliferation, but not apoptosis in stenosed (failed) saphenous vein grafts from patients with previous coronary artery bypass surgery (Kavurma, Schoppet et al. 2008). TRAIL has been shown to be a chemoattractant for monocytes (Wei, Wang et al. 2010) and aortic wall macrophages (Bumdelger, Kokubo et al. 2016). However, hitherto the effects of TRAIL on PASMC biology had not been studied.

The data from this chapter extend prior observations that TRAIL is abundantly expressed on normal human and rodent aortic and pulmonary arterial SMCs (Gochuico, Zhang et al. 2000; Secchiero, Zerbinati et al. 2004; Kavurma, Schoppet et al. 2008). Importantly these data are the first to observe increased TRAIL mRNA is expression on PASMCs from patients with PAH. We were unable to examine for TRAIL protein expression directly on PASMCs from PAH patients due to sample unavailability. However our prior observation of TRAIL immunostaining within concentric and plexiform lesions from patients with advanced IPAH (Lawrie, Waterman et al. 2008) collectively introduce a role for TRAIL in influencing VSMC behavior in pathology of the pulmonary circulation.

3.5.3 TRAIL receptor expression profile in PASMCs

These experiments also identified that PASMCs from human lung express all known TRAIL receptors, although only the membrane receptors, TRAIL R1 and R3 were significantly over expressed on PASMCs from patients with IPAH. Interestingly a significant upregulation of the soluble TRAIL-R5 receptor (osteoprotegerin, TNFRSF11B) was observed. Our group has described a pathogenic role for OPG in PAH (Lawrie, Waterman et al. 2008; Condliffe, Pickworth et al. 2012; Lawrie, Hameed et al. 2012). The finding that TRAIL-R1 and R3 are upregulated on human PASMCs is similar to that reported for aortic VSMCs using qPCR (Kavurma, Schoppet et al. 2008).

However not all studies have observed a similar receptor expression. At a protein level only TRAIL-R1 and R2, but not R3 or R4 were detectable using flow cytometry (Secchiero, Zerbinati et al. 2004). Similarly, selective expression of TRAIL R1 and R2 on aortic VSMCs has been reported by others, although the authors did not report if they examined for TRAIL-R3 and R4 expression in addition to death receptors (Gochuico, Zhang et al. 2000; Keogh, Harris et al. 2007). Nevertheless, TRAIL R2 was only detectable on normal human PASMC using FACS analysis for all receptors (Song, Choi et al. 2011). This would suggest that cell type, level of expression (protein or mRNA) as well as the method employed may have influenced the expression pattern observed. Preliminary analysis revealed immunostaining for all TRAIL receptors and in particular TRAIL-R3 in pathological lesions of IPAH, *(personal communication, Dr Mark Southwood, University of Cambridge).*

3.5.4 Mitogenic Effects of TRAIL on PASMC *in-vitro*

Despite differences in TRAIL receptor levels our observation that soluble recombinant TRAIL induced proliferation of PASMCs support previous *in-vitro* findings of the effects of TRAIL (at similar concentrations) on systemic VSMCs (Secchiero, Zerbinati et al. 2004; Kavurma, Schoppet et al. 2008; Chan, Prado-Lourenco et al. 2010; Song, Choi et al. 2011; Bumdelger, Kokubo et al. 2016) and add to the body of literature favoring a mitogenic effect of TRAIL on VSMCs. Additionally TRAIL has been shown to promote the survival of vascular endothelial cells and promote angiogenesis (Secchiero, Gonelli et al. 2003; Secchiero, Gonelli et al. 2004).

However in contrast, two studies have noted TRAIL directly induced apoptosis of aortic VSMCs, which may be have related to the method used to detect apoptosis, the expression pattern of

TRAIL receptors or possibly the dose of TRAIL used. For example in two of the three studies reporting only expression of TRAIL death receptor (TRAIL R1/2) on aortic SMCs, both were also associated with the finding of soluble TRAIL induced apoptosis of VSMCs, an effect blocked by antibodies to these specific receptors (Gochuico, Zhang et al. 2000; Keogh, Harris et al. 2007). In contrast a third group observed an identical TRAIL R1 and R2 expression yet the aortic VSMCs were resistant to TRAIL mediated cytotoxicity at concentrations up to 2000ng/ml, whereas 100ng/ml induced apoptosis in the HL60 tumour cell line. Apoptosis was evaluated with flow cytometry for PI/annexinV staining and also by confirming intact procaspase levels on VSMCs (Secchiero, Melloni et al. 2004).

The concentration with which we have observed TRAIL induced proliferation of PASMCs (30-100ng/ml) is similar to others reporting a similar effect (Kavurma, Schoppet et al. 2008) but also to that inducing apoptosis in aortic VSMCs (100ng/ml) although only modest levels (~10-15%) of apoptosis were observed after 24hours (Gochuico, Zhang et al. 2000). However, a two-three fold increase in apoptosis was noted after exposing aortic SMCs to rhTRAIL (10-1000ng/ml) for 60 hours (Keogh, Harris et al. 2007). In both aforementioned studies the presence of apoptosis was demonstrable by detection of PARP (a peptide cleaved during apoptosis) and in the latter study, additionally through use of time lapse microscopy and demonstration of a reduction in levels of apoptosis after treatment with a pan-caspase inhibitor (zVAD-fmk). In contrast, similar concentrations of recombinant TRAIL (10-1000ng/ml) were unable to elicit significant apoptosis, as measured by DNA fragmentation (Nesterov, Ivashchenko et al. 2002). A differential effect of TRAIL on VSMCs growth has been only been described by one group; at low doses (0.1-100ng/ml for 24-hours) rhTRAIL induced proliferation (with no corresponding increase in apoptosis) whilst at a higher dose (400ng/ml) rhTRAIL solely induced apoptosis of aortic VSMCs (Kavurma, Schoppet et al. 2008).

TRAIL is a membrane anchored protein, that can also be cleaved by cysteine proteases to release a soluble form that is biologically active (Zauli and Secchiero 2006). Atherosclerotic plaque rupture and consequent thrombosis is the underlying pathological substrate for clinical syndromes of acute myocardial and cerebral infarction. VSMC apoptosis is one of the major cellular events that can promote plaque rupture, and in this context, TRAIL expressed on CD4 T-cells induced VSMC apoptosis (Sato, Niessner et al. 2006). Furthermore a physiological role for TRAIL in the vascular remodeling encountered during pregnancy has been identified. Trophoblast derived TRAIL induced VSMC apoptosis and regulated spiral artery adaptation during placental development (Keogh et al CR 2007). Interestingly Fas Ligand, an archetypal

apoptosis inducing cytokine of the TNF family and upon which TRAIL was categorised (by virtue of sharing ~40% sequence homology) has been shown to induce PASMC apoptosis and elevated serum levels in patients with advanced PAH are observed (Zhang, Fantozzi et al. 2003; Akagi, Nakamura et al. 2013).

3.5.5 Diverse Effects of TRAIL: Role of Non-Canonical Signaling

Thus the effects of TRAIL *in-vitro* at least may relate to cell type, surface expression, disease context, dose and patterns of receptor expression (Kavurma and Bennett 2008). Albeit, the majority of *in-vitro* mechanistic studies however support a mitogenic role for TRAIL in VSMCs and this assertion were strengthened by *in-vivo* data from mice deficient for TRAIL. TRAIL expression in VSMC was upregulated following vascular injury and associated exclusively with upregulation in markers of proliferation but not apoptosis. $TRAIL^{-/-}$ mice were significantly protected from neointimal hyperplasia following wire induced femoral arterial injury. This was shown to be dependent on upstream FGF2 and NFκB signaling, whilst TRAIL also stimulated FGF-2 release highlighting positive feedback mechanism (Chan, Prado-Lourenco et al. 2010). FGF2 has been shown to have a role in experimental PAH; reduced expression of two protective micro RNAs (miR-424 and miR-503) due to reduced endothelial Apelin expression stimulated increased FGF2 gene and protein expression on patient derived PAECs. FGF2 resulted in hyperproliferation of PAEC and stimulated PASMC proliferation in a paracrine manner. (Kim, Kang et al. 2013)

Additionally it was reported for the first time that PDGF-BB induced VSMC proliferation and migration required TRAIL. This response was significantly attenuated in VSMC from $TRAIL^{-/-}$ mice. The authors elegantly identified that the TRAIL dependant mitogenic effects of PDGF-BB were regulated at a transcriptional level (Azahri, Di Bartolo et al. 2012).

These reports are very interesting and relevant to TRAIL, because a role for FGF2 (Izikki, Guignabert et al. 2009; Tu, Dewachter et al. 2011; Kim, Kang et al. 2013; Ricard, Tu et al. 2014) and PDGF (Barst 2005; Perros, Montani et al. 2008) have already been implicated in PAH.

Finally we have observed that mitogenic effects of TRAIL in PASMCS are dependent upon ERK1/2 signaling. ERK1/2 signaling has consistently been linked to vascular proliferation in PAH (Schermuly, Dony et al. 2005; Hansmann, de Jesus Perez et al. 2008; Yang, Davies et al. 2008; Morecroft, Doyle et al. 2011; Kwapiszewska, Markart et al. 2012).

ERK1/2 is the archetypal member of the MAPK signaling pathway and plays a central role in regulating cell proliferation and differentiation. It is activated sequentially by upstream kinases following receptor stimulation by several growth factors including PDGF and other tyrosine kinases. ERK1/2 is rapidly stimulated with subsequent translocation to the nucleus where it activates multiple transcription factors (Cargnello and Roux 2011; Roskoski 2012). Consistent with reported physiology we observed peak levels of activated ERK1/2 in PASMCs (phosphorylated p44/42) within 5 minutes of stimulation with recombinant human TRAIL and were able to detect nuclear expression using immunofluorescence (data not shown). Moreover inhibiting ERK1/2 (by blocking the upstream Kinase MEK1/2 with PD98059) significantly reduced TRAIL induced proliferation and migration of PASMCs. ERK signaling has previously been shown to divert TRAIL towards non apoptotic signaling (Tran, Holmstrom et al. 2001; Söderström, Poukkula et al. 2002) and pro-survival in vascular endothelial cells (Secchiero, Gonelli et al. 2003; Secchiero, Gonelli et al. 2004).

Further discussion on putative mechanistic links between existing pathways in PAH and TRAIL are covered in Chapter 1 of this thesis.

3.6 CHAPTER SUMMARY

The cytokine TRAIL and its surface receptors are upregulated on PASMCs from the lungs of patients with advanced PAH. This finding supports our group's prior observation of TRAIL immunostaining in concentric and plexiform lesions from these patients. TRAIL stimulated the proliferation and migration of PASMCs in culture. The membrane receptors TRAIL R1 and TRAIL-R3 were also upregulated on PASMCs and blocking TRAIL R3, but not R1 attenuated the proliferative effects of TRAIL *in-vitro*. Moreover these findings add to a growing literature on the relevance of the ERK1/2 signaling cascade to PAH, as it was necessary for mediating the mitogenic and chemotactic effects of TRAIL in culture.

Collectively these data develop and extend a role for TRAIL in modulating VSMC biology and importantly are the first to introduce a pathogenic effect for TRAIL in PAH. They support the concept that TRAIL is overproduced within the pulmonary vascular wall of patients with PAH and promotes pulmonary arterial remodeling, possibly through autocrine and paracrine effects. Collectively these findings are clinically relevant and important because they have led to the identification of a potentially novel driver of disease that appears to be linked to existing disease pathways and hopefully may offer an additional therapeutic avenue for treating PAH. Ongoing work in our department is characterising the gene and protein expression profile of TRAIL stimulated PASMCs.

CHAPTER 4: TRAIL IS NECESSARY AND SUFFICIENT FOR THE DEVELOPMENT OF EXPERIMENTAL PULMONARY HYPERTENSION

4.1 INTRODUCTION

In the previous chapter it was shown that TRAIL and its receptors were upregulated on PASMCS from patients with severe IPAH and that TRAIL was a pro-proliferative and migratory stimulus for normal human PASMCs *in-vitro*. These findings advanced our prior observation of TRAIL immunostaining within concentric and plexiform vascular lesions from these patients. Collectively these findings suggested a possible role for TRAIL in disease. However the patients from whom the PASMCs had been isolated were suffering from advanced disease and were undergoing lung transplantation. Therefore it was unclear if these findings reflected merely an epiphenomenon of endstage disease or whether they indicated a pathogenic role for TRAIL. To further elucidate this, my next series of investigations were performed utilising rodent models of PAH.

Classic and widely used models of PAH in rodents have been induced either with hypoxia (hypoxia induced PH-HPH) or the plant derived toxin, monocrotaline (MCT). Both pre-clinical models display several, (but not all) features of the human disease including medial hypertrophy, adventitial remodeling, vascular inflammation and in the case of the MCT model, severe right heart failure that is invariably fatal (Stenmark, Meyrick et al. 2009; Pak, Janssen et al. 2010; Tang, West et al. 2012).

A metabolic model of PAH induced by feeding a high fat western diet to male mice deficient in Apolipoprotein-E ($ApoE^{-/-}$) has been reported (Hansmann, Wagner et al. 2007). Although only a modest elevation of RVSP was observed, $ApoE^{-/-}$ mice fed a high fat diet (typically one that mimics a “western diet” in composition) have been widely used to study the pathogenesis of atherosclerosis (Getz and Reardon 2006; Maeda 2011). This model is routinely employed in our department and of the several diets in use, one in particular (eponymously referred to as a “Paigen” diet) is highly atherogenic, compared to a standard western high fat diet (Chamberlain, Francis et al. 2009). We utilised the aggressive nature of the Paigen diet, as an adaptation of the model of western diet induced PAH (Hansmann, Wagner et al. 2007) as a third preclinical model in which to study PAH.

My specific aims in this chapter were to determine:

- The expression of TRAIL in pulmonary arterioles from rodents with MCT induced PAH.
- If inhibition of TRAIL attenuated MCT induced PAH in rats.
- If TRAIL deficient mice were protected from hypoxia induced PH.
- If TRAIL deficiency protected $ApoE^{-/-}$ mice from Paigen diet induced PAH.

Through genetic deletion, pharmacological inhibition and over expression I will demonstrate in this chapter that TRAIL plays a direct pathogenic role in all three of these experimental models of PAH.

4.2 MATERIALS AND METHODS

Experimental protocols for the rodent studies are provided in this section. Detailed methods on rodents, diets, interventions and phenotyping are provided in chapter 2 of the thesis.

4.2.1 Monocrotaline time course and disease prevention protocol

Male Sprague Dawley rats (starting weight 200-250g, n=70) received a single subcutaneous injection of either monocrotaline (MCT) at 60mg/kg or an equivalent volume of 0.9% saline (control) (Section 2.1). They were divided into equal groups (n=7/group/time point) and phenotyped after 2, 7, 14, 21 or 28 days. **(Fig. 4.1A)**.

To investigate whether TRAIL was required for the development of disease a prevention study was performed. Rats (starting weight 200-240g) were injected with MCT (60mg/kg) and randomly assigned (n=4/gp) to implantation of an osmotic pump (Alzet® 2002 mini-pump, 200µl reservoir, 0.5µl/hr delivery rate for 2 weeks) containing one of the following interventions to target TRAIL: a polyclonal goat anti-mouse IgG TRAIL antibody (anti-TRAIL, #AF1121), recombinant Fc mouse TRAIL R2 (rmTRAILR2, #729-DR, amino acids 53-177 of the extracellular domain) or recombinant mouse Osteoprotegerin, (rmOPG, #MAB459-MO, amino acids 22-401). An isotype antibody was used as control (Goat IgG isotype, #AB-108-C, all R&D systems, Europe) and all interventions were delivered for 14 days (≈84ng/hr or ≈0.4ng/h/g for active treatments and 100ng/hr for control). Disease phenotyping was performed 7 days later (21 days after MCT) as described below (Fig 4.2A).

4.2.3 Chronic hypoxia experiments

Male TRAIL$^{-/-}$ and wild-type mice (C57BL/6 background, 8-13 weeks old, n=6-7/group) were placed in a sealed chamber (Coy Laboratory products Inc., Michigan, US) containing 10% oxygen for 2 weeks. The concentration of oxygen was maintained by controlling the inflow rate of oxygen and nitrogen using an automated hardware system supplied with the chambers. The concentration of Carbon Dioxide (<0.5%) was maintained with Soda lime pellets (#72073, Sigma-Aldrich, Dorset, UK) which acted as a CO_2 absorber. Concentration of gases was displayed electronically by hardware supplied with the chamber apparatus. Gas cylinders were purchased through our local hospital pharmacy (Royal Hallamshire Hospital, Sheffield, UK)

from BOC Industrial gases (Sheffield, UK). The hypoxia chambers were opened briefly (<5 min) and only once during the study period (day 7) for transfer of mice to fresh cages containing new bedding, food and water as well as for replenishing Soda lime. Control mice (normoxia group) were placed in the same room exposed to ambient room air (FiO2 21%). All mice were fed standard chow. After two weeks mice were phenotyped as described.

4.2.4 Paigen diet induced model of PAH

Male ApoE$^{-/-}$, TRAIL$^{-/-}$ and ApoE$^{-/-}$/TRAIL$^{-/-}$ knockout mice (8-12 weeks old, n=6-8/group) were fed standard chow or a high fat diet containing cholate (Paigen diet) (refer to section 2.3.1 for further details). All mice underwent terminal phenotyping 8 weeks after commencing the diet as described.

In subsequent experiments ApoE$^{-/-}$/TRAIL$^{-/-}$ mice (n=4/group) were implanted with subcutaneous osmotic micro-pumps (Alzet® 1004 model, Durect Corp, U.S) delivering either recombinant mouse TRAIL (rmTRAIL, #315-19, PeproTech Inc. NJ, U.S.A) at 10ng/hr (~0.4ng/g/h) or placebo (equivalent volume of phosphate buffered Saline-PBS). Mice were then fed Paigen and underwent standard phenotyping after 8 weeks.

4.2.5 Phenotyping: Haemodynamics, RVH and morphometric lung analysis.

Details for each component of the phenotypic evaluation of rodents are provided in chapter 2. Where indicated Pulmonary artery acceleration time (PAAT), which is inversely related to PA pressure, was measured by transthoracic echocardiography prior to invasive haemodynamic assessment (section 2.5). Closed chest cardiac catheterisation was performed with the appropriate high fidelity micromanometer catheter (pressure volume) to measure right and left heart pressures, and to derive cardiac index (section 2.6). Estimation of right ventricular hypertrophy (RVH), lung histology (ABEVG) immunohistochemistry (for α-SMA, vWF, TRAIL, PCNA and TUNEL) were performed on paraffin embedded lung sections (5µm thick) as described (Sections 2.7-2.9). The degree of pulmonary arteriolar remodeling (ratio of media to cross sectional area and percentage vessels thickened) and, where indicated levels of vascular proliferation and apoptosis were quantified as described (section 2.10).

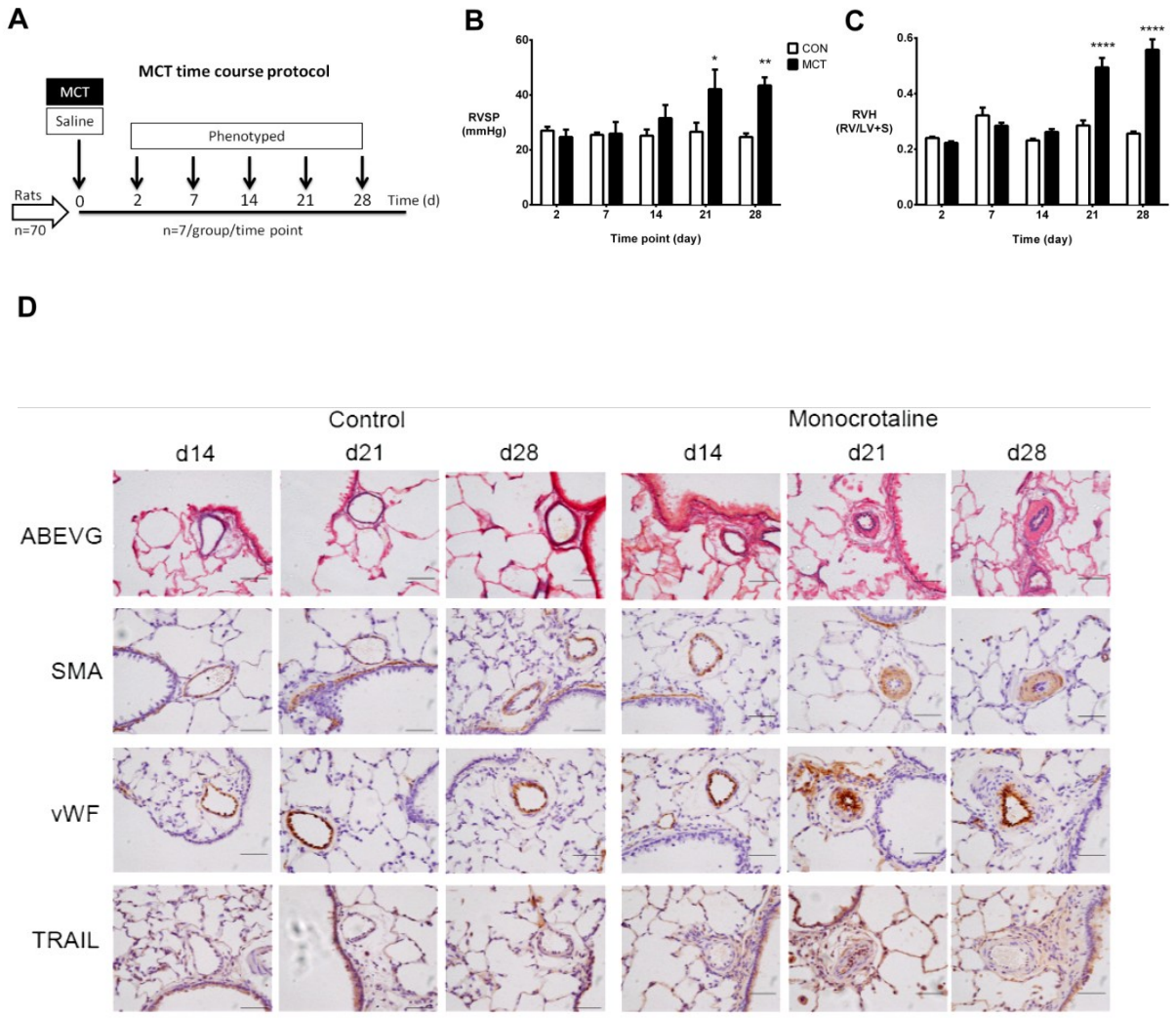
4.3 RESULTS

4.3.1 TRAIL expression in pulmonary arterioles increases with development of PAH in the MCT rat model

Given that TRAIL expression was upregulated in endstage human lung removed during transplantation, I first sought to determine the temporal and spatial expression of TRAIL during disease development and progression. A time course study of disease was initiated in the MCT rat model **(Fig. 4.1 A)**. Consistent with extensive prior literature, MCT treated rats developed PAH, as defined by significantly elevated right ventricular systolic pressure (RVSP) **(Fig. 4.1B)** and development of right ventricle hypertrophy (RVH) **(Fig. 4.1C)** from day 21, when compared to controls.

Accompanying the MCT induced haemodynamic changes were progressive pulmonary arteriolar remodeling from day 14 **(Fig. 4.1 D)**. The percentage of vessels that were muscularised (partly or fully) progressively increased from this time point (data not shown). Medial hypertrophy (α-SMA immunostaining), adventitial remodeling (ABEVG and vWF staining) and reduction in vessel lumen (vWF staining) were visible by day 21 **(Fig. 4.1 D)**.

TRAIL immunoreactivity was observed in epithelial and endothelial cells in saline treated rats whereas in MCT treated rats additional immunostaining in medial and perivascular tissue was evident from day 21 **(Fig. 4.1 D)** and this coincided with the aforementioned peak in RVSP, RVH and vascular remodeling.



<u>Figure 4.1</u> - TRAIL expression in pulmonary arterioles increases with pulmonary vascular remodeling in the monocrotaline rat model.

Schematic diagram of the study protocol **(A)** Bar graphs show RVSP **(B)** RVH **(C)** and representative photomicrographs of serial lung sections from saline and monocrotaline treated rats 14, 21 and 28 days after injection **(D).** Sections were stained with Alcian Blue Elastic van Gieson (ABEVG) or immunostained for α-smooth muscle actin (α-SMA), von Willebrand factor (vWF) or TRAIL. TRAIL expressing cells are present within the media and peri-vascular regions of remodelled small pulmonary arteries. **Error bars represent mean +/- SEM, n=7 animals/group/timepoint. *=p<0.05, **=p<0.01, ****=p<0.001 compared to saline treated controls. All images are representative of rats at each time point and are presented at their original magnification x400. Scale bar represents 50 µm.**

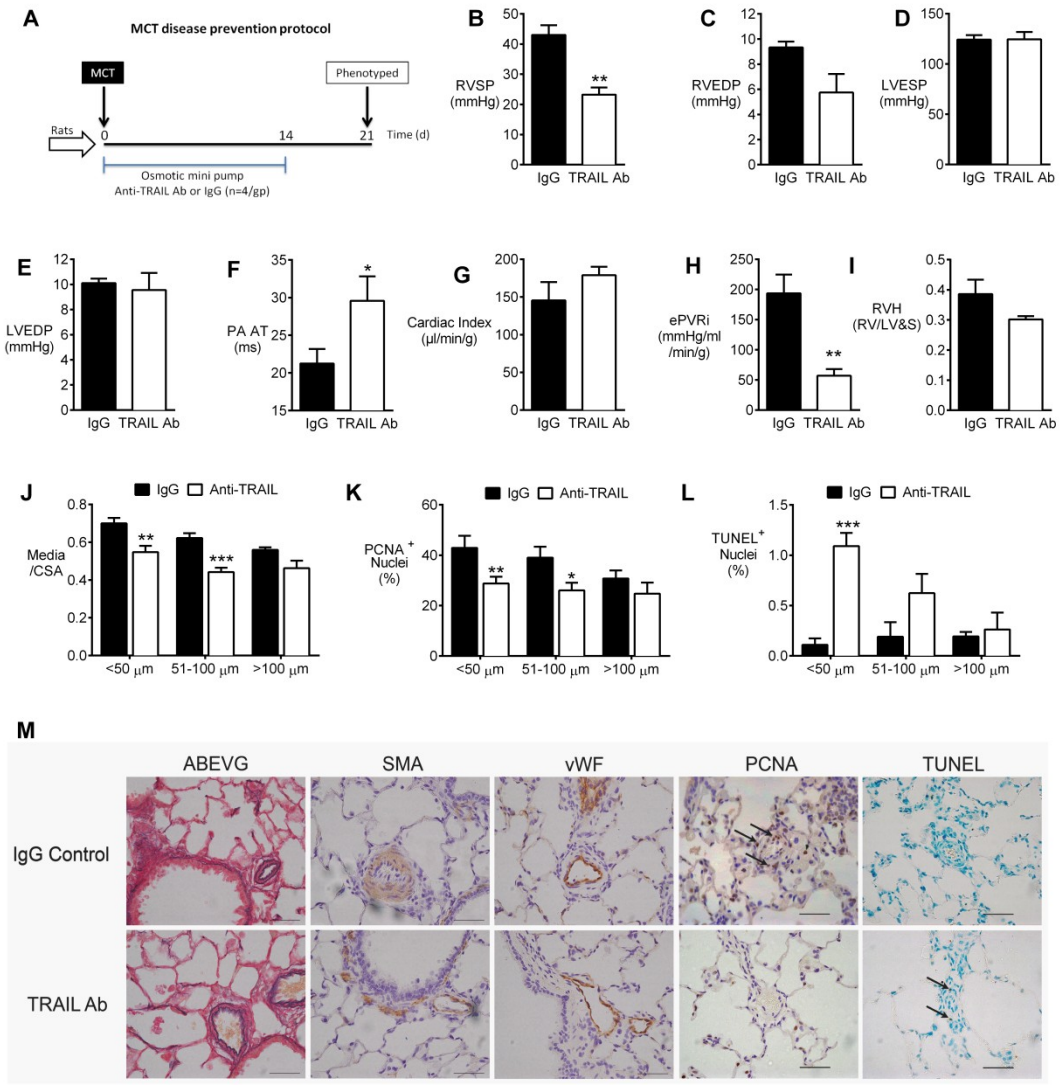
4.3.2 An anti-TRAIL antibody prevents the development of PAH in the MCT rat model

As TRAIL expression appeared to increase with disease progression in rats, I next sought to determine if inhibiting TRAIL could attenuate the development of MCT induced PAH. Rats were injected with MCT and immediately assigned to receive an osmotic mini pump delivering either an anti-TRAIL antibody or an isotype control (n=4/group) for 14 days **(Fig. 4.2 A)**.

21 days after MCT injection, rats treated with an IgG isotype antibody (control) had developed PAH, as defined by an elevated RVSP, right ventricular end-diastolic pressure (RVEDP), reduced Pulmonary artery acceleration time (PAAT) and an increased pulmonary vascular resistance index (ePVRi) (see Figure 4.2).

In contrast, rats treated with an anti-TRAIL antibody for only two weeks, had a normal RVSP **(Fig 4.2 B)**, RVEDP **(Fig 4.2C)** , PAAT **(Fig 4.2F)** , higher cardiac index **(Fig 4.2G)** with lower ePVRi **(Fig. 4.2H)** and RVH **(Fig. 4.2I)** 21 days after MCT injection. No significant differences in left ventricular haemodynamics were observed **(Fig. 4.2 D-E)**. Unlike rats treated with the anti-TRAIL antibody, those treated with either rmTRAIL-R2 (extracellular) or rmOPG (TRAIL R5) were not protected with an RVSP that was not significantly different from controls (data not shown).

Accompanying the favorable haemodynamics noted in anti-TRAIL treated rats, there were significant reductions in pulmonary vascular remodeling as measured by the media/cross-sectional area in arterioles of <50µm and between 50-100µm in size **(Fig. 4.2 J**). This was associated with a corresponding reduction in levels of cellular proliferation **(Fig. 4.2K &M)**. There was also an increased level of apoptosis **(Fig. 4.2 L & M)** in the smallest pulmonary arterioles (<50µm). No increase in apoptosis was observed in systemic tissue from these animals (spleen and aorta, data not shown).



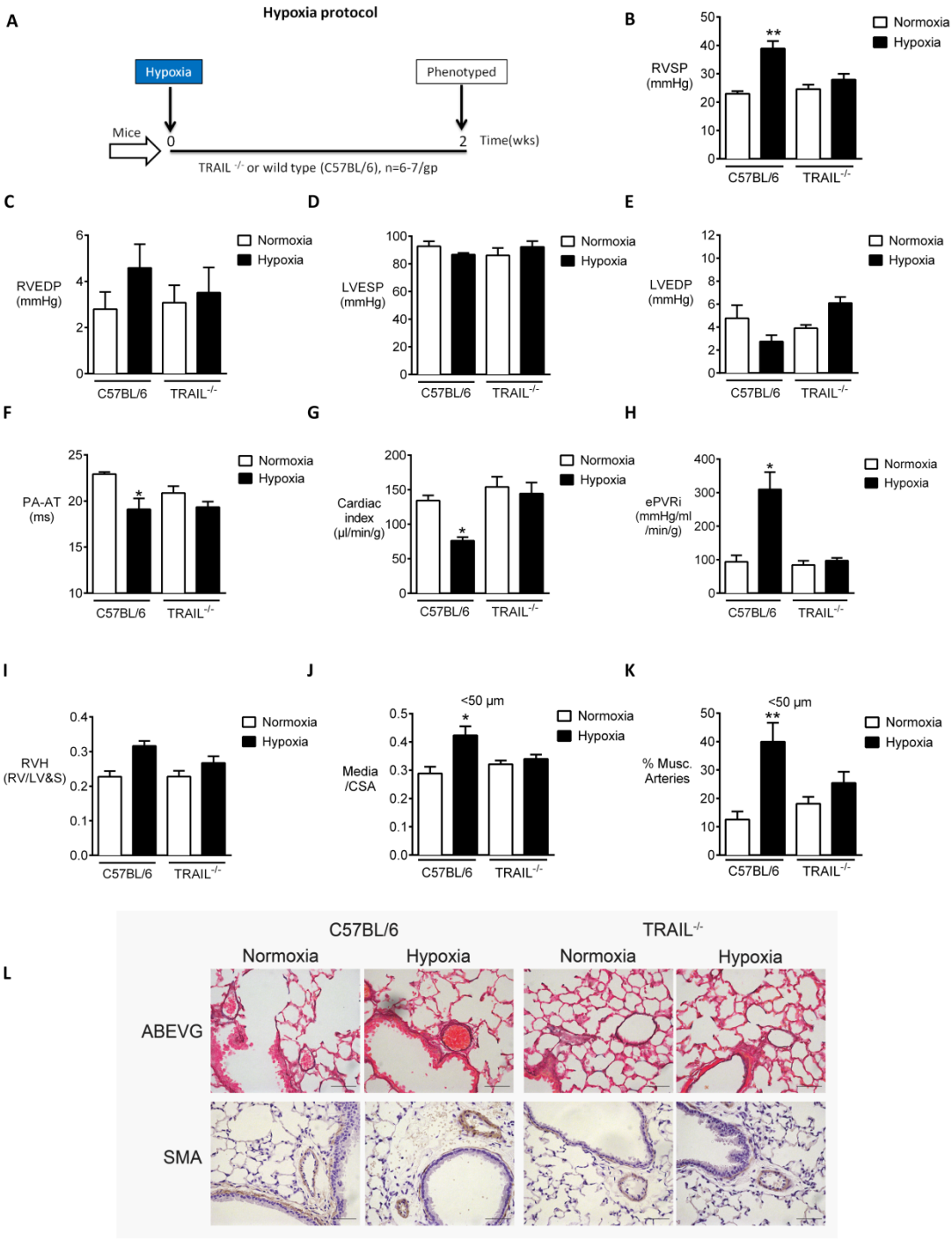
<u>Figure 4.2</u>- Anti-TRAIL antibody treatment prevents the development of PAH at day 21 in the monocrotaline rat model.

Schematic diagram of the study protocol is shown in (A). Bar graphs show (B) right ventricular end-systolic pressure (RVSP) (C) right ventricular end-diastolic pressure (RVEDP), (D) left ventricular end-systolic pressure (LVESP) (E) left ventricular end-diastolic pressure (LVEDP), measured in mm Hg. (F) Pulmonary artery Acceleration time (PA AT), (G) cardiac index, (H) estimated Pulmonary Vascular Resistance index (ePVRi) and (I) right ventricular hypertrophy (RVH). (J) The degree of medial wall thickness as a ratio of total vessel size (Media/CSA), quantification of the percentage of (K) proliferating cells (PCNA positive), and (L) apoptotic (TUNEL positive) separated into pulmonary arteries less than 50 µm (<50 µm) in diameter, vessels from 51 to 100 µm (51-100 µm) in diameter and vessels larger that 100 µm (>100µm) in diameter. (M) Representative photomicrographs of serial lung sections from IgG and anti-TRAIL treated monocrotaline induced rats 21 days after injection. Sections were stained with Alcian Blue Elastic van Gieson (ABEVG) or immunostained for α-smooth muscle actin (α-SMA), von Willebrand factor (vWF), proliferating cell nuclear antigen (PCNA) or Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). **Bars represent mean +/- SEM, n=4 animals per group, *=p<0.05, **=p<0.01, ***=p<0.001 compared IgG treated rats. Arrows point to PCNA or TUNEL positive cells. All images are presented at their original magnification x400, scale bar represents 50 µm.**

4.3.3 TRAIL$^{-/-}$ mice are protected from hypoxia induced pulmonary hypertension

To determine whether TRAIL was relevant in hypoxia induced PH, I exposed TRAIL$^{-/-}$ mice to sustained hypoxia (FiO_2 10%) for two weeks **(Fig. 4.3 A).** Compared to normoxia, wild type (C57BL/6) control mice exposed to hypoxia developed a significant PH phenotype as defined by; elevated RVSP (38.9mmHg+/- 2.6mmHg; **Fig. 4.3B**), increased pulmonary vascular resistance (ePVRi; **(Fig. 4.3 H)**, development of right ventricular hypertrophy (RVH; **Fig.4.3I**) with a corresponding reduction in PAAT **(Fig. 4.3F)** and cardiac index **(Fig. 4.3G).** These haemodynamic changes were linked directly to visibly and quantitatively greater (adverse) vascular remodeling within pulmonary arterioles of <50μm diameter **(Fig. 4.3 J-L).**

In contrast, TRAIL$^{-/-}$ mice exposed to identical hypoxic conditions were protected from the haemodynamic and microscopic changes observed in hypertensive control mice **(Fig. 4.3 B-L).** No significant differences in left ventricular haemodynamics were observed across all groups **(Fig. 4.3 D-E).**



<u>Figure 4.3</u> - TRAIL$^{-/-}$ mice are protected from chronic hypoxia induced PH.

Schematic diagram of the study protocol (A) Bar graphs show (B) right ventricular end-systolic pressure (RVSP) (C) right ventricular end-diastolic pressure (RVEDP), (D) left ventricular end-systolic pressure (LVESP) (E) left ventricular end-diastolic pressure (LVEDP), measured in mm Hg. (F) Pulmonary artery Acceleration time (PA AT), (G) cardiac index, (H) estimated Pulmonary Vascular Resistance index (ePVRi) and (I) right ventricular hypertrophy (RVH). (J) The degree of medial wall thickness as a ratio of total vessel size (Media/CSA), and (K) the percentage of thickened pulmonary arteries less than 50 µm in diameter. (L) Representative photomicrographs of serial lung sections from C57BL/6 and TRAIL$^{-/-}$ mice following either 2 weeks exposure to room air or hypoxia. Sections were stained with Alcian Blue Elastic van Gieson (ABEVG) or immunostained for α-smooth muscle actin (α-SMA**). Bars represent mean +/- SEM, n=6-7 animals per group, *=p<0.05, **=p<0.01 compared normoxic mice. All images are presented at their original magnification x400, scale bar represents 50 µm.**

4.3.4 TRAIL deficiency protects ApoE$^{-/-}$ mice from Paigen diet induced PAH

Following the initial report of ApoE$^{-/-}$ mice developing mild PAH (mean RVSP ≈29mmHg) after feeding on a high fat western diet for 11 weeks (Hansmann, Wagner et al. 2007), we reported that feeding of a more aggressive high fat diet (the Paigen diet) for only 8 weeks produced a more severe PAH phenotype in ApoE$^{-/-}$ mice (Lawrie, Hameed et al. 2011). I thus wanted to also determine if TRAIL was pathogenic in this model of severe PAH **(Fig. 4.4A).**

Compared to chow controls, ApoE$^{-/-}$ mice fed Paigen diet for 8 weeks developed severe PAH, as defined by; significantly increased RVSP (50 +/- 4 vs 26 +/-2 mmHg; **Fig. 4.4B**) RVEDP **(Fig. 4.4C)** and ePVRi **(Fig.4.4H)**, reduced PAAT **(Fig. 4.4F)** and cardiac index **(Fig. 4.4G)**. Significant pulmonary vascular remodeling was observed in these mice including heavily muscularised arterioles and obliterative neointimal lesions were observed in 5-10% of vessels **(Fig. 4.4K)** characterized by dysregulated elastin and collagen deposition. Interestingly despite significantly elevated pulmonary vascular haemodynamics, ApoE$^{-/-}$ mice did not develop any significant RVH **(Fig. 4.4I).**

Remarkably however, ApoE$^{-/-}$ mice that were also deficient for TRAIL (ApoE$^{-/-}$ /TRAIL$^{-/-}$) were protected from the development of PAH when fed a Paigen diet, as evidenced by near normal RVSP (27.8+/-3.2 mmHg; **Fig. 4.4B**) and normal ePVRi **(Fig. 4.4H)** and other haemodynamic indices **(Fig. 4.4 C-J).** The normal haemodynamic phenotype observed in ApoE$^{-/-}$ /TRAIL$^{-/-}$ mice was directly associated with a lack of any appreciable evidence of vascular remodeling and was in fact rather similar to that observed in chow fed animals. This was in stark contrast to severe pulmonary arteriolar remodeling encountered in Paigen fed ApoE$^{-/-}$ mice **(Fig. 4.4 K).**

Finally there was no evidence of a PAH phenotype in TRAIL$^{-/-}$ mice fed either chow or Paigen diet **(Fig. 4.4B-J)**. No significant differences in left ventricular and aortic pressures were observed between all groups of mice **(Fig. 4.4 D-E).**

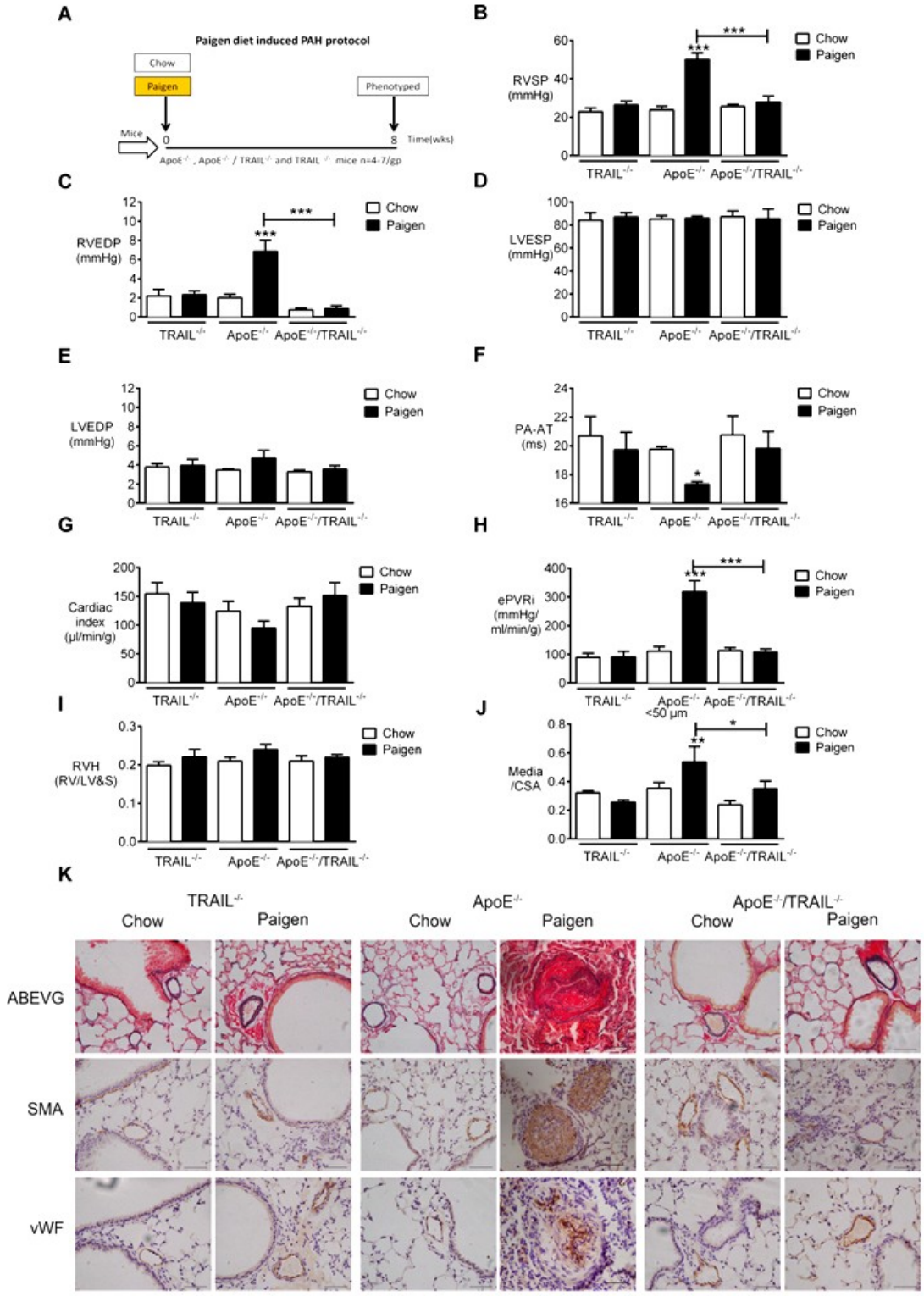


Figure 4.4 – ApoE$^{-/-}$/TRAIL$^{-/-}$ mice are protected from the development of PAH.

Figure 4.4 – ApoE$^{-/-}$/TRAIL$^{-/-}$ mice are protected from the development of PAH.

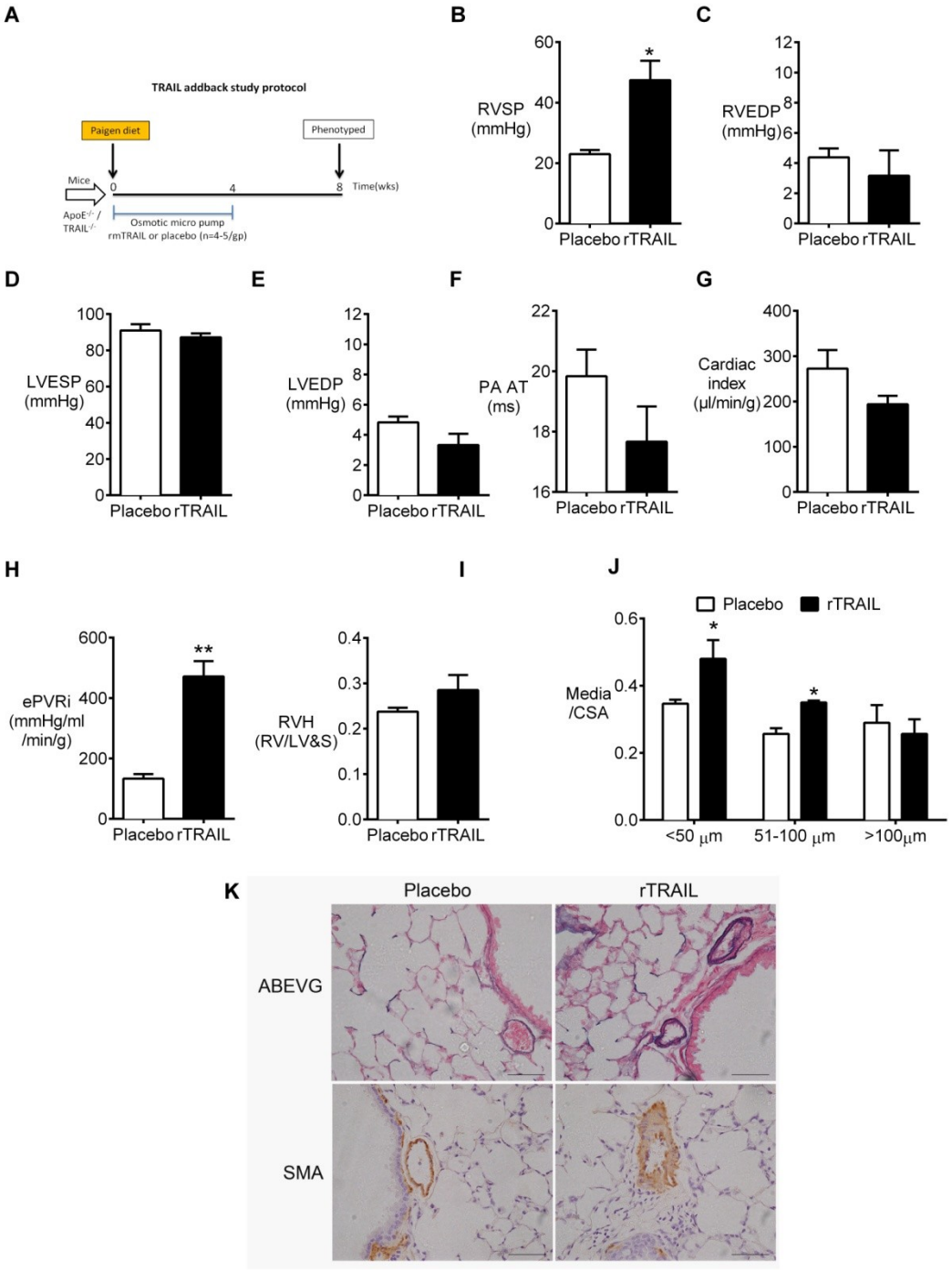
Schematic diagram of the study protocol (A**)** Bar graphs show (B) right ventricular end-systolic pressure (RVSP) (C) right ventricular end-diastolic pressure (RVEDP), (D) left ventricular end-systolic pressure (LVESP) (E) left ventricular end-diastolic pressure (LVEDP), measured in mm Hg. Pulmonary artery Acceleration time (PA AT), (F) cardiac index, (G) estimated Pulmonary Vascular Resistance index (ePVRi) and (H) right ventricular hypertrophy (RVH). (I) The degree of medial wall thickness as a ratio of total vessel size (Media/CSA) in pulmonary arteries less than 50 μm in diameter. (J) Representative photomicrographs of serial lung sections from TRAIL$^{-/-}$, ApoE$^{-/-}$ and ApoE$^{-/-}$/TRAIL$^{-/-}$ mice fed on either regular chow or Paigen diet for 8 weeks. Sections were stained with Alcian Blue Elastic van Gieson (ABEVG) or immunostained for α-smooth muscle actin (α-SMA), von Willebrand factor (vWF**). Bars represent mean +/- SEM, n=4-6 (7 in one group for RVSP) animals per group, *=p<0.05, **=p<0.01, ***=p<0.001 compared chow fed mice. All images are presented at their original magnification x400, scale bar represents 50 μm.**

4.3.5 Exogenous TRAIL restores a disease phenotype in ApoE$^{-/-}$/TRAIL$^{-/-}$ mice

As genetic deletion of TRAIL protected ApoE$^{-/-}$ mice from Paigen induced PAH, I hypothesized that exogenous TRAIL would revert ApoE$^{-/-}$ /TRAIL$^{-/-}$ mice from their protected phenotype. Therefore ApoE$^{-/-}$ / TRAIL$^{-/-}$ (8-14 weeks old) mice were implanted with an osmotic micro pump to deliver either recombinant murine TRAIL or an equivalent volume of saline, for four weeks. All mice were fed a Paigen diet for 8 weeks before phenotyping **(Fig. 4.5A).**

Compared to ApoE$^{-/-}$/ TRAIL$^{-/-}$ receiving placebo, those that received rmTRAIL for only four weeks, developed PAH as defined by a significantly elevated RVSP (mean 47.5+/- 5mmHg vs. 23+/- 1.3mmHg, **Fig. 4.5B**), ePVRi (471 +/- 88 mm Hg/ml/min/g, **Fig. 4.5H**) with levels similar to those encountered in ApoE$^{-/-}$ fed Paigen **(Fig. 4.4B)**. Furthermore in rmTRAIL treated mice, there was a trend for a reduced PAAT **(Fig. 4.5F)** and Cardiac index **(Fig. 4.5G)**. Again no significant differences in left ventricular/aortic haemodynamics were observed **(Fig. 4.5 D-E).**

Consistent with haemodynamics supporting PAH, significant pulmonary vascular remodelling was observed in arterioles of <50µm and 50-100µm from mice treated with rmTRAIL **(Fig. 4.5 J-K)**. These data further support a causative role of TRAIL in the development of PAH in this murine model.



<u>Figure 4.5</u> - Addition of recombinant TRAIL to ApoE$^{-/-}$/TRAIL$^{-/-}$ mice re-established the development of PAH.

Figure 4.5 - Addition of recombinant TRAIL to ApoE$^{-/-}$/TRAIL$^{-/-}$ mice re-established the development of PAH Schematic diagram of the study protocol (A) Bar graphs show (B) right ventricular end-systolic pressure (RVSP) (C) right ventricular end-diastolic pressure (RVEDP), (D) left ventricular end-systolic pressure (LVESP) (E) left ventricular end-diastolic pressure (LVEDP), measured in mm Hg. (F) Pulmonary artery Acceleration time (PA AT), (G) cardiac index, (H) estimated Pulmonary Vascular Resistance index (ePVRi) and (I) right ventricular hypertrophy (RVH). (J) The degree of medial wall thickness as a ratio of total vessel size (Media/CSA) in pulmonary arteries less than 50 μm in diameter. (K) Representative photomicrographs of serial lung sections from ApoE$^{-/-}$/TRAIL$^{-/-}$ mice fed Paigen diet for 8 weeks who received either recombinant TRAIL (rTRAIL) or saline (placebo) by osmotic micro-pump for 4 weeks. Sections were stained with Alcian Blue Elastic van Gieson (ABEVG) or immunostained for α-smooth muscle actin (α-SMA). **Bars represent mean +/- SEM, n=4-5 animals per group, *=p<0.05 , **p=(<0.01) compared placebo treated mice. All images are presented at their original magnification x400, scale bar represents 50 μm.**

4.4 DISCUSSION

The salient novel findings from the investigations I have detailed in this chapter are:

1) TRAIL is expressed within remodeled pulmonary arterioles in rats with MCT induced PAH and this temporally coincided with elevation of pulmonary haemodynamics.
2) Treatment with an anti-TRAIL antibody prevented PAH in the MCT rat model.
3) TRAIL deficient mice were protected from hypoxia induced PH.
4) $ApoE^{-/-}$ mice deficient for TRAIL were protected from Paigen diet induced PAH.
5) Exogenous TRAIL was sufficient to restore a disease phenotype in $ApoE^{-/-}/TRAIL^{-/-}$ mice.

4.4.1 The use animal models to study PAH

Studying human PAH has been challenging for a number of reasons and include; a lack of availability of pathological lung tissue in part due to the severity of disease at presentation precluding lung biopsy and fewer patients undergoing lung transplantation. In addition the disease develops over several years and often is diagnosed at an advanced stage thus limiting evaluation during the earlier phases of disease.

The lack of availability of lung tissue from early-stage disease represents a major limiting factor for dissecting the initiating mechanisms of human PAH. Similar to many branches of translational medicine, animal models have provided valuable tools for improving our understanding of the molecular and cellular disease mechanisms as well as providing an avenue for preclinical drug discovery (Stenmark, Meyrick et al. 2009). There are several animal models available for investigating PH and it is widely acknowledged that no single model recapitulates all of the pathological and clinical features of the disease. Thus investigators recommend that more than one model be used when characterising new pathways or testing new therapies in preclinical studies (Stenmark, Meyrick et al. 2009; Pak, Janssen et al. 2010; Ryan, Bloch et al. 2011; Voelkel and Gomez-Arroyo 2011; Sutendra and Michelakis 2013).

4.4.2 Defining a role for TRAIL in classical (hypoxia & monocrotaline) models of PAH

In this chapter I have provided the first evidence that TRAIL is both necessary and sufficient for the development of disease in three rodent models of pulmonary hypertension.

Firstly in rats with monocrotaline induced PAH, TRAIL expression was evident within the media and perivascular regions of remodeled pulmonary arterioles on immunohistochemistry and this coincided with the peak in haemodynamics and vascular remodeling. Endothelial injury and inflammation are considered to be relevant to both human PAH (Hassoun, Mouthon et al. 2009; Soon, Holmes et al. 2010; Price, Wort et al. 2012) and the MCT model (Voelkel and Tuder 1994; Bhargava, Kumar et al. 1999; Kimura, Egashira et al. 2009; Cuttica, Langenickel et al. 2010; Price, Montani et al. 2011). TRAIL has been shown to modulate the inflammatory response (Adam, Paul et al. 2009; Hoffmann, Zipp et al. 2009; Nguyen, Cudrici et al. 2009; Jin, Chae et al. 2010; Malyszko, Przybylowski et al. 2011; Rethi and Eidsmo 2012). Therefore in addition to the mitogenic effects of TRAIL on PA-SMCs described in chapter 3, it is plausible that TRAIL is promoting local pro-inflammatory effects which contribute to local vascular remodelling. However it is also emerging that TRAIL may exert anti-inflammatory and immune modulating properties (Secchiero, Corallini et al. 2005; Jin, Chae et al. 2010; McGrath, Marriott et al. 2011; Marcuzzi, Secchiero et al. 2012; McGrath, Lawrie et al. 2012; Steinwede, Henken et al. 2012) highlighting that the other cell and disease factors can determine the effects of TRAIL (Benedict and Ware 2012).

My next major finding reported in this chapter is that inhibiting TRAIL using an anti-TRAIL neutralising antibody (but not recombinant TRAIL receptors R2 and R5) prevented rats from developing MCT induced PAH. In addition to normal pulmonary haemodynamics (RVSP, Cardiac output and ePVRi) anti-TRAIL treated rats had significantly lower vascular remodeling (medial hypertrophy) in the lung. Consistent with TRAILs pro-proliferative effect on PASMCs *in-vitro* (chapter 3) it was reassuring to observe that the protected phenotype observed in anti-TRAIL treated rats was associated with significantly reduced proliferation in pulmonary arterioles **(Fig. 4.2K)**. Additionally however I observed small but significantly greater levels of apoptosis in these vascular lesions **(Fig. 4.2L)**. Although I have not directly evaluated apoptosis of PASMC *in-vitro*, it is tempting to speculate that phenotypically altered vascular wall cells (including α-SMA expressing PASMCS) may undergo apoptosis following withdrawal of the trophic factor TRAIL. TRAIL has been reported to display both proliferative and apoptotic effects on VSMCs *in-vitro* depending on the dose, (Kavurma, Schoppet et al. 2008), so it is

possible that antibody neutralization of TRAIL may have altered the levels or bioavailability of TRAIL locally which may have induced a direct/indirect apoptotic effect on PASMCs.

4.4.3 TRAIL and hypoxia in PH

Prior to my work, only one study had investigated TRAIL after hypoxia and pulmonary vascular inflammation and observed that TRAIL gene expression was reduced (1.62 fold) after 7 days of exposure to hypoxia in rats, but not at an earlier (day 1) or later (day 21) timepoint. In addition no significant differences were observed after return to normoxia (Burke, Frid et al. 2009). Thus a clear role for TRAIL in hypoxia induced pulmonary hypertension had yet to been established.

My data are the first to clearly define a role of TRAIL to hypoxia induced PH because I have demonstrated that C57BL/6 mice lacking TRAIL ($TRAIL^{-/-}$) are protected from hypoxia induced pulmonary hypertension, as defined by normal haemodynamics and reduced vascular remodeling and build on the findings observed in the MCT rat model.

Subsequent to the completion of my studies, independant investigators have reported significantly increased (3 fold) expression of TRAIL in lungs and serum TRAIL protein in wild type (C57BL/6) mice exposed to hypoxia (Liu, Yang et al. 2015). Furthermore TRAIL gene/protein expression has been shown to be significantly increased in human COPD lungs (broncholaveloar lavage and parenchyma) and in an established murine model of COPD (induced with cigarette smoke) whilst antibody inhibition of TRAIL reversed experimental disease (Haw, Starkey et al. 2016).

4.4.4 TRAIL and pulmonary vascular disease in $ApoE^{-/-}$ mice.

Thus so far, my data support a pathogenic role for TRAIL in the two classical models of disease (MCT models PAH whereas hypoxia models PH due to lung disease). The third model I used in this study is a modification of the metabolic model of PAH described using $ApoE^{-/-}$ mice which develop mild PAH (mean RVSP 28.9+/- 0.6 vs 23.6 +/- 0.6 mmHg in controls) after feeding a high fat western diet for 11 weeks (Hansmann, Wagner et al. 2007). The authors demonstrated that these mice had reduced adiponectin levels and were insulin resistant. Treatment with the PPARγ activator, Rosiglitazone increased adiponectin levels, improved insulin sensitivity and reversed PAH. This study took advantage of prior observations noting lung tissue from patients

with PAH have reduced mRNA expression of both ApoE (Geraci, Moore et al. 2001) and PPARγ (Ameshima, Golpon et al. 2003; Matsuda, Hoshikawa et al. 2005).

Furthermore Insulin resistance, diabetes and other features of the metabolic syndrome are increasingly becoming recognized as clinical features of pulmonary hypertension (Movahed, Hashemzadeh et al. 2005; Robbins, Newman et al. 2009; Zamanian, Hansmann et al. 2009; Heresi, Aytekin et al. 2010; Pugh, Robbins et al. 2011) and subsequently have been explored in preclinical rodent models of PAH (Agard, Rolli-Derkinderen et al. 2009; Lopez-Lopez, Moral-Sanz et al. 2011; Moral-Sanz, Menendez et al. 2011; Moral-Sanz, Lopez-Lopez et al. 2012; West, Niswender et al. 2013; Kelley, Baust et al. 2014).

There are several possible mechanisms leading to vascular diseases such as atherosclerosis or pulmonary hypertension in $ApoE^{-/-}$ mice. Briefly, these mice display; a marked pro-inflammatory state due to hyperlipidaemia, cytokine activation which consequently leads to oxidative stress, endothelial dysfunction and importantly reduced nitric oxide bioavailability. Furthermore in addition to regulating circulating lipid levels, apolipoprotein E possess many anti-inflammatory (non-lipid) properties (Davignon 2005) such as reducing oxidative stress, improving NO bioavailability whilst also inhibiting endothelial cell activation and VSMC proliferation and links to caveolin 1 (Yue, Bian et al. 2012) which is linked to PAH (Austin, Ma et al. 2012). ApoE has been shown inhibit vascular smooth muscle proliferation induced by PDGF (Ishigami, Swertfeger et al. 1998; Ishigami, Swertfeger et al. 2000; Zeleny, Swertfeger et al. 2002). ApoE can bind to the LDL receptor-related protein (LRP) and block the mitogenic effects of PDGF (Zhu and Hui 2003) (through receptor endocytosis and degradation).

With reference to pulmonary vasculature, it has been shown that human PASMCs produce ApoE which inhibited PDGF-β induced PASMC proliferation. More importantly the authors described a novel anti proliferative mechanism for normal BMPR2 signaling that required PPAR-γ (Hansmann, de Jesus Perez et al. 2008). Serendipitously it was discovered that only the type II BMP receptor, BMPR2 (and not type I) accelerated atherosclerosis in $ApoE^{-/-}$ mice also heterozygous for BMPR2 ($ApoE^{-/-}$ / $BMPR2^{-/+}$) This was mechanistically linked to heightened vascular inflammation and ROS production (Kim, Song et al. 2013). Female $ApoE^{-/-}$ mice injected with monocrotaline develop severe pulmonary hypertension which was attenuated with estrogen therapy (Umar, Partow-Navid et al. 2017). These authors have also highlighted a role for oxidised lipids in promoting pulmonary hypertension through a number of mechanisms (Ross, Hough et al. 2015; Sharma, Ruffenach et al. 2016). Recently plasma

proteomic profiling identified ApoE levels as a valuable independent prognostic biomarker in PAH (Rhodes, Ghataorhe et al. 2017).

ApoE$^{-/-}$ mice were originally developed and remain widely used to study the development of atherosclerosis. These mice develop atherosclerosis even when fed standard chow, however feeding of a high fat diet leads to marked hypercholesterolaemia that markedly enhances the development of atheroma (Maeda 2011). Our department has widely used this strain for this purpose and often uses a particular variety of diet (Paigen diet) that is more atherogenic compared to a western diet (Paigen, Morrow et al. 1987; Getz and Reardon 2006; Chamberlain, Francis et al. 2009). The more pronounced atherogenic effect is attributable to the cholate (0.5%) within this diet which induces greater levels of hyperlipidaemia (cholate facilitates the emulsification and increases absorption of intestinal fat) and consequent vascular inflammation.

After feeding ApoE$^{-/-}$ mice a Paigen diet for 8 weeks, they developed severe pulmonary hypertension, as defined by elevated RV systolic pressure **(Fig. 4.4B)**, ePVRI **(Fig. 4.4H)**, marked vascular remodeling **(Figs. 4.4 J-K)**. Moreover our group has reported that ApoE$^{-/-}$ mice fed Paigen mice have increased circulating levels of the pro-inflammatory cytokines IL-1β and IL-6. Furthermore we demonstrated increased TRAIL expression in the lung and in line with previous findings in humans and rodent models (hypoxia and MCT) reduced expression of BMPR2 (Lawrie, Hameed et al. 2011).

The finding of normal left ventricular haemodynamics **(Fig. 4.4 D-E)** and echocardiographic indices of LV function (stroke volume, fractional shortening and LV fractional area change- data not shown) collectively are in favor of supporting that this is a valid model of pre-capillary PH (i.e. PAH). In addition to the shorter duration of feeding and greater severity of pulmonary haemodynamics, the extent of pulmonary vascular remodeling with our Paigen diet model are noteworthy when compared to that after a western diet (Hansmann, Wagner et al. 2007).

Remarkably however I observed that ApoE$^{-/-}$ mice with genetic deletion of TRAIL when fed Paigen were virtually protected from the development of this severe PAH phenotype. Furthermore in this chapter I have shown that this protected phenotype could be reversed with administration of a recombinant TRAIL peptide **(Fig. 4.5)**. My data thus support a pathological role for TRAIL in the development of PAH in this novel murine model of severe

PAH and add to the pathogenic effect of TRAIL in both hypoxia and MCT induced pulmonary hypertension.

My findings in this chapter support a pathogenic role for TRAIL in experimental PAH. However as discussed in chapter 1 TRAIL appears to have a protective role in the development of atherosclerosis in mice fed western type high fat diets. $ApoE^{-/-}$/ $TRAIL^{-/-}$ mice have reduced aortic atheroma burden when fed chow or the milder western diet for 8 weeks but this difference was not evident after 12 weeks of diet (Watt, Chamberlain et al. 2011). Importantly however when fed an aggressive Paigen diet, these same mice were not protected from atherosclerosis (personal communication Prof. Sheila Francis, Dept Cardiovascular Science, University of Sheffield, unpublished data). These observations would suggest that in $ApoE^{-/-}$ mouse at least, TRAIL has a more pronounced pathogenic effect on pulmonary vascular remodeling compared to aortic atherosclerosis.

The Paigen diet is certainly more aggressive in terms of atherosclerosis severity (Chamberlain, Francis et al. 2009) and extent of pulmonary vascular disease. Indeed whilst studying PAH, we have observed that when younger (age <8weeks) $ApoE^{-/-}$ mice were studied, up to 25% of them died often within 4-7 weeks of feeding. These mice display outward signs of illness through reduced activity, shivering, and abnormal fur. An explanation for this deterioration is presently not clear nor whether these mice have severe PH, heart failure or an alternative cause such as infection or sepsis. On the few instances where it was possible to perform echocardiography on these sick mice I have noted low cardiac output with globally impaired LV function. The right ventricle was not however dilated (which would suggest the absence of RV failure). Interestingly mice deficient for ApoE and a scavenger receptor for HDL (SR-BI) fed a Paigen diet develop severe hyperlipidaemia, obstructive coronary lesions, myocardial infarction, cardiac dysfunction and fibrosis and often die prematurely (Zhang, Picard et al. 2005; Nakagawa-Toyama, Zhang et al. 2012).

Several reports have suggested that the ingredient cholate in the Paigen diet formulation is responsible for the formation of lung granulomas in $ApoE^{-/-}$ mice thus proposing it as a model of sarcoidosis (Samokhin, Bühling et al. 2010; Samokhin, Gauthier et al. 2011). Although we observed some early granulomas with low frequency within the lungs of our mice, we detected no difference in these across our mouse genotypes (data not shown), suggesting that the two lung pathologies are not intrinsically linked, at least at the earlier time points used for this study (8 weeks on diet compared with 16 weeks in the studies cited). Our findings from the

hypoxia and monocrotaline models provide additional proof to support that the effects of TRAIL are pathogenic in PAH irrespective of the stimulus and model used.

Finally given the aforementioned anti-inflammatory properties of ApoE, studies report heightened lung inflammation and pathological features of emphysema in $ApoE^{-/-}$ fed a high fat diet (Naura, Hans et al. 2009; Ouyang, Huang et al. 2015; Yao, Gordon et al. 2016). Interestingly TRAIL deficiency appears to protect mice (although not studied in $ApoE^{-/-}$ mice) from experimental COPD (Haw, Starkey et al. 2016).

4.4.5 Links between TRAIL and features of the metabolic syndrome in PAH

High fat feeding of mice induces significant vascular inflammation, glucose intolerance, insulin resistance and almost all diets (including the western but not Paigen) lead to obesity. The obesity and diabetes pandemics are widely linked to several systemic cardiovascular diseases (Reaven 2011) but are also being appreciated to play a part in pulmonary vascular disease (Fouty 2008; Benson, Pugh et al. 2012; Pugh, Newman et al. 2013; Kelley, Baust et al. 2014). Subsequent work has expanded the metabolic hypothesis of PAH by demonstrating that C57BL/6 mice with BMPR2 mutations ($BMPR2^{R899x}$) mice develop obesity and a PAH phenotype following a high fat diet (West, Niswender et al. 2013; Kelley, Baust et al. 2014). A disproportionally higher prevalence of obesity has been observed in patients with IPAH (Burger, Foreman et al. 2011). Interestingly however similar to the obesity survival paradox in heart failure a similar survival benefit for obese patients has been observed in PAH (Zafrir, Adir et al. 2013).

A consistent link between diabetes and development of vascular disease is apparent and a role for TRAIL in contributing significantly to the latter is emerging. However a growing body of experimental data suggests that TRAIL may have protective effects on the development of obesity and auto-immune type 1 diabetes (Di Bartolo, Chan et al. 2011; Bernardi, Zauli et al. 2012; Blumer and Steinberg 2012). Published data link TRAIL to protection from cytokine induced islet cell apoptosis through up regulation of the decoy TRAIL receptors R3 and R4 (Ou, Metzger et al. 2002) and Tissue inhibitor of metalloproteinase 1 (TIMP-1) (Kang, Park et al. 2010). Co-incidentally TIMP1 was shown to be up-regulated in human PAH lungs (Lepetit, Eddahibi et al. 2005) and to aggravate experimental PAH (Vieillard-Baron, Frisdal et al. 2000).

However the relationship of TRAIL with type II diabetes is less clear at present (reviewed in (Harith, Morris et al. 2013).

Thus an emerging paradigm linking insulin resistance, obesity and diabetes (spectrum of metabolic syndrome) to PAH on the one hand and my observation that TRAIL drives Paigen induced PAH on the other, contrasts with evidence suggesting that TRAIL protects from diabetes. It is not entirely clear how to reconcile these discordant experimental findings. Although Paigen HFD fed mice are hyperinsulinaemic and have a proinflammatory state they do not put on the same weight as western HFD fed counterparts because of the ketogenic properties of this diet (Chamberlain, Francis et al. 2009) . It thus may be of some interest to see whether $ApoE^{-/-}$ / $TRAIL^{-/-}$ are protected from western diet induced PAH or whether some of that protection is offset by TRAILs contribution to diabetes and obesity.

4.4.6 Lack of RVH in the Paigen diet model of PAH: Limitation or opportunity for further exploration?

The marked reduction in pulmonary hypertension (as judged by RVSP) and vascular remodeling observed in rodents with inactivated TRAIL from both hypoxia and MCT prevention studies was associated with reduced magnitude of RVH. Surprisingly however despite the greater haemodynamics, I could not demonstrate any appreciable RVH (as defined by the RV/LV+septum weight ratio) in the $ApoE^{-/-}$ mice fed Paigen. This in contrast to an elevated Fulton index (0.41+/-0.03) reported in $ApoE^{-/-}$ mice fed a western diet after developing modest PAH (RVSP 28.9+/-0.6mmHg) (Hansmann, Wagner et al. 2007).

Although this may be viewed as limitation of the Paigen model, I believe however that this observation, if representing a true biological phenomenon is potentially very important for a number of reasons. Firstly prognosis of patients in PAH (and other forms of PH) is greatly determined by right ventricular function (Sutendra and Michelakis 2013). Indices of RV performance have been shown to consistently be reliable predictors of adverse outcome even despite modern therapies in PAH (Reda E 2011; van de Veerdonk, Kind et al. 2011). Therefore identifying key mechanisms that govern favorable RV remodeling is an important step towards therapeutically augmenting RV function.

It is unlikely that methodological errors in determination of RVH (during specimen dissection and weighing) would explain the apparent lack of RVH in Paigen fed ApoE$^{-/-}$ mice because I used a standardized approach in our lab with which I have observed RVH in non diet models of PH (e.g. c57BL/6 mice exposed to hypoxia). Furthermore given the aforementioned paradox I subsequently noted that ApoE$^{-/-}$ mice placed in hypoxia for 2 weeks developed RVH (RV/LV+S=0.36, vs 0.25 in normoxia, n=3, data not shown) suggesting that this strain of mouse per se does not lack the ability to develop RVH but perhaps that it may be modulated by the Paigen diet. This observation has been independently confirmed recently in female ApoE$^{-/-}$ mice developing PAH and RVH (RV/LV+S=0.33) following monocrotaline injection. The PAH and RVH were severe in older female mice (RVSP 63+/- 10mmHg, RV/LV+S =0.53) (Umar, Partow-Navid et al. 2017) Interestingly studies in isolated cardiomyocytes from hypercholesterolaemic ApoE $^{-/-}$ mice (fed a western diet) have shown them to be more resistant to the effects of ischaemia reperfusion injury (Dworschak, d'Uscio et al. 2005). Furthermore we reported a modest degree of RVH (equivalent to levels seen with hypoxia) in ApoE$^{-/-}$/IL1R1$^{-/-}$ mice fed Paigen which we have shown to develop a severe PAH phenotype (mean RVSP 75mmHg yet RVH ~0.31) compared to ApoE$^{-/-}$ when fed Paigen (Lawrie, Hameed et al. 2011). Additionally we noted that this strain along with ApoE$^{-/-}$ mice developed only mild RV dilatation (as measured by echo and MRI) yet had preserved RV contractility (Lawrie, Hameed et al. 2011). Finally recent studies from our lab in ApoE$^{-/-}$ mice fed Paigen diet have confirmed similar PH haemodynamics whilst again confirming no apparent RVH (Renshall, Arnold et al. 2018).

Currently I am unable to explain this apparent paradox. However given that RVH is a compensatory response by cardiomyocytes in the face of an increased afterload (PVR) to preserve/augment ventricular performance, it is tempting to hypothesise that in this model the high fat diet (specifically Paigen) may favorably modulate myocardial substrate metabolism and energetics such that the need for increasing myocyte size is delayed or even not required.

Some data would lend support to this because a ketogenic diet (which is essentially what the Paigen diet is) has been shown to preserve myocardial fatty acid oxidation (the preferential energy substrate in adult mammalian hearts) in mice (Wentz, d'Avignon et al. 2010) whereas a western diet has been shown induce abnormal myocardial fatty acid oxidation and contractile dysfunction in rats (Wilson, Tran et al. 2007). These findings may explain the higher degree of RVH with a lower RVSP in ApoE$^{-/-}$ fed a western diet compared to our Paigen fed model. There is also evidence to support beneficial effects of a high fat diet on attenuating the development of left ventricular hypertrophy and dysfunction (Okere, Young et al. 2006; de Wilde, Mohren et

al. 2008; Chess, Khairallah et al. 2009; Berthiaume, Bray et al. 2010; Christopher, Huang et al. 2010).

Deranged RV myocardial energetics have increasingly being recognised in PAH whereby a switch to glycolysis from oxidative phosphorylation has been observed (Piao, Fang et al. 2010; Sutendra, Bonnet et al. 2010; Hemnes, Brittain et al. 2013; Ryan, Piao et al. 2013; Sutendra and Michelakis 2014). Finally our findings in the $ApoE^{-/-}/IL1R1^{-/-}$ mice lend further support to the idea that a favorable state of myocardial substrate utilization may be occurring in the hearts of $ApoE^{-/-}$ mice because cardiac function is preserved with only a small degree of RV hypertrophy in the face of a disproportionally higher pressure overload. Interestingly there is evidence of impaired RV myocardial fatty acid oxidation and lipotoxicity in PAH (Hemnes, Brittain et al. 2013; Talati and Hemnes 2015; Brittain, Talati et al. 2016; Talati, Brittain et al. 2016).

4.4.7 Diverse effects of TRAIL reflect our incomplete understanding of biology

The incompletely characterized yet diverse functions of TRAIL and in particular its immunomodulatory properties along with disease context may partly explain some of the variation in the observed findings (Falschlehner, Schaefer et al. 2009; Benedict and Ware 2012). Furthermore non-canonical TRAIL signaling is slowly being appreciated and may likely account for the diverse pathophysiological effects of the TRAIL signaling system (Azijli, Weyhenmeyer et al. 2013; Lalaoui and Silke 2017). Finally as discussed in Chapter 1 (p.51) splice variants of TRAIL have been described yet the precise function and expression of these is still incomplete and their contribution to the apparently diverse effects of TRAIL have not been studied.

4.5 CHAPTER SUMMARY

In this chapter I have demonstrated that TRAIL plays a pathogenic role in the development of PAH using three rodent models of PAH. The trigger for PAH in these models vary, yet the pathophysiological mechanisms, albeit incompletely understood, share in common well accepted abnormalities in PAH; endothelial dysfunction, pro-inflammatory response, dysfunctional nitric oxide signaling and aberrant cell growth. The next crucial question is whether targeting of TRAIL offers any therapeutic potential for halting or reversing disease in humans with PAH, in whom disease is often diagnosed at an advanced stage.

CHAPTER 5: REVERSING PAH WITH AN ANTI-TRAIL ANTIBODY

5.1 INTRODUCTION

In the previous chapter I demonstrated through pharmacological inhibition, genetic deletion and over expression, that TRAIL was necessary for the development of disease in three rodent models of disease (monocrotaline, hypoxia and the Paigen diet).

I next sought to determine if inhibiting TRAIL would be beneficial in retarding disease progression or ideally induce disease regression in rodents with established disease. The importance of this hypothesis is very pertinent to the development of clinical therapies in PAH because, as discussed in chapter 1, patients with PAH often continue to have a poor survival despite the development of pulmonary vasodilator therapies over the past decade. The unfavorable prognosis is due to a number of reasons and include the insidious yet aggressive nature of the disease, delayed diagnosis in most patients (and thus presentation at clinically and pathologically advanced stages of disease) and finally because currently available therapies do not sufficiently alter aberrant cellular growth which continues to occur in patients despite treatment with pulmonary vasodilators. Thus there remains an unmet need for "anti-remodeling "therapy in PAH.

Given the potential contribution of TRAIL to the development of PAH, I hypothesised that TRAIL could potentially be a novel therapeutic target for reversing PAH. Thus in my next series of investigations I sought to establish whether an anti-TRAIL antibody would

1) Improve survival and slow disease progression in rats with established MCT PAH.
2) Rescue ApoE$^{-/-}$ mice from severe Paigen diet induced PAH.

5.2 MATERIALS AND METHODS

Experimental protocols for the rodent studies are provided in this section. Detailed methods on rodents, diets, interventions and phenotyping are provided in chapter 2 of the thesis.

5.2.1 Monocrotaline disease reversal protocol

To investigate the effects of blocking TRAIL on halting disease progression and prolonging survival a reversal study was performed (Fig 5.1A schematic). Male Sprague Dawley rats (start weight 200-240g, n=12) received a single subcutaneous injection of monocrotaline (MCT) at 60mg/kg (Section 2.1). After 21 days, they were randomly assigned (n=6/gp) to implantation of an osmotic pump (Alzet® 2002 mini-pump, 200µl reservoir, 0.5µl/hr delivery rate for 2 weeks) containing either a polyclonal goat IgG anti-mouse TRAIL antibody (anti-TRAIL, #AF1121) or an isotype antibody as control (Goat IgG isotype, #AB-108-C, both R&D systems, Europe). Both interventions were delivered for 14 days (≈84ng/hr or ≈0.4ng/hr/g for active treatments and 100ng/hr for control). Disease phenotyping was performed when rats displayed outward signs of overt right heart failure (RHF) at which point they were euthanised. Overt RHF was defined by loss of body weight (either >5%/24h or >10%/48h), lethargy, cyanosis and/or respiratory distress as previously described (Merklinger, Jones et al. 2005; de Man, Handoko et al. 2012).

5.2.2 Disease reversal protocol in ApoE$^{-/-}$ fed Paigen diet.

ApoE$^{-/-}$ mice (8-12 weeks old, n=12) were fed a high fat diet containing Cholate (Paigen diet) for 8 weeks. After 8 weeks, mice were implanted with subcutaneous osmotic pumps (Alzet® 1004 micro pump, Durect Corp, U.S) and randomly assigned to treatment with either an anti-TRAIL antibody (as above) at 20ng/hr (~0.8ng/h/g) or placebo (equivalent volume of phosphate buffered Saline-PBS). Mice were fed Paigen diet for a further 4 weeks before undergoing standard phenotyping after a total of 12 weeks on diet (see Fig 5.2A for schematic illustration). Where indicated mice that displayed outward signs of RHF were euthanized.

5.2.3 Phenotyping: Haemodynamics, RVH and morphometric lung analysis.

Details for each component of the phenotypic evaluation of rodents are provided in chapter 2. Where indicated Pulmonary artery acceleration time (PAAT), which is inversely related to PA pressure, was measured by transthoracic echocardiography prior to invasive haemodynamic assessment (section 2.5). Closed chest cardiac catheterisation was performed with the appropriate high fidelity micromanometer catheter (pressure volume) to measure right and left heart pressures, and to derive cardiac index (section 2.6). Estimation of right ventricular hypertrophy (RVH), lung histology (ABEVG) immunohistochemistry (for α-SMA, vWF, TRAIL, PCNA and TUNEL) were performed on paraffin embedded lung sections (5µm thick) as described (Sections 2.7-2.9). The degree of pulmonary arteriolar remodeling (ratio of media to cross sectional area and percentage vessels thickened) and, where indicated levels of vascular proliferation and apoptosis were quantified as described (section 2.10).

5.3 RESULTS

5.3.1 Treatment with an anti-TRAIL antibody improves survival and partially slows disease progression in rats with established MCT induced PAH.

Rats were injected with MCT and allowed to develop PAH. 21 days later they were assigned to therapy with either a mouse anti-TRAIL antibody or IgG isotype control antibody (~0.4ng/g/hr). Antibodies were delivered for 14days via osmotic mini pumps **(Fig. 5.1A).** Rats were euthanised when they displayed signs of RHF (between days 26-36) as defined in section 5.2.1. Compared to controls, rats treated with an anti-TRAIL antibody had a significantly improved survival **(Fig.5.1B)**. I did not observe any significant differences in cardiac haemodynamics **(Fig. 5.1 C-G)** or right ventricular hypertrophy **(Fig. 5.1J)** between the two groups, although anti-TRAIL treated rats displayed a non-significant trend for a lower ePVRI **(Fig. 5.1G)** and higher cardiac index **(Fig 5.1H).** They did however demonstrate a modest but significant reduction in pulmonary vascular remodeling as measured by the media/cross-sectional area in pulmonary arterioles **(Fig. 5.1K)** This was associated with significant quantitative reduction in levels of cellular proliferation **(Fig. 5.1L)** although no significant effect was demonstrable on apoptosis as measured by TUNEL **(Fig. 5.1M).**

<u>Figure 5.1</u>- Anti-TRAIL antibody improved survival and pulmonary arteriolar remodelling in rats with established MCT induced PAH.

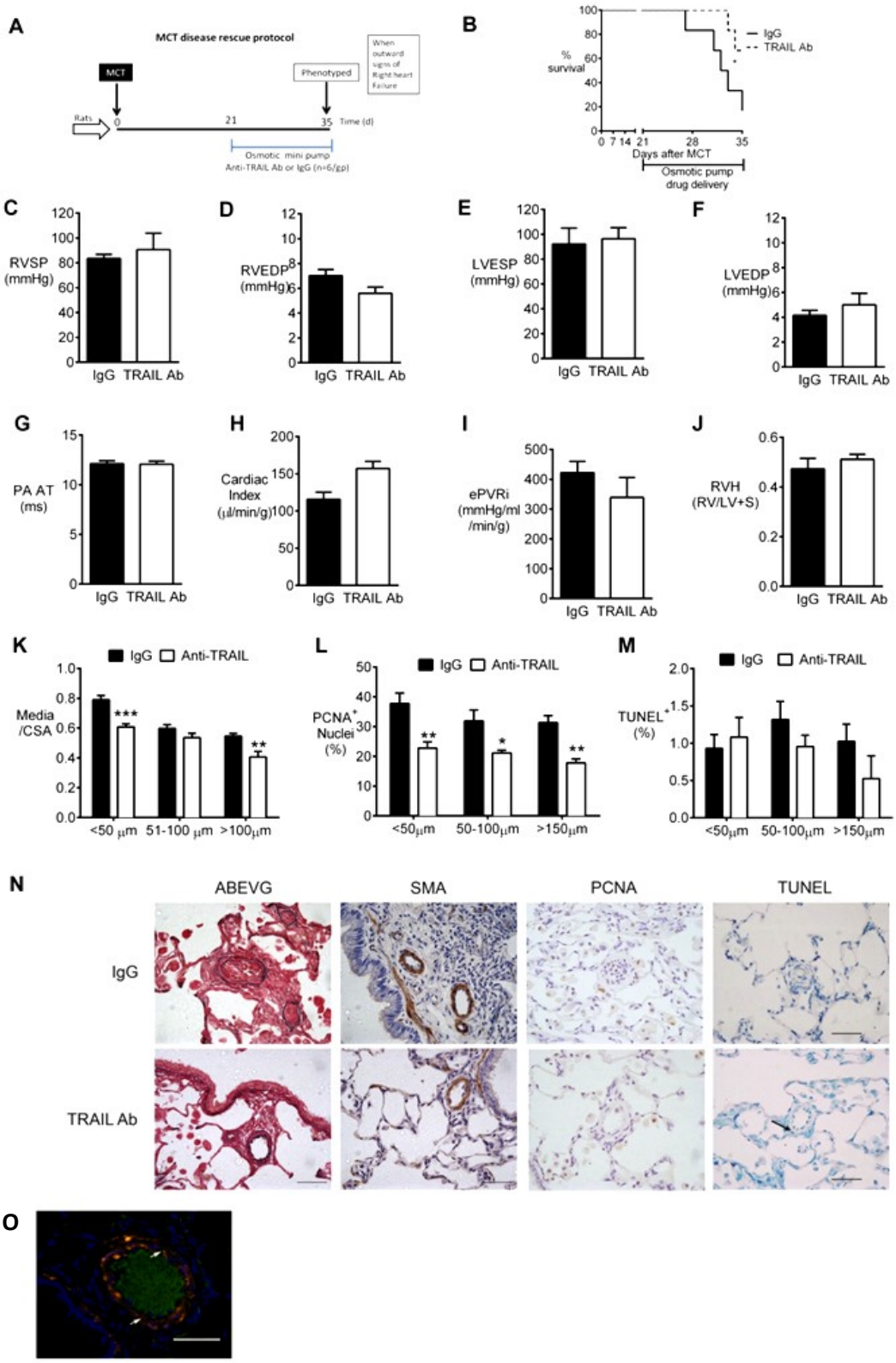


Figure 5.1- Anti-TRAIL antibody improves survival and pulmonary arteriolar remodelling in rats with established MCT induced PAH.

Schematic diagram of the study protocol is shown in (A). (B) Kaplan-Meier plot of survival in IgG and anti-TRAIL–treated rats. (C–E) Bar graphs show RVESP (C), RVEDP (D), LVESP (E), and LVEDP (F), measured in mm Hg. (G–I) PA AT (G), cardiac index (H), ePVRi (I), and RVH (J). (K) The degree of medial wall thickness as a ratio of total vessel size (Media/CSA). (L and M) Quantification of the percentage of proliferating cells (PCNA positive; L), and apoptotic (TUNEL positive; M) separated into pulmonary arteries <50 μm in diameter, vessels from 51 to 100 μm in diameter, and vessels >100 μm in diameter. (N) Representative photomicrographs of serial lung sections from MCT-injected rats treated with either IgG isotype control or an anti-TRAIL antibody. Sections were stained with Alcian Blue Elastic Van-Gieson (ABEVG) or immunostained for α-SMA, PCNA, or TUNEL. (O) Representative confocal microscopy image showing apoptotic cells (green) and SMC (red) with dual-positive cells (yellow) highlighted by white arrows. **Error bars represent mean ± SEM, *n* = 6 animals per group. *, P < 0.05; **, P < 0.01; ***, P < 0.001 compared with IgG-treated MCT-injected rats. Arrows point to TUNEL-positive cells. Bars, 50 μm. All images are presented at their original magnification x400, scale bar represents 50 μm.**

5.3.2 Treatment with an anti-TRAIL antibody reverses haemodynamics and pulmonary arteriolar remodeling in ApoE-/- mice with Paigen diet induced PAH

Data from the rat reversal study suggested that there was partial slowing of disease progression, but not reversal with anti-TRAIL treatment. I next examined whether we could achieve a greater response and induce disease regression using the ApoE$^{-/-}$ mouse model. This approach had two advantages: (1) use of species-specific antibody and (2) implantation of a micro-pump allowing delivery of a twofold higher dose (~0.8 vs. 0.4 ng/h/g) of the anti-TRAIL antibody for twice the duration (4 wk).

ApoE$^{-/-}$ mice were fed a Paigen diet for 8 weeks and then randomly assigned to therapy with either a mouse anti-TRAIL antibody or IgG isotype control antibody (~0.8ng/g/hr). Antibodies were delivered for 4 weeks via osmotic micro pumps whilst remaining on the diet **(Fig. 5.2A).** Paigen fed ApoE$^{-/-}$ mice treated with IgG control, now 12 weeks on diet, displayed even higher RVESP compared to those after 8 weeks on diet (85+/- 25 vs 50+/-3.5 mmHg, **Fig. 5.2B & Fig 4.4B**). Two mice (one from each group) were euthanized before the 12 week period (at week 10) due to illness, but their data was included in the final analyses as per the assigned group so as not bias any effects of treatment.

ApoE$^{-/-}$ mice treated with control IgG isotype developed severe PAH, as defined by increased RVESP (85+/-25mmHg, **Fig.5.2B**), RVEDP **(Fig. 5.2C),** reduced PAAT **(Fig. 5.2D),** cardiac index **(Fig. 5.2E)** and increased ePVRi **(Fig. 5.2F)**. Remarkably despite very high RVESP with levels similar to systemic (LV) pressures control treated mice again did not develop RVH **(Fig. 5.2G-I)** as discussed in chapter 4 (Section 4.4)

In stark contrast mice treated with anti-TRAIL had no significant evidence of a PAH phenotype as judged by significantly reduced RVESP, to near normal levels (30+/-3 mmHg, **Fig. 5.2B**), RVEDP **(Fig. 5.2C)** and ePVRi **(Fig. 5.2F)**. A trend for higher cardiac Index was also observed **(Fig. 5.2E)**. No significant differences were noted in left ventricular pressures **(Fig. 5.2 G-H)**.

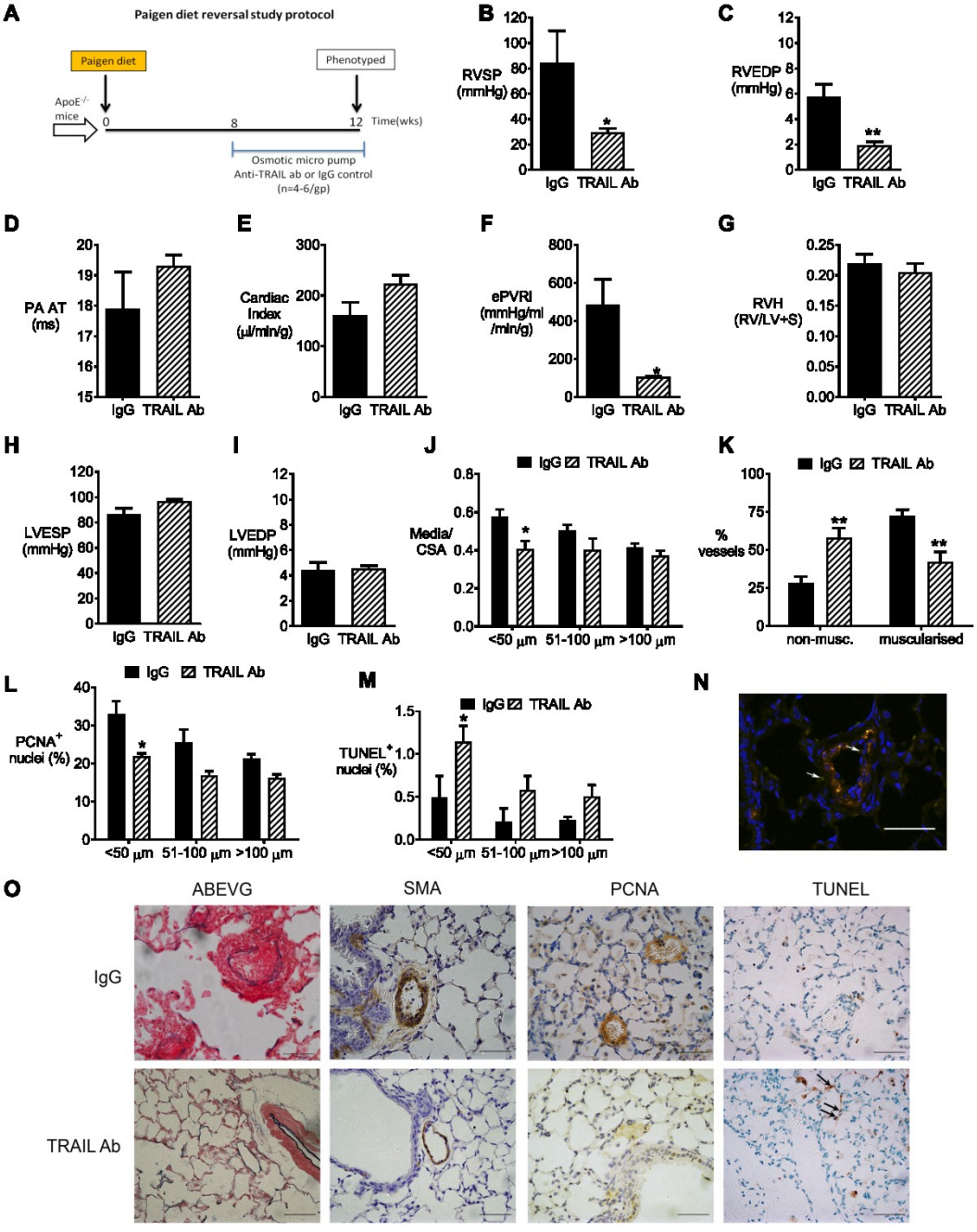
Reversal of right heart haemodynamics observed in anti-TRAIL treated mice was associated with a significant reduction in pulmonary vascular remodeling. Anti-TRAIL treatment led to significant reduction in medial hypertrophy **(Fig. 5.2 J, O)** and the proportion of muscularised arterioles **(Fig. 5.2 K and O)** in the smallest pulmonary arterioles (<50μm). Similar to the rat MCT reversal study there was also a significant reduction in vessel proliferation as measured

by levels of immunostaining for PCNA **(Fig. 5.2L and O).** However unlike the MCT reversal study, anti-TRAIL treatment in was associated with increased levels of apoptotic cells within pulmonary arterioles **(Fig. 5.2M and O)** as was observed in the prevention studies described in chapter 4. Confocal microscopy identified that remaining apoptotic cells present within the media of the small pulmonary arteries were SMA positive **(Fig. 5.2N).**

Figure 5.2: Anti-TRAIL antibody treatment of established PAH in the Paigen diet–fed ApoE$^{-/-}$ mouse induces disease regression.

Schematic diagram of the study protocol is shown in (A) Bar graphs show (B) right ventricular end-systolic pressure (RVSP) (C) right ventricular end-diastolic pressure (RVEDP), measured in mm Hg. (D) Pulmonary artery Acceleration time (PA AT), (E) cardiac index, (F) estimated Pulmonary Vascular Resistance index (ePVRi) and (G) right ventricular hypertrophy (RVH). (H) left ventricular end-systolic pressure (LVESP) (I) left ventricular end-diastolic pressure (LVEDP) measured in mmHg, (J) The degree of medial wall thickness as a ratio of total vessel size (Media/CSA), (K) relative percentage of muscularised small pulmonary arteries and arterioles in <50 µm vessels, quantification of the percentage of (L) proliferating cells (PCNA positive), and (M) apoptotic (TUNEL positive) separated into pulmonary arteries less than 50 µm (<50 µm) in diameter, vessels from 51 to 100 µm (51-100 µm) in diameter and vessels larger that 100 µm (>100µm) in diameter. (O) Representative photomicrographs of serial lung sections from ApoE$^{-/-}$ mice fed on Paigen diet for 12 weeks. Sections were stained with Alcian Blue Elastic van Gieson (ABEVG) or immunostained for α-smooth muscle actin (α-SMA), proliferating cell nuclear antigen (PCNA) or Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). (N) Representative confocal microscopy image showing apoptotic cells (green) and SMC (red) with dual positive cells (yellow) highlighted by white arrows. **Bars represent mean +/- SEM, n=4-6 animals per group, *=p<0.05, **=p<0.01, compared IgG treated Paigen diet fed mice. Arrows point to TUNEL positive cells. All images are presented at their original magnification x400, scale bar represents 50 µm.**

<u>Figure 5.2:</u> Anti-TRAIL antibody treatment of established PAH in the Paigen diet–fed ApoE$^{-/-}$ mouse induces disease regression.



5.4 DISCUSSION

In this chapter I have demonstrated that inhibiting TRAIL with a mouse antibody led to:

1) Improved survival, reduced medial hypertrophy and proliferation in small pulmonary arterioles in rats with established MCT induced PAH.

2) Rescue of $ApoE^{-/-}$ mice from Paigen diet induced severe PAH as judged by normalization of right heart haemodynamics, significantly reduced vessel remodeling associated with reduced proliferation and increased apoptosis within small pulmonary arterioles.

5.4.1 The Challenge for developing new drugs in PAH

The holy grail of pharmacological therapy in PAH is disease reversal. None of the currently available drugs to date have been shown to favorably alter the pathology of this disease significantly. In 1996 i.v Prostacyclin was shown to improve survival in PAH (Barst , Rubin et al. 1996). Despite lacking a clear mechanism of action it has been a welcomed addition for improving survival for many, but not all patients (Barst 2010). Moreover there is evidence that the subsequent generation of targeted oral pulmonary vasodilator therapies improve short term prognosis in patients with PAH (approx 2% absolute risk reduction) (Galie, Manes et al. 2009).

Somewhat surprisingly though, post mortem studies in patients with PAH treated with "modern therapies" such as prostacyclin analogues, endothelin and PDE5 receptor antagonists reveal progressive pathological changes despite treatment (neointimal proliferation, plexiform lesions and perivascular inflammation) (Rich, Pogoriler et al. 2010; Pogoriler, Rich et al. 2012; Stacher, Graham et al. 2012). Thus the debate regarding when (rather than if), the stage at which this invariably progressive and fatal disease becomes irreversible (pharmacologically at least) still continues (Wagenvoort 1988; Sakao, Tatsumi et al. 2010; West 2011; Dorfmüller and Humbert 2012).

For many patients the use of combination pulmonary vasodilator therapies, including intravenous prostacyclin analogs has allowed patients to either no longer require transplantation or "buy" additional time before transplantation. However transplantation

remains a destination therapy for a significant number of patients with PAH either because they are inadequate clinical responders and/or continue to deteriorate. Currently double lung (+/- heart) transplantation offers the only promise of normalising pulmonary haemodynamics and improving RV function. However clinical care before and after transplantation still remains complex and challenging in many ways. In addition there remains a shortage of suitable long donors, stringent listing criteria, significant mortality whilst on the waiting list and transplantation is associated with adverse outcomes in the first year. Even amongst first year survivors, 5 year and 10 year survival is approximately 50% and 35% respectively (Gottlieb and A. Corris 2012).

Although the role for transplantation is undoubted and active efforts to increase organ donation promising, there remains an unmet clinical need to further improve the medical management of the disease. Identification of key cellular and molecular targets causally linked to pulmonary vascular remodeling should lead to the development of novel classes of therapy that can fundamentally alter disease biology and improve clinical outcomes.

5.4.2 Effects of blocking TRAIL in experimental PAH

Building on the evidence provided in earlier chapters of this thesis supporting a pathogenic role for TRAIL in human PAH, PASMC proliferation *in-vitro* and in preclinical models the next and probably most challenging task was to determine if inhibiting TRAIL could halt progression or reverse disease.

In the MCT rat model I have showed that a goat anti-mouse TRAIL antibody was able to improve survival, reduced medial hypertrophy and cellular proliferation but did not significantly improve catheter haemodynamics. These data suggested there was partial disease slowing. However in mice the same antibody displayed impressive efficacy. ApoE$^{-/-}$ mice fed Paigen diet for 12 weeks developed severe PAH whilst anti-TRAIL treated mice had near normal haemodynamics (mean RVESP≈ 85mmHg vs 29mmHg). This greater efficacy was likely a result of a higher dose and use of species specific antibody in the mouse. As highlighted in chapter 4 the Paigen model of PAH displays more pathological features of human PAH compared to the MCT model.

5.5 CHAPTER SUMMARY

The data presented in this chapter provide evidence for disease slowing in the MCT rat and disease reversal in the Paigen mouse model and extend the pathogenic role for TRAIL identified in earlier chapters and highlight potential for pursuing TRAIL inhibition as a rational therapeutic strategy for exploration in human PAH.

CHAPTER 6: NON BONE MARROW DERIVED CELL TRAIL DRIVES PAH PATHOGENESIS.

6.1 INTRODUCTION

Inflammatory and immune mechanisms are increasingly being recognised in the pathogenesis of PAH (Perros, Dorfmuller et al. 2007; Tamosiuniene, Tian et al. 2011; Ormiston, Chang et al. 2012; Price, Wort et al. 2012; George, Oliver et al. 2014; Rabinovitch, Guignabert et al. 2014). TRAIL expression can be upregulated on several immune/inflammatory cells including Lymphocytes (Ehrlich, Infante-Duarte et al. 2003) Monocytes (Griffith, Wiley et al. 1999; Wei, Wang et al. 2010) dendritic cells (Fanger, Maliszewski et al. 1999) and NK cells (Kayagaki, Yamaguchi et al. 1999) particularly after stimulation by interferon (Kayagaki, Yamaguchi et al. 1999). Furthermore TRAIL is upregulated on endothelial, vascular smooth muscle and PA smooth muscles cells as already described. Given that TRAIL is expressed by a variety of circulating inflammatory and vascular cells, both of which have been implicated in PAH it would be mechanistically valuable to identify the major source/location of TRAIL which I have shown to drive disease pathogenesis. Such information could aid in refining the development of targeted therapies to TRAIL and for example may help govern whether to best deliver them systemically or locally (e.g. as nebulised therapy).

Thus my aim in this final series of investigations was to determine the relative importance of tissue expression of TRAIL compared to that from bone marrow derived cells (BMDC) in the pathogenesis of PAH. Therefore studies were performed in TRAIL chimeric mice created after bone marrow transplantation in the setting of the Paigen fed $ApoE^{-/-}$ model of severe PAH.

6.2 MATERIALS AND METHODS

Experimental protocols for the rodent studies are provided in this section. Detailed methods on rodents, diets, interventions and phenotyping are provided in chapter 2 of the thesis.

6.2.1 Bone marrow transplantation (BMT) protocol

We used a BMT protocol that has been successfully used in our department (Chamberlain, Evans et al. 2006; Evans, Jackman et al. 2009). Young male ApoE$^{-/-}$ and ApoE$^{-/-}$/TRAIL$^{-/-}$ donor mice, aged 4-6 weeks were sacrificed and their femurs and tibias were harvested under sterile conditions in a class II laminar flow hood. Marrow from the bones was flushed (under sterile conditions) with RPMI-1640 media (containing sodium bicarbonate, 10% (V/V) fetal bovine serum-FBS, but no phenol red) with a 26G needle and syringe. The cell-media solution was passed through a 40 μm cell strainer and then centrifuged at 500G for 5 minutes. The supernatant was removed and the cell pellet re-suspended in Hank's Buffered Salt Solution (HBSS) containing 10% (v/v) FBS and placed on ice until the tail vein injections. Cells were counted on a haemocytometer, using 1% (v/v) acetic acid to lyse the red blood cells. The average yield from each preparation produced was approximately 20 x 10^6 cells/ml.

Recipient male ApoE$^{-/-}$ and ApoE$^{-/-}$/TRAIL$^{-/-}$ mice, aged 6-8 weeks, received sub lethal whole body irradiation (Cesium 137) totaling 11 Grays (1100 rads) split into two doses, 4 hours apart. Irradiated mice were then injected with 200 μL of bone marrow cells via the tail vein. All mice were housed individually in ventilated cages in rooms dedicated for post BM transplanted mice. They were fed standard chow for six weeks and also received neomycin (1mmol/L final concentration) and polymyxin B (1000 USP units/mL) in drinking water as antimicrobial prophylaxis. Four groups of chimeric mice were created and are summarised in the table 6.1.

6 weeks later , following bone marrow reconstitution on regular chow, mice were assigned to either chow or Paigen diets (8 experimental groups) for a further 8 weeks before undergoing standard phenotypic evaluation as described (See **Fig. 6. 1 A** for schematic illustration).

BMT Group	Donor Genotype	Recipient Genotype	TRAIL Expression		Objective
			BM Cells	Tissue (non-BM)	
1	$ApoE^{-/-}$	$ApoE^{-/-}$	+	+	Positive control (when fed Paigen)
2	$ApoE^{-/-}$	$ApoE^{-/-}/TRAIL^{-/-}$	+	-	Is BM derived TRAIL important for developing PAH?
3	$ApoE^{-/-}/TRAIL^{-/-}$	$ApoE^{-/-}$	-	+	Is tissue (lung) TRAIL important for developing PAH?
4	$ApoE^{-/-}/TRAIL^{-/-}$	$ApoE^{-/-}/TRAIL^{-/-}$	-	-	Negative control

Table 6.1. Bone marrow chimeric mice groups studied

6.2.2 Phenotyping: Haemodynamics, RVH and morphometric lung analysis.

Details for each component of the phenotypic evaluation of rodents are provided in chapter 2. Where indicated Pulmonary artery acceleration time (PAAT), which is inversely related to PA pressure, was measured by transthoracic echocardiography prior to invasive haemodynamic assessment (section 2.5). Closed chest cardiac catheterisation was performed with the appropriate high fidelity micromanometer catheter (pressure volume) to measure right and left heart pressures, and to derive cardiac index (section 2.6). Estimation of right ventricular hypertrophy (RVH), lung histology (ABEVG) immunohistochemistry (for α-SMA, vWF, TRAIL) were performed on paraffin embedded lung sections (5μm thick) as described (Sections 2.7-2.9). The degree of pulmonary arteriolar remodeling (ratio of media to cross sectional area and percentage vessels thickened) was quantified as described (section 2.10).

6.2.3 TRAIL expression (mRNA and protein)

qPCR was performed for TRAIL gene expression and western Immunoblotting for TRAIL protein on whole lung homogenates as described in chapter 2.

6.3 RESULTS

6.3.1 TRAIL expression in ApoE$^{-/-}$ and double knockout mice

Taqman PCR was used to measure gene expression on circulating cells isolated from whole blood RNA (non-irradiated mice) and confirmed expression of TRAIL in ApoE$^{-/-}$ mice and its absence in ApoE$^{-/-}$/TRAIL$^{-/-}$ mice **(Fig. 6B).** TRAIL gene expression was increased in non-irradiated Paigen fed ApoE$^{-/-}$ mice compared to those on chow **(Fig. 6B).** Western Immunoblotting demonstrated a similar pattern of expression for TRAIL protein in lung tissue from recipient ApoE$^{-/-}$ and ApoE$^{-/-}$/TRAIL$^{-/-}$ mice **(Fig. 6C).**

6.3.2 Tissue TRAIL (lung) is a major driver of disease pathogenesis in PAH

Four groups of chimeric mice were generated by BM transplantation (BMT). ApoE$^{-/-}$ BM was transplanted (BMT) into sub lethally irradiated ApoE$^{-/-}$ /TRAIL$^{-/-}$ mice (ApoE$^{-/-}$ into ApoE$^{-/-}$ /TRAIL$^{-/-}$) to produce chimeras where TRAIL was only expressed within circulating BMDC, and into ApoE$^{-/-}$ mice as a positive control for the procedure (ApoE$^{-/-}$ into ApoE$^{-/-}$). Conversely, ApoE$^{-/-}$ /TRAIL$^{-/-}$ BM was transplanted into sublethally irradiated ApoE$^{-/-}$ mice (ApoE$^{-/-}$ /TRAIL$^{-/-}$ into ApoE$^{-/-}$) to generate chimeras where TRAIL was only expressed in non BMDCs and thus lung tissue and into ApoE$^{-/-}$ /TRAIL$^{-/-}$ (ApoE$^{-/-}$ /TRAIL$^{-/-}$ into ApoE$^{-/-}$ /TRAIL$^{-/-}$) mice as a negative control. 6 weeks after BM reconstitution, mice were randomly placed on either regular chow or Paigen diet. The conversion rate of BMT was 94.9 ± 2.6%. Chow-fed mice from all BMT groups served as a control for the irradiation and transplant procedure and displayed no PH phenotype **(Fig. 6 D-K).**

ApoE$^{-/-}$ into ApoE$^{-/-}$ mice fed Paigen diet developed PAH as evidenced by increased RVESP **(Fig. 6D)** , ePVRi **(Fig. 6I)** and an increase in both frequency **(Fig. 6K)** and the degree **(Fig 6. L-M)** of pulmonary arteriolar thickening. In contrast ApoE$^{-/-}$/TRAIL$^{-/-}$ into ApoE$^{-/-}$/TRAIL$^{-/-}$ fed Paigen were protected from PAH and collectively these findings recapitulated those I observed in non-irradiated Paigen fed ApoE$^{-/-}$ and ApoE$^{-/-}$/TRAIL$^{-/-}$ **(Ch 4, Fig. 4.4).** They also confirmed that these two BMT groups were valid positive and negative controls respectively.

The remaining two groups of chimeric mice with expression of TRAIL limited to either tissue or circulating BMDC displayed disease symptoms after feeding of the Paigen diet and were sacrificed for analysis at 6 weeks, rather than 8 week time point. Mice with TRAIL on

circulating BMDC, but not in tissue (ApoE into ApoE$^{-/-}$/TRAIL$^{-/-}$) displayed a trend for a raised RVESP **(Fig. 6 D)** and ePVRI **(Fig. 6I)** however this did not reach statistical significance. Although these mice did have significant increase in vessel remodeling **(Fig. 6K)** this was insufficient to raise RVESP. Conversely chimeric mice with no TRAIL on circulating BMDCs but TRAIL within tissue (ApoE$^{-/-}$/TRAIL$^{-/-}$ into ApoE$^{-/-}$) developed a significant increases in RVESP **(Fig. 6D)** , ePVRI **(Fig. 6I)** with corresponding increases in the number of muscularised arterioles **(Fig. 6K)** as well as the degree of medial hypertrophy in pulmonary arterioles with diameters of <50µm **(Fig. 6L)** and between 50-100µm **(Fig. 6M).** The vessel remodeling was significantly greater that observed in negative control and ApoE$^{-/-}$ into ApoE$^{-/-}$/TRAIL$^{-/-}$ mice whilst comparable to levels observed in the positive control group**.** A trend for reduced cardiac index was also noted similar to the positive control group **(Fig.6H)**

As noted previously no differences in left ventricular haemodynamics were observed across all groups (**Fig. 6F-G)** and despite developing PAH those mice again did not develop any RVH, further suggesting that these mice are (favorably) resistant to the development of RVH **(Fig. 6J).**

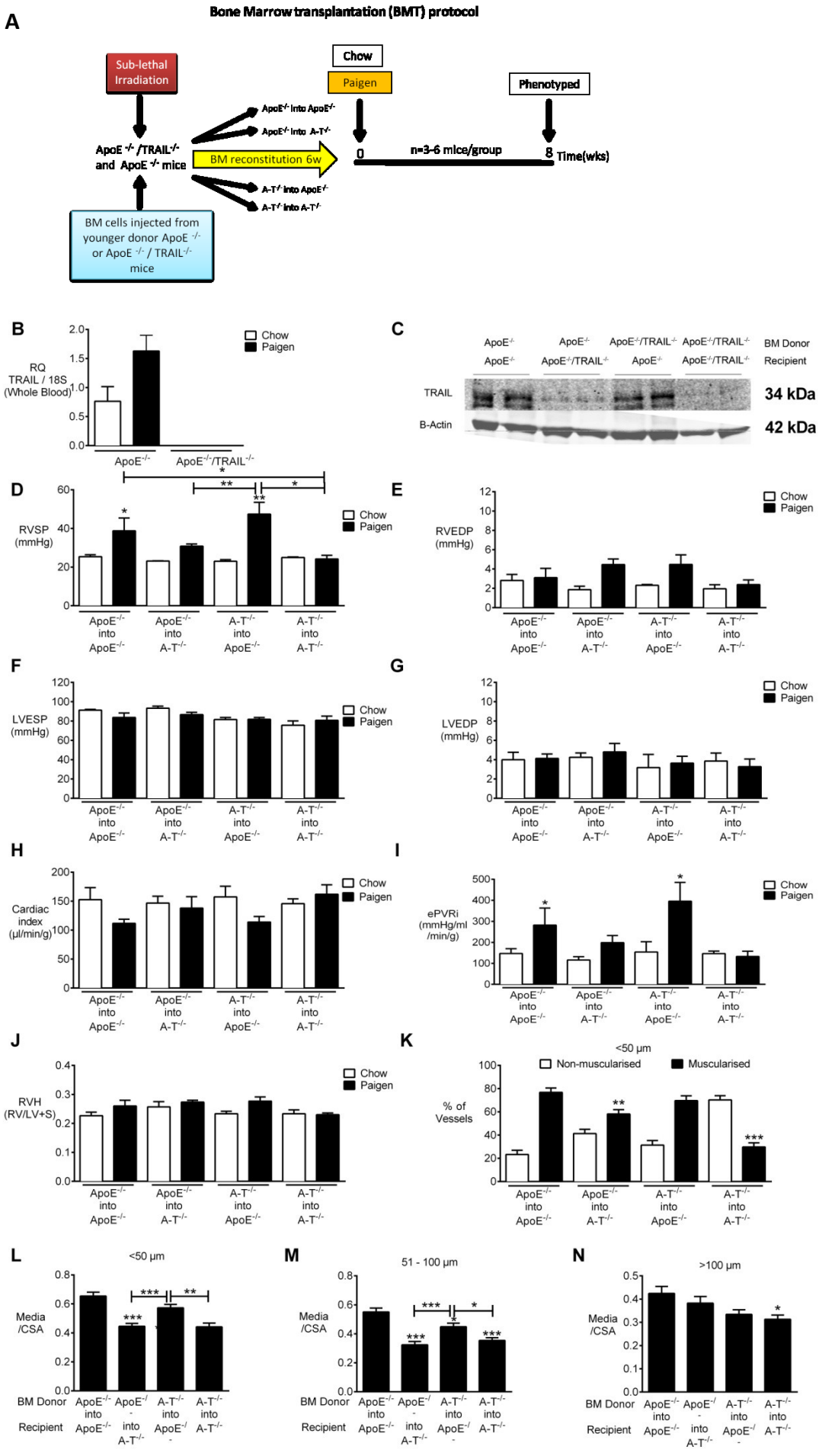


Figure 6- For legend see next page

Fig. 6 Non bone marrow (immune) derived cellular TRAIL drives PAH in the Paigen diet–fed ApoE$^{-/-}$ mouse.

Schematic diagram of the study protocol is shown in (A) TaqMan expression of TRAIL in whole blood from ApoE$^{-/-}$ and ApoE$^{-/-}$/TRAIL$^{-/-}$ mice using ΔΔCT with 18S rRNA as the endogenous control gene (B). Four groups of chimeric mice were generated by BMT. ApoE$^{-/-}$ BM was transplanted into irradiated ApoE$^{-/-}$/TRAIL$^{-/-}$ mice (ApoE$^{-/-}$ into ApoE$^{-/-}$/TRAIL$^{-/-}$) to produce chimeras where TRAIL was only expressed within circulating cells, and into ApoE$^{-/-}$ mice as a positive control for the procedure (ApoE$^{-/-}$ into ApoE$^{-/-}$). Conversely, ApoE$^{-/-}$/TRAIL$^{-/-}$ BM was transplanted into irradiated ApoE$^{-/-}$ mice (ApoE$^{-/-}$/TRAIL$^{-/-}$ into ApoE$^{-/-}$) to generate chimeras where TRAIL was only expressed within the vessel wall, and into ApoE$^{-/-}$/TRAIL$^{-/-}$ (ApoE$^{-/-}$/TRAIL$^{-/-}$ into ApoE$^{-/-}$/TRAIL$^{-/-}$) mice as a negative control. (C) Western immunoblot for TRAIL in whole lung lysates from representative chimeric mice. (D–G) Bar graphs show RVESP (D), RVEDP (E), LVESP (F), and LVEDP (G), measured in mmHg. (H-J) Cardiac index (H), ePVRi (I), and RVH (J). (K) Relative percentage of muscularised small pulmonary arteries pulmonary arteries <50 μm in diameter, and the degree of medial wall thickness as a ratio of total vessel size (Media/CSA). (L–N) Pulmonary arteries <50 μm in diameter (L), vessels from 51 to 100 μm in diameter (M), and vessels >100 μm in diameter (N). **Error bars represent mean ± SEM, *n* = 3–6 animals per group. *, P < 0.05; **, P < 0.01; ***, P < 0.001, compared to chow-fed equivalent mice unless otherwise stated.**

6.4 DISCUSSION

In this chapter I have demonstrated that in chimeric mice with TRAIL restricted to tissue but not bone marrow derived cells (BMDCs) developed significant Paigen diet induced PAH, suggesting that local tissue derived TRAIL is pathophysiologically the most important.

PAH is a complex disease and numerous cell types and mediators having been implicated in its pathogenesis. Generally they can be divided into local tissue resident (lung) and circulating cells whilst accepting that communication between these two compartments is likely dynamic and fluid. Although there is no doubt that local vascular cells are crucial to the pathology of PAH, in recent years there has been growing recognition for bone marrow derived progenitor cells as contributors to vascular homeostasis and disease (Sainz and Sata 2006) including pulmonary hypertension (Frid, Brunetti et al. 2006; Stevens, Phan et al. 2008; Yeager, Frid et al. 2011). However at present the literature contains conflicting reports describing both protective and pathogenic roles for such cell types highlighting the complex nature of the pathophysiology of PAH.

Although I observed no abnormal phenotype in all groups of chow fed knockout mice, interestingly there have been several case reports of patients (mainly in paediatric and young adult patients) developing PAH and PVOD after having undergone bone marrow transplantation for hematological malignancies (Bentur, Cullinane et al. 1991; Limsuwan, Pakakasama et al. 2006; Limsuwan, Pakakasama et al. 2011; Dandoy, Hirsch et al. 2013) Furthermore patients with chronic myelofibrosis can develop PAH (Adir and Humbert 2010) and a previous report identified abnormalities in myeloid precursors in patients as well as in unaffected family members in familial PAH suggesting that there were intrinsic abnormalities of the myeloid cell lineage (Farha, Asosingh et al. 2011).

TRAIL can be expressed and produced by a number of vascular cells including vascular smooth muscle cells (Secchiero, Zerbinati et al. 2004; Kavurma, Schoppet et al. 2008) endothelial cells (Favre, Mancuso et al. 2003; Fu, Zhu et al. 2003; Viemann, Goebeler et al. 2006; O'Brien, Richardson et al. 2007) and inflammatory cells including Dendritic cells (Fanger, Maliszewski et al. 1999), monoctyes (Griffith, Wiley et al. 1999), T cells, B cells (Ehrlich, Infante-Duarte et al. 2003), mast cells (Berent-Maoz, Salemi et al. 2010) and neutrophils (Cassatella 2006) all of which have been reported to play some role in PAH. With respect to TRAIL, my findings in this chapter would support that non bone marrow derived is the predominant driver of the pulmonary arteriolar remodeling and consequent adverse haemodynamics in experimental

PAH. My data however do not completely rule out a role for BMDC derived TRAIL because mice with TRAIL expressed on BMDCs but not tissue (ApoE$^{/-}$ into ApoE$^{-/-}$/TRAIL$^{-/-}$) developed some muscularisation of pulmonary arterioles although this did not lead to a significant increase in RV haemodynamics (RVESP or ePVRI).

During these analyses, we observed that some lungs displayed signs of alveolar septa thickening and mild fibrosis, and subsequently modified Ashcroft scoring (Hubner, Gitter et al. 2008) was performed to all lung sections in a blinded fashion (kindly by Prof. M Whyte, Dept. of Academic Respiratory Medicine, University of Sheffield). Although only mild changes were observed (maximum of 2 from an 8-point scale), interestingly mice with only TRAIL on circulating cells (ApoE$^{-/-}$ into ApoE$^{-/-}$ /TRAIL$^{-/-}$) had no fibrosis and all scored 0 on the modified Ashcroft score (data not shown). These ancillary findings are noteworthy given that a protective role for TRAIL in experimental drug induced lung fibrosis has been reported (McGrath, Lawrie et al. 2012).

TRAIL is a type II transmembrane protein that is predominantly surface bound but can also be cleaved to produce an active soluble form and exert a paracrine effect (Mariani and Krammer 1998; Schneider, Holler et al. 1998; Manzo, Nebbioso et al. 2009). Cysteine proteases such as Calpain, cathepsisn and papain mediate enzymatic cleavage and are abundant within vascular tissue (Chapman, Riese et al. 1997; Cheng, Shi et al. 2012). Calpain in particular has been reported to exert pathogenic effects in experimental pulmonary vascular remodeling (Ma, Han et al. 2011). Cleavage of TRAIL by cysteine proteinases such calpain may play an important role in allowing paracrine effects of cleaved soluble TRAIL on multiple cells types. This may also explain why the systemic delivery of recombinant soluble form of TRAIL, without direct presentation by another cell, to Paigen diet–fed ApoE$^{-/-}$ /TRAIL$^{-/-}$ mice (Chapter 4 **Fig. 4.5**) resulted in the development of a PAH phenotype suggesting that soluble/cleaved levels within the vessel wall are critically important. Furthermore the ability of TRAIL to be shed may in part account for the elevated levels of serum TRAIL observed in both mice with hypoxia induced PH and patients with PAH in whom higher levels were associated with more advanced disease (Liu, Yang et al. 2015). Ongoing work in our department with mice that have specific inactivation of TRAIL in smooth muscle cells will help to further characterise the significance of local TRAIL to experimental disease pathogenesis.

6.5 CHAPTER SUMMARY

My findings from this chapter support the view that the predominant source of TRAIL driving PAH in the ApoE$^{-/-}$ murine model of PAH is not bone marrow derived and may be from the lung itself. This adds further mechanistic insight into the pathogenic role for TRAIL already described in chapters 3-5. In addition these data would suggest that future anti-TRAIL therapies could be preferentially be delivered to the lung by nebulised or inhaled preparations.

CHAPTER 7 GENERAL DISCUSSION

7.1 SUMMARY OF MAJOR FINDINGS

The work presented in this thesis, details for the first time a significant role for TRAIL in pulmonary hypertension. The original and novel findings can be summarised as follows:

1. Gene expression of TRAIL and receptors TRAIL R1 and R3 were increased in PA-SMCs isolated from patients with PAH.
2. TRAIL protein expression (by immunohistochemistry) was evident within lesions from human PAH and two rodent models of PAH (Paigen diet and MCT).
3. Recombinant TRAIL was a mitogen (proliferative and migratory) for human PA-SMCs *in-vitro* (through ERK signaling) and attenuated by an anti-TRAIL R3 antibody.
4. Genetic deletion of TRAIL prevented mice from developing hypoxia induced PH.
5. Genetic deletion of TRAIL protected $ApoE^{-/-}$ mice from developing diet induced PAH whilst recombinant TRAIL restored a disease phenotype in these mice.
6. Antibody blockade of TRAIL prevented the development of MCT induced PAH in rats. This was associated with reduced proliferation and increased apoptosis within pulmonary vascular lesions.
7. An anti-TRAIL antibody reduced pulmonary vascular remodeling and improved survival in rats with established MCT induced PAH
8. In $ApoE^{-/-}$ mice with established PAH, an anti-TRAIL antibody profoundly reversed pulmonary vascular haemodynamics and remodeling. Disease reversal was associated with significantly reduced levels of proliferation and increased apoptosis within vascular lesions.
9. Bone marrow transplant experiments in chimeric mice supported a role for locally derived TRAIL (non bone marrow derived) as the major driver for pulmonary vascular remodeling in Paigen diet induced PAH.

7.2 FURTHER EVIDENCE SUPPORTING A ROLE FOR TRAIL IN PAH.

I have provided evidence that supports a pathogenic role for TRAIL in three animal models. At the time of undertaking this work our laboratory was not utilising the Sugen-Hypoxia rat model of PAH. This increasingly used model features neointimal proliferation with angio-obliterative lesions and is now widely regarded as the most optimal rodent model of human PAH and a "benchmark" for study (Ciuclan, Bonneau et al. 2011; Al Husseini, Bogaard et al. 2012; Van Hung, Emoto et al. 2014). In truth however there is no perfect single animal model to study PAH and the use of several models , each with different disease triggers and pathophysiology is recognized as a valid approach as judged by the growing number of reports adopting this strategy.

To further support a role for TRAIL in PAH, our lab has subsequently confirmed my observations in the Sugen-hypoxia mouse model (Dawson, Arnold et al. 2014). They showed reduced BMPR2 and increased TRAIL gene expression in lungs from wild type mice. Wild type mice developed a severe disease phenotype whereas $TRAIL^{-/-}$ mice were completely protected from Sugen-Hypoxia induced PAH as evidenced by normal haemodynamics (RVSP ≈25mmHg vs ≈60mmHg), normal PVR, absence of RVH, significantly reduced pulmonary arteriolar remodeling and reduced levels of vascular proliferation (PCNA immunostaining). An advantage of the murine sugen-hypoxia model of PAH compared with the rat model is that it permits use of knockout mice to characterise the role of candidate genes (Ciuclan, Bonneau et al. 2011). These additive findings in the Sugen-hypoxia model are noteworthy given that TRAIL has been shown to induce endothelial apoptosis and inhibit angiogenesis (Chen and Easton 2010).

An independent group has reported increased soluble levels of TRAIL in serum from patients with PH, including PAH and higher levels correlated with markers of worse disease (functional class, exercise duration and PA pressure). Additionally serum TRAIL levels were reduced following treatment with pulmonary vasodilators in a small subset of patients undergoing repeat haemodynamic evaluation. In hypoxic mice, serum TRAIL and lung mRNA levels of TRAIL were significantly increased after hypoxia compared to normoxic control mice. Mice treated with the prostacyclin analogue, Treprostinil, demonstrated a reduction in serum TRAIL levels after treatment, although the lack of a control group in the mouse intervention study and small numbers of patients (n=9) with repeat RHC data precludes firm conclusions on the significance of a reduction in serum TRAIL levels after therapy and its utility as a biomarker of disease (Liu, Yang et al. 2015).

Levels of TRAIL expression have shown to be elevated in lung tissue from patients with COPD and an established murine of COPD which was associated with heightened cellular and molecular inflammation. These features were significantly reduced in TRAIL$^{-/-}$ mice and also after treatment with anti-TRAIL antibody in mice with established COPD highlighting a pathogenic role for TRAIL in COPD (Haw, Starkey et al. 2016). An independent study identified that MMP12 mediated PASMC proliferation was mediated via TRAIL (Kelly 2015).

7.3 SUMMARY OF TRAIL PATHOPHYSIOLOGY IN PAH

The corpus of my work has defined a pathogenic role for TRAIL in rodent models of disease. I have used classical models of disease (hypoxia and monocrotaline) which are regarded as single hit models as well as the ApoE$^{-/-}$ mouse model with multiple insults including inflammation and deranged metabolism. I have shown that TRAIL plays a pathogenic role in all three models and inhibition of TRAIL can prevent, slow progression and even reverse disease in one or more models.

The main strengths of the approach used in this thesis are that we have used several animal models of disease to confirm a pathogenic role for TRAIL. The phenotyping has been comprehensive in terms of recording invasive right and left heart haemodynamics (via the superior method of a "closed chest technique"). Echocardiography was performed, utilizing a dedicated high frequency preclinical imaging system to evaluate disease. RVH was estimated using accepted Fulton method, and we performed histological and immunohistochemical analysis of pulmonary vascular remodeling. Furthermore levels of proliferation and apoptosis were assessed in lung tissue of rodents in the intervention studies. All histological/immunohistochemical analyses were performed in a blinded fashion. Moreover studies in rodents were performed *after* establishing relevance of TRAIL in human disease by observing upregulated expression of TRAIL and its receptors R1 and R3 on PASMCs from patients with PAH (albeit endstage disease) and the mitogenic effects of TRAIL on human PA-SMCs *in-vitro*. This collective approach is commensurate with recent recommendations by established investigators in the field of PAH (Bonnet, Provencher et al. 2017).

When interpreted in the context of prior data describing a mitogenic role for TRAIL in systemic vascular biology, potential links between TRAIL and existing PAH disease mechanisms (summarized and discussed in detail in chapters 1, 3 and 4) and our recent findings from the Sugen Hypoxia mouse model (section 7.2 above) all collectively provide a strong basis for regarding TRAIL as a conserved pathophysiological mediator in PAH.

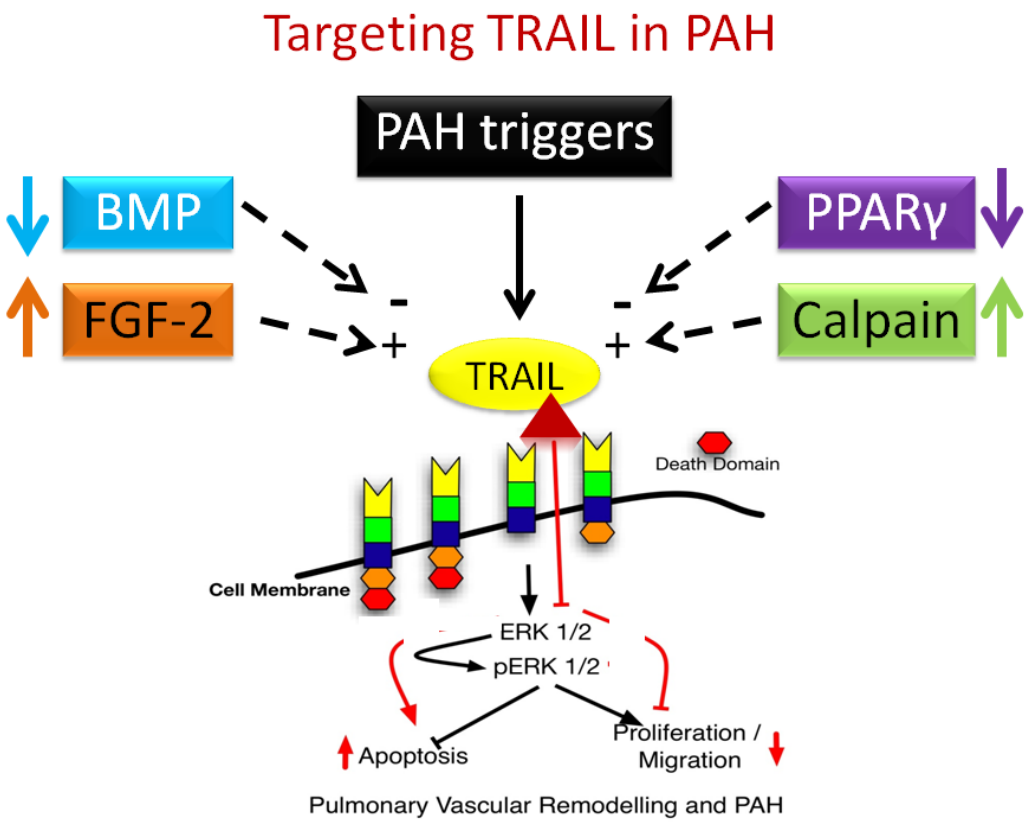


Figure 7.1 Proposed mechanism of TRAIL in PAH. Schematic illustration how existing PAH pathways can link to TRAIL (based on the literature) and how TRAIL signalling via ERK promotes PASMC proliferation. Blocking TRAIL can halt these events and prevent and reverse experimental disease.

A proposed schematic illustration regarding the pathophysiology of TRAIL in PAH is summarized in **Fig. 7.1**. TRAIL is produced and released by resident lung cells, (PASMCs, endothelial cells and inflammatory cells) and modulated by existing PAH disease pathways. TRAIL promotes aberrant PASMC growth through an autocrine and paracrine manner, likely mediated via the TRAIL R3 receptor and intracellular ERK signaling. This drives pulmonary arteriolar remodeling leading to pulmonary hypertension. Inhibition of TRAIL signaling significantly interrupts this implicit sequence of events and consequently prevented and reversed experimental PAH. Reduced proliferation and increased apoptosis levels were observed in pulmonary vascular lesions. Although several growth factors and cytokines have been shown to play a role in, it is plausible to hypothesise that some growth factors such as TRAIL may predominate in the downstream cytokine orchestra promoting a proliferative phenotype of PA smooth muscle cells. Thus inhibiting TRAIL signaling as an “anti-remodelling” strategy in PAH is conceptually appealing and worthy of further study.

7.4 CHASM BETWEEN PRECLINICAL RESEARCH AND SUCCESSFUL HUMAN TRANSLATION

"Essentially, all models are wrong, but some are useful." George E.P. Box.

Box, G. E. P. (1979), "Robustness in the strategy of scientific model building", in Launer, R. L.; Wilkinson, G. N., Robustness in Statistics, Academic Press, pp. 201–236.

Limitations of preclinical models have been increasingly rehearsed and reliance on them as spring board for clinical translation, both in PAH and wider afield has been fraught with many disappointments. There is growing emphasis on improving the robustness and validity of findings from preclinical research. The ARRIVE guidelines advocate robust study methodology including use/reporting of study size/power calculations, blinding and randomization as well as matching for age and sex in rodents, as mandated in human clinical trials (Kilkenny, Browne et al. 2010; van der Worp, Howells et al. 2010; Ioannidis 2012; Landis, Amara et al. 2012; Peers, South et al. 2014; Begley and Ioannidis 2015). This has been endorsed by the pulmonary vascular research community (Lythgoe, Rhodes et al. 2016; Bonnet, Provencher et al. 2017). Although my investigations predated the publication of the ARRIVE guidelines, I have fulfilled several key criteria. Furthermore a consistent pathogenic role for TRAIL has been observed in four animal models.

Lythgoe *et al.* 2016, illustrate examples and lessons from drug targets, identified primarily in bench preclinical research that have failed to reach fruition for humans with PAH. They reaffirm the importance of shifting the focus beyond pulmonary vasoconstriction/ vasodilatation towards targeting underlying vascular remodeling whilst also acknowledging the challenges in successfully attaining this. These lessons are a welcomed reminder and the authors provide an important checklist for optimal drug design in experimental human PAH. At this stage, the concept of inhibiting of TRAIL in humans as a therapeutic strategy is untested and would require investigating/establishing the optimal dose, route of delivery, measures of efficacy as well as ensuring acceptable safety and tolerability in humans.

Thus I believe that any excitement about my positive findings should be tempered and rightly so given concerns about the low yield of translating such findings into clinically successful therapies for use in human disease. This is evidently manifested in the significantly high failure rate of modern therapies (50% due to lack of efficacy and 25% for safety) (Arrowsmith and Miller 2013; Hwang, Lauffenburger et al. 2016).

7.5 THERAPEUTIC IMPLICATIONS OF TARGETING TRAIL IN HUMANS

Generally speaking, successful pharmacological therapies must demonstrate clinical efficacy, safety and tolerability. At this stage, the concept of inhibiting of TRAIL in humans as a therapeutic strategy is untested and will require investigating/establishing the optimal dose, route of delivery, measures of efficacy as well as ensuring acceptable safety and tolerability in humans. Clues to guide the latter can be drawn from existing literature, particularly as the receptors for TRAIL receptors are ubiquitously expressed (spleen, lung, prostate, heart and lung) and the broad reported functions of TRAIL. As highlighted in the introductory chapter, a role for TRAIL has been reported in Immune surveillance, inflammation, cell growth, differentiation and survival. These have implications for situations such as tumour growth, metastases, immune cell function and autoimmunity. These diverse functions of TRAIL reflect the complex nature of signaling by TRAIL and the increasing recognition of non-apoptotic (non-canionical) TRAIL signaling. Thus any strategy targeting TRAIL must factor these aspects when evaluating efficacy and safety. However, given the generally poor outlook of many patients with PAH, some of these complications may not be evident in the short to medium term but are likely to be more important in the longer term. Similar concerns are evident with drugs that have been regarded as a "double edged sword" with clear examples being immunosuppression in solid organ transplantation and inhibitors of TNFα in chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease.

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7.6 AREAS MERITING FURTHER STUDY OF TRAIL IN PAH

My data suggest a pathogenic role for TRAIL in PAH and highlight its potential as a novel therapeutic target. However additional investigations are necessary before a more definitive role for TRAIL can be established and thus permit a successful attempt at therapeutic exploitation. Important unanswered questions include the following;

1) Does genetic/genomic variation in TRAIL alter susceptibility to PAH?
2) What is the relationship between BMPR2 signaling and TRAIL?
3) Can circulating TRAIL levels be used as a disease biomarker in PAH?
4) What is the pattern of TRAIL receptor expression in human PAH lungs?
5) What effect does TRAIL have on endothelial cell physiology? Does it influence the development of neointimal proliferation and plexiform lesions? What is the "cross talk" with smooth muscle cells?
6) What role does TRAIL have on modulating right ventricular structure and function?
7) What is the optimal strategy to inhibit the TRAIL system in PAH? (Ligand vs receptors)
8) What effect does TRAIL have on the metabolic state/Warburg phenotype in PAH?

7.6.1 Does genetic variation in TRAIL alter susceptibility to PAH?

Genetic variation in TRAIL has been linked with susceptibility to multiple sclerosis (López-Gómez, Fernández et al. 2011), osteoporotic fractures (Zhang, Liu et al. 2011), Asthma (Isi, Oral et al. 2010; Weckmann, Kopp et al. 2010) and fatty liver disease (Yan, Xu et al. 2009). An initiative by the Vanderbilt group established a list of 209 genes relevant to human PAH after undertaking biological functional and network analyses. (Zhao, Austin et al. 2014) Within this list TRAIL (TNFSF10) was identified and ranked 99th using this integrated systems based approach. Unlike Genome wide association studies (GWAS) in common complex diseases such as coronary disease when studies include tens of thousands of cases, GWAS in PAH have been hampered due small patient numbers as a result of disease rarity. Thus, the power to detect genetic variants with small effects is significantly limited especially when disease prevalence is low. It would however be worth taking a candidate gene based approach to determine whether variations in TRAIL/receptor gene or promoters are linked to disease.

7.6.2 What is the relationship between BMPR2 pathway and TRAIL signaling?

Given the pivotal role of dysfunctional BMPR2 signaling in PAH, it would be of major interest to explore the impact TRAIL may have on modulating this. Potentially TRAIL could be an additional hit in the permissive setting created by genetic or functional deficient BMPR2 signaling. Experiments using PA cells (smooth muscle and endothelial cells) from PAH patients harboring pathogenic BMPR2 mutations could be stimulated *ex-vivo* with TRAIL to explore the mitogenic properties of TRAIL and its receptors.

7.6.3 What effect does TRAIL have on endothelial cell physiology in PAH?

Our lab has observed TRAIL immunostaining in plexiform lesions from human PAH (Lawrie, Waterman et al. 2008) and established that TRAIL knockout mice were protected from Sugen-Hypoxia induced PAH in mice (Dawson, Arnold et al. 2014) highlighting a potential link between TRAIL and angioproliferative disease in PAH. To further support this notion, TRAIL, and its receptors including OPG are expressed on microvascular ECs from infantile haemangiomas and the OPG-TRAIL (and TRAIL R3) pathway promoted the proliferative anti-apoptotic phenotype of these cells (Vishvanath, Itinteang et al. 2011). Moreover TRAIL expression has also been linked to angiogenesis within the lung (Favre, Mancuso et al. 2003) and studies in TRAIL$^{-/-}$ mice have supported its role in ischaemia induced angiogenesis and neovascularisation (Hubert, Davies et al. 2009; Di Bartolo, Cartland et al. 2015; Cartland, Genner et al. 2016). Clearly it would be of significant value to determine whether anti-TRAIL antibodies could be successful in reversing Sugen-Hypoxia induced PAH in rats, a model in which disease reversal is regarded as being “difficult”.

Early EC apoptosis is viewed as one of the early events in PAH whilst the development of apoptosis resistant and proliferative ECs are key features of advanced lesions. Given that TRAIL has been shown to induce both induce endothelial cell apoptosis and survival it is tempting to speculate that TRAIL could regulate one or both in PAH which may be dependent on the stage of disease. Cell culture work testing the effects of TRAIL on normal and PAH patient derived pulmonary microvascular EC in monoculture and co-culture with PASMCs could be used to explore this area further.

7.6.4 Can circulating TRAIL levels be used as a disease biomarker in PAH?

As already highlighted in chapter 1, levels of serum TRAIL appear to be inversely linked to a variety of cardiovascular disease states. Preliminary data from our group showed a similar pattern in idiopathic PAH (Watt, Soon et al. 2009) whereas more recent data suggest TRAIL levels are increased in PAH (Liu, Yang et al. 2015) whilst others observed no difference in the PAH related to congenital heart diseases (Brun, Holmstrøm et al. 2009; Brun, Ueland et al. 2011). Interestingly other members of the TNF superfamily have been linked to PAH with reduced serum levels of TWEAK (Filusch, Zelniker et al. 2011; Jasiewicz, Kowal et al. 2014) and increased FAS Ligand (Akagi, Nakamura et al. 2013) being reported.

These studies however involved small patient numbers. Given the ongoing national PAH biobank data collected across the UK PAH centers it would be relatively easy to measure circulating levels of TRAIL and its receptors in larger cohorts to determine any value as a disease biomarker (diagnostic and prognostic). Establishing the utility of this approach would also permit disease monitoring should anti-TRAIL therapies be explored.

7.6.5 What effect does TRAIL have on the metabolic /Warburg phenotype in PAH?

Hitherto the relationship between hypoxia and TRAIL has almost exclusively been explored in the context of cancer biology with which the pathogenesis PAH shares some resemblance (Rai, Cool et al. 2008; Guignabert, Tu et al. 2013; Sutendra and Michelakis 2013). Given that there remains a significant appeal for TRAIL as an anti-tumour agent, a number of studies have investigated the effects of the hypoxic tumour microenvironment on the apoptotic effects of TRAIL. It is recognized that many solid tumours have upregulated levels of the hypoxia sensitive transcription factor HIF-1α (Hypoxia Inducible Factor 1-alpha) (Greijer and van der Wall 2004; Zhao, Butler et al. 2013) which is linked to the development of resistance to chemotherapeutic agents including TRAIL (Mayes, Campbell et al. 2005; Jeong, Moon et al. 2010).

Furthermore upregulation of HIF-1α is directly linked to other hallmarks of cancer (Hanahan and Weinberg 2011); notably an altered metabolic cellular phenotype favoring glycolysis (Warburg effect), altered mitochondrial physiology and ATP production all of which contribute to the development of apoptosis resistant hyperproliferative cells . In recent years these features are also now increasingly recognized as important phenotypes in PAH (Archer,

Gomberg-Maitland et al. 2008; Dromparis, Sutendra et al. 2010; Sutendra, Bonnet et al. 2010; Sutendra, Dromparis et al. 2011; Dromparis, Paulin et al. 2013; Sutendra and Michelakis 2014).

HIF-1α has been shown to inhibit TRAIL mediated apoptosis of tumour cell lines *in-vitro* through several mechanisms, including upregulation of antiapoptotic factors such as BCL-2/IAP (Park, Billiar et al. 2002), preventing the translocation of the pro-apoptotic mediator Bax (Kim, Park et al. 2004) or via upregulation of the "decoy death receptor" TRAIL R3 (Pei, Wu et al. 2010). This latter finding is noteworthy because, I have shown upregulated TRAIL-R3 expression and it mediating the proliferative effects of TRAIL on human PASMCs.

Interestingly upregulation of BCL-2 has been reported in PAH serum (Akın, Alehan et al. 2015), on PAH derived endothelial cells (Benza, Williams et al. 2016) and linked to an irreversible disease pattern on lung biopsy from PAH related to congenital heart disease (Levy, Maurey et al. 2007). BCL-2 suppression permits TRAIL induced apoptosis of vascular endothelial (Alladina, Song et al. 2005) and lung alveolar cells (Morissette, Vachon-Beaudoin et al. 2008; Morissette, Parent et al. 2011). Collectively this may help to explain why TRAIL displays a pro-proliferative rather than an apoptotic effect on smooth muscle cells in pulmonary vascular disease.

Additionally HIF-1α has intricate links with p53 (Obacz, Pastorekova et al. 2013) which is known to regulate TRAIL (Morissette, Vachon-Beaudoin et al. 2008; Zhao, Lu et al. 2012; Meijer, Kruyt et al. 2013). Finally both HIF-1α (Lai and Law 2004; Kwapiszewska, Wygrecka et al. 2008; Zhang, Wu et al. 2009; Fijalkowska, Xu et al. 2010; Rey and Semenza 2010; Farha, Asosingh et al. 2011) and p53 (Mizuno, Bogaard et al. 2011; Mouraret, Marcos et al. 2013) have been implicated in pulmonary hypertension. Therefore determining the mechanistic links between HIF-1α, p53, BCL-2 and TRAIL in PAH is an area that potentially merits further study. This may shed light on why mitochondrial membranes are hyperpolarized in PASMCs and PAECs leading to a pro-proliferative and anti-apoptotic vascular cell phenotype in PAH (Paulin and Michelakis 2014). Given that TRAIL mediated apoptosis via the intrinsic pathway involves mitochondria recent studies are shedding light on its involvement in disturbed mitochondrial physiology in cancer (Akita, Suzuki-Karasaki et al. 2014; Suzuki-Karasaki, Ochiai et al. 2014; Suzuki-Karasaki, Fujiwara et al. 2015).

7.7 CONCLUDING REMARKS

Following initial observations of TRAIL in human PAH lesions and increased gene expression in diseased PASMCs, I have shown a pathogenic role for TRAIL in hypoxia, monocrotaline and high fat Paigen diet induced rodent models PAH. This phenotype has been further confirmed more recently by our group in the Sugen-Hypoxia model. These consistent results observed across multiple models would favor that TRAIL is a conserved pathophysiological driver of disease. Intervention studies in mice and rats highlight the potential for TRAIL as a novel target for an anti-proliferative strategy in human PAH.

However as has been rather evident elsewhere, this latter observation is not without caution as failure to make it to the bedside is an all too common occurrence in several translational research initiatives. PAH is no exception given the complex nature of the disease and it would not be unconceivable to propose there will be no single “wonder drug” ever likely to treat this disease. Rather a strategy utilising multiple therapies targeting key aspects of the pathobiology of disease (personalised to each patient) is probably the one most likely to succeed.

Despite improvements in the diagnosis and therapy over the past two decades there remains substantial morbidity and mortality associated with PAH. The work presented in this thesis is an original contribution to the ongoing challenge to cure PAH and highlights potential for targeting the TRAIL pathway. My findings justify further study with the ultimate goal of developing anti-TRAIL based therapies.

CHAPTER 8 REFERENCES

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Barbara Girerd, Jason Weatherald, David Montani, Marc Humbert
European Respiratory Review Sep 2017, 26 (145) 170037; DOI: 10.1183/16000617.0037-2017

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