IMPERIAL COLLEGE LONDON

Department of Experimental Medicine

ADMA METABOLISM AND CHRONIC HYPOXIA-INDUCED PULMONARY HYPERTENSION

A thesis presented for the degree of Doctor of Philosophy by

Lucio Iannone

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Declaration

I, Lucio Iannone confirm that the work presented in this thesis is my own. Where information has been derived from external sources or data obtained by others, I can confirm this has been indicated in the text.

Lucio Iannone

November 2014

Abstract

Rationale: Elevated asymmetric dimethylarginine (ADMA) levels contribute to the pathogenesis of hypoxia-induced pulmonary hypertension. Chronic hypoxia decreases the activity of the enzymes metabolising ADMA, dimethylarginine dimethylaminohydrolases (DDAH1 and DDAH2) but the mechanisms responsible are not understood.

Objective: To elucidate the physiological significance and the mechanism of hypoxia-induced downregulation of ADMA metabolism.

Methods and Results: Exposure of human pulmonary artery endothelial cells (HPAECs) to hypoxia inhibited both DDAH1 and DDAH2 gene and protein expression, reduced DDAH activity and increased ADMA levels. In contrast, in human pulmonary artery smooth muscle cells (HPASMCs) only DDAH2 was reduced while ADMA levels remained unchanged. This endothelium-specific regulation of DDAH1 and ADMA resulted from NFκB-dependent, microRNA-21 (miR-21)-mediated degradation of DDAH1 mRNA. Down regulation of DDAH1 activity contributed to hypoxia-induced endothelial barrier dysfunction and HPASMC proliferation and was prevented by overexpression of DDAH1 and miR-21 blockade. Conversely, overexpression of miR-21 mimicked the effects of hypoxia. DDAH1 overexpressing transgenic mice exposed to 2 weeks hypoxia, showed attenuated pulmonary hypertension and vascular remodelling, compared with wildtype controls. Importantly, inhibition of miR21 in vivo prevented the hypoxia-induced reduction in pulmonary DDAH1 expression and attenuated the development of pulmonary hypertension. Lung tissue samples from hypoxic mice and treatment-naïve IPAH patients also showed reduced DDAH1 expression and increased miR-21 levels, compared with controls.

Conclusion: Down regulation of DDAH1 expression by miR-21 in the pulmonary vascular endothelium has a key role in the pathogenesis of hypoxia-induced pulmonary hypertension and may be of broader significance in pulmonary hypertension.

Acknowledgments

Dedicated to Daniel, the best thing I have ever done.

I would like to express my immense gratitude to my supervisors Beata Wojciak-Stothard, James Leiper and Lan Zhao, who have been inspiring, and providing excellent guidance throughout these 3 years. I am especially thankful to Beata, who's patience and support have made an important impact on me to accomplish this project, and James for his passionate approach, for his ability to stimulate my curiosity and especially for the capability to make me work until late just to answer his scientific questions.

I would like to show special gratitude to Prof. Martin Wilkins for providing important support and advice throughout this project (and also for the Monday morning football chats). Special thanks should go also to other PH group members for the great time both in the lab and at the pub during the last 3 years.

As for anything I have achieved in life, I am immensely indebted to my family who have always supported me and for their positive influence.

Finally, to Morena my wife, whom I married during the last year of this project. She had a real experience of the natural drama of a PhD student with all of its highs and lows… and did not run away. Despite many ruined weekends and evenings, she has always provided moral support, encouragement, food, laughs and jokes throughout. I would not have done this without you.

Abbreviations

ADMA	Asymmetric dimethylarginine
AGT- II	Angiotensin-II
ANP	Atrial natriuretic peptide
ATP	Adenosine triphosphate
BH_4	Tetrahydrobiopterin
BMP	Bone morphogenetic protein
BMPR2	Bone morphogenetic protein receptor 2
bp	Base pair
BrdU	Bromodeoxyuridine
BSA	Bovine specific albumin
BSA	Bovine specific albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribose nucleic acid
cGMP	Cyclic guanosine monophosphate
CPE	Cytopathic Cytopathic effect
Ct	qPCR fluorescence threshold
CTEPH	Chronic thromboembolic pulmonary hypertension
D7-ADMA	2,3,3,4,4,5,5-D7-asymmetric dimethylarginine
DAPI	4',6-diamidino-2-phenylindole
DDAH	Dimethylarginine dimethylaminohydrolase
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
DTT	Dithiothreitol
ECM	Extracellular matrix
EDCF	Endothelium-derived constriction factor
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial NOS/NOSIII
ET-1	Endothelin-1
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factors
FITC	Fluorescein isothiocyanate
FPH	Familial pulmonary hypertension
FXR	Farnesoid X receptor
GDF	Growth and differentiation factors
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HEK 293	Human embryonic kidney cells

HIF-1	Hypoxia-inducible factor 1
HPAECs	Human pulmonary arterial endothelial cells
HPASMCs	Human pulmonary arterial smooth muscle cells
HPLC	High performance liquid chromatography
HPLC	High performance liquid chromatography
HPV	Hypoxic pulmonary vasoconstriction
HRP	Horseradish peroxidase
HUVECs	Human umbilical vein endothelial cells
IL-	Interleukins
iNOS	Inducible NOS
IPH	Idiopathic pulmonary hypertension
IP3	Inositol 1,4,5-trisphosphate
IV	Intravenous
LC-MS	Liquid chromatography-mass spectrometry
LNA	Locked nucleic acid
L-NMMA	N(G)-monomethyl-L-arginine
MCT	Monocrotaline
miR/miRNA	MicroRNA
MLC	Myosin light chain
MRE	MicroRNA Recognition Element
mRNA	Messenger RNA
NF-kb	Nuclear factor kB
NK	Natural killer
nm	Nanometers
nNOS	Neuronal NOS
NO	Nitric oxide
NOS	Nitric oxide synthase
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAH	Pulmonary arterial hypertension
PAP	Pulmonary arterial pressure
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDE-5	Phosphodiesterase-5
PDEGF	Platelet-derived endothelial cell growth factor
pGC	Particulate guanylyl cyclase
PH	Pulmonary hypertension
PI	Propidium Iodide
PKA	Protein kinase A
PKG	Protein kinase G
PRMT	Protein methyl transferase
PVDF	Polyvinylidene difluoride

PVR	Pulmonary vascular remodelling
qPCR	Quantitative PCR
Rho	Ras homologous
RNA	Ribonucleic acid
ROCK	Rho Kinase
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-PCR
RV	Right ventricle
RVH	Right ventricular hypertrophy
RVSP	Right ventricular systolic pressure
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SDS	Sodium dodecyl sulphate
SERC	SR Ca2+-ATPase
Ser188	Serine 188
sGC	Soluble guanylyl cyclase
$t_{1/2}$	Half-life
TBP	Tata binding protein
TGF-β	Transforming growth factor β
TNF-α	Tumor necrosis factor alpha
T-reg	Regulatory T cell
UTR	Untranslated region
VASP	Vasodilator-stimulated phosphor-protein
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
α-SMC	Alpha-smooth muscle actin

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Tables

1. Introduction

1.1 Pulmonary Circulation

The main function of the pulmonary circulation is to deliver oxygen to the blood and free it of carbon dioxide. Cardiovascular function is essential to the survival of higher organisms since every cell requires nutrition and gas exchange. The circuit starts with the pulmonary artery, which extends from the right ventricle and carries blood with low oxygen content to the lungs, where it branches into two arteries, one for each lung, and then into arterioles and capillaries. The primary site of gas exchange is the vascular/alveolar interface in the pulmonary capillaries, located deep within the lung. Once blood is oxygenated in the lung, it is pumped by the heart throughout the body, where exchange of gas within the tissue occurs via capillary beds. Then, carbon dioxide-rich blood is returned to the lungs via the vena cava for the respiratory/circulatory cycle to begin again. The walls of pulmonary arteries and veins are significantly thinner than the walls of corresponding vessels in the rest of the body. Therefore, the pressure in this part of the system is only one sixth of that in the systemic circulation. In the pulmonary circulation, the roles of arteries and veins are the contrary of what they are in the systemic circulation. The blood in the arteries is less oxygenated; meanwhile blood in the veins is oxygen-rich. Carbon dioxide is removed from the blood in the pulmonary arteries across capillary walls and leaves the body through the mouth and nose (Young 1940).

1.1.1. Anatomy and Function of Pulmonary Vasculature

Anatomically the post-capillary blood vessels are composed of three layers – the tunica intima, the tunica media and the tunica adventitia. The tunica intima forms the innermost layer and contains a monolayer of endothelial cells (ECs). The tunica media forms the middle layer of the vessel and comprises smooth muscle cells (SMCs) organized in a circumferentially-arranged manner. The tunica adventitia comprises primarily fibroblasts and connective tissue, which have the role of providing a structural support and holding the vessel in position. These layers are separated by the internal and external elastic laminae, while the internal lamina consists in a fenestrated layer of elastic tissue that is the outermost part of the intima of an artery, the external lamina beneath the smooth muscle delimits the tunica media from the tunica adventitia(Hatabu, Gefter et al. 1991).

The pulmonary circulation has a large surface area (~120m^2) with the essential function of gas exchange and important roles in host defence and maintenance of blood homeostasis. The pulmonary vascular bed is unique as it receives all cardiac output, maintains a low blood pressure, and is exposed to high mechanical stress and the highest oxygen tension of all vascular beds. The airways branch to form bronchi, bronchioli, terminal bronchioli and respiratory bronchioli leading to the alveolar ducts, which incorporate the majority of the vast surface area of the lung. The pulmonary vessels also branch to form smaller and less muscularised arteries leading to the capillary bed, where the lung gas exchange occurs (Stan 2009). Consistent with vessel formation in other tissues, angiogenesis and vasculogenesis are considered to work together to form the pulmonary vascular system. Growth factors such as the platelet-derived endothelial cell growth factor (PDEGF), vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF) stimulate the growth of the new endothelium in a bed of extracellular matrix (ECM) (Stenmark and Mecham 1997). In addition, the various components of ECM, including collagen, elastin, fibronectin, and proteoglycans are involved in a number of functions, such as tensile strength, tissue architecture, elasticity, proliferation and cell migration. Larger vessels are supported by the development of a smooth muscle layer whereas more distal vessels remain completely non-muscularised (Hislop and Pierce 2000) and while layers of elastin provide vessels with elasticity, the collagen complex provides rigidity.

1.1.2. Hypoxia and the pulmonary circulation

The cellular responses to oxygen are essential for physiological mediator release, cellular metabolism, proliferation and survival. The lung has three sources of oxygen: inspired gas, bronchial circulation and venous blood returning to the lung via the right ventricle. Generally, hypoxia is defined as a condition in which the body is deprived of adequate oxygen supply. In the lung, hypoxia is a pathologic condition caused by a deficiency of oxygen in the atmosphere, where the blood oxygen saturation becomes less than 90% (based on the oxyhemoglobin desaturation curve).

Acute hypoxia is defined as a rapid (minutes-hours) decrease in tissue oxygen, while chronic hypoxia is usually a slow (hours-days) reduction in tissue oxygenation. In the lung, the endothelium of large pulmonary arteries is exposed to mixed alveolar and venous oxygen. Thus, it is normal to find heterogeneity in the response of the endothelium to hypoxia, depending on the type of blood vessel. In general, acute hypoxia causes acute hypoxic pulmonary vasoconstriction crucially required to assure optimized adaptation of the perfusion to ventilation, while chronic hypoxia causes sustained pulmonary vasoconstriction and pulmonary vascular remodelling, leading to development of pulmonary hypertension (Ghofrani, Voswinckel et al. 2006). Several lines in the literature implicate hypoxia as a stimulus for altered pulmonary vascular functions; each of the resident vascular cell types can face alterations in proliferation, expression of growth factors, matrix protein production, cytokines and receptors, and each resident cell type plays a specific role in the remodelling response (Stenmark, Fagan et al. 2006).

1.2. Pulmonary Hypertension (PH)

In healthy adults, mean pulmonary arterial pressure (mPAP) is approximately 15 mmHg at rest, while in pulmonary hypertension (PH) there is an elevation of the resting mean pulmonary artery pressure (PAP) above 25mmHg. Increased pulmonary vascular resistance (PVR) that requires the right ventricle to raise PAP to maintain cardiac output causes the failure of the afterload-intolerant right ventricle, ultimately leading to death (Simonneau, Robbins et al. 2009). PH is a multifactorial, progressive disease, with a significant mortality and morbidity rate. It is characterised by increased pulmonary vasoconstriction and structural remodelling of the pulmonary vasculature, which vary in degree of importance in individual patients. Thrombotic lesions and inflammation are frequently involved in the pathology of PH (Tuder and Voelkel 1998)

The structural remodelling seen in the pulmonary vessels in PH comprise medial thickening due to smooth muscle cell hyperplasia and hypertrophy (Hislop and Reid 1976), formation of a neointima composed of smooth muscle cells and the occurrence of plexiform and other obstructive lesions due to endothelial and smooth muscle cell proliferation (Yi, Kim et al. 2000). Increased recruitment, proliferation and differentiation of pericytes and fibroblasts has also been implicated in muscularization of distal pulmonary arteries (Chan, Currie et al. 1987)

The vasoconstriction of the pulmonary arteries in PH has been attributed to reduced expression/activity of voltage-gated potassium channels on vascular smooth muscle cells and endothelial dysfunction characterised by reduced production of vasodilators such as nitric oxide (NO) and prostacyclin and increased production of vasoconstrictors such as endothelin-1 (ET-1) and serotonin (5-HT) (Humbert, Morrell et al. 2004).

1.2.1. Classification of PH

The PH classification has gone through a series of changes since the 1st classification proposed in 1973 that identified only two categories, primary and secondary PH, depending on the presence or absence of risk factors (Hatano and Strasser 1975). During the 2nd world symposium on PH in France (Evian, 1998), a new classification was suggested to create categories of PH that shared similar pathogenesis, clinical futures and therapeutic options. The 3rd PH conference in Italy (Venice, 2003) proposed only minor changes, with the

exception of the introduction of the idiopathic (IPH), familial (FPH) and associate PH. In 2008 the 4th world symposium on PH held in California (Dana Point), suggested a new classification in order to accurately reflect the literature over the past 5 years and clarify some areas that were ambiguous (Simonneau, Robbins et al. 2009). The current Dana Point classification is presented in Table 1.

GROUP	***TYPE***
1	**Pulmonary arterial hypertension (PAH)**
1.1	**Idiopathic pulmonary arterial hypertension**
1.2	**Heritable pulmonary arterial hypertension (known as familial pulmonary arterial hypertension)**
1.3	**Drug-induced and toxin-induced forms of pulmonary arterial hypertension**
1.4	**Pulmonary arterial hypertension associated with identified diseases (HIV, schistosomiasis infections)**
1.5	**Persistent pulmonary hypertension of the newborn**
2	**Pulmonary hypertension resulting from left heart disease**
3	**All forms of pulmonary hypertension associated with alveolar hypoxia and/or disorders of the respiratory system**
4	**All thrombo-embolic diseases leading to pulmonary hypertension**
5	**Pulmonary hypertension with unclear multifactorial mechanisms (diseases affecting directly the pulmonary vessels)**

Table 1: Update clinical classification of pulmonary hypertension (Dana Point, 2008)

The pathogenesis of group 1, pulmonary arterial hypertension (PAH), includes a variety of diseases that share numerous pathophysiological and prognostic features. Although rare, idiopathic PAH (IPAH; group 1.1) is a disease in which there is not a family history or recognised risk factor for PAH. In heritable PAH (group 1.2; known as familial PAH), loss-of-function mutations in the transforming growth factor β/bone morphogenetic protein (TGF-β/BMP) receptor superfamily have been identified as a principal mechanism. Drug-induced and toxin-induced forms of PAH are included in group 1.3, while group 1.4 contains PAH associated with identified diseases, for example, HIV infections. Persistent pulmonary

hypertension of the newborn is incorporated in group 1.5, while patients with pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis are classified as being in group 1.3. PH resulting from left heart disease probably represents the most frequent cause of PH (Simonneau, Robbins et al. 2009). Left-sided ventricular diseases might produce an increase in the left atrial pressure, with passive retrograde transmission of the pressure leading to increase in pulmonary arterial pressure (PAP). The Group 3 includes all forms of PH associated with hypoxia condition or respiratory disorders. Predominantly, the cause of group 3 PH is alveolar hypoxia secondary to lung disease, impaired control of breathing, or residence at high altitude. The group 4 includes PH caused by obstruction of pulmonary arterial vessels due to thromboembolism or tumours. Chronic thromboembolic pulmonary hypertension (CTEPH) represents a common cause of PH. The group 5 consists of several forms of PH in which the multifactorial mechanisms are unclear (Simonneau, Robbins et al. 2009).

Overall, PH affects less than 5/100,000 people in Europe and has a prevalence of 15-50 patients/million of the population. The prognosis of PH remains poor with high mortality rate. The estimated median survival for untreated PH patients is around 2.8 years (National Audit for Pulmonary Hypertension, 2013). Despite the advances in understanding and improved treatment options for PH in recent years, a recent multi-centre report showed 1 and 3 year survival rates of approximately 83% and 58% respectively in PH populations (Humbert, Sitbon et al. 2010). However, for end-stage PH patients who failed to respond to the current therapeutics and interventions, there is no choice but to consider cardiopulmonary transplantation as the final option (Keogh, Mayer et al. 2009).

1.2.2. Responses of Pulmonary Circulation to Hypoxia

In most tissues of the human body, the response to hypoxia is vasodilation. By widening the blood vessels, the tissue allows a greater perfusion. By contrast, in the lungs, the response to alveolar hypoxia is vasoconstriction, diverting pulmonary blood flow from areas of low oxygen to areas of high oxygen availability (Motley, Cournand et al. 1947). This response consists of 2 phases. The phase 1 of hypoxic pulmonary vasoconstriction (HPV) is transient (seconds), endothelium-independent and it is triggered by inhibition of voltage-dependent potassium channels in pulmonary artery SMCs (PASMCs) that leads to membrane depolarization and opening of voltage-dependent calcium channels (Aaronson, Robertson et al. 2006). The increase in intracellular calcium concentration initiates the contraction by stimulating phosphorylation of the myosin light chain.

Phase 2 of HPV is endothelium-dependent and may last from hours to days, depending on the duration of hypoxia. The phase 2 of HPV is sustained by the activation of Rho-kinase in PASMC, which inhibits the de-phosphorylation of the myosin light chain by myosin light chain phosphatase, thereby allowing the contraction to proceed even when the calcium (Ca^{2+}) concentration remains constant, in a process known as calcium sensitization of arterial smooth muscle contraction (Hilgers and Webb 2005). During phase 2, the endothelium modulates HPV by releasing vasorelaxants such as nitric oxide (NO) or prostacyclin, or vasoconstrictors such as endothelin-1 (ET-1) or angiotensin-II (AGT- II). Increased release of endothelium-derived constriction factor (EDCF) from hypoxic lung, different from ET-1 has also been postulated (Robertson, Ward et al. 2001).

Although the exact mechanism by which hypoxia is sensed and translated into vasoconstriction in the pulmonary circulation is not well understood, the involvement of reactive oxygen species (ROS) has been suggested (figure 1).

Briefly, hypoxia can inhibit mitochondrial oxidative phosphorylation and increase ROS formation, which in turn, leads to increased intracellular Ca^{2+} levels and Rho kinase-mediated Ca^{2+} sensitization followed by vasoconstriction (Sylvester, Shimoda et al. 2012) (figure 1).

The pulmonary vascular response depends on the duration of the stimulus. Indeed, acute (intermittent) hypoxia was shown to increase oxidative stress, inflammation and expression of genes involved in ion transport and homeostasis (Giordano 2005; Rojas, Figueroa et al.

2006; Elahi, Kong et al. 2009). Chronic (or sustained) hypoxia was shown to increase proliferation of medial vascular smooth muscle cells and excessive deposition of ECM components by adventitial fibroblasts (Meyrick and Reid 1983). Furthermore, chronic hypoxia induces pulmonary hypertension in humans and animals (Zhao, Mason et al. 2001).

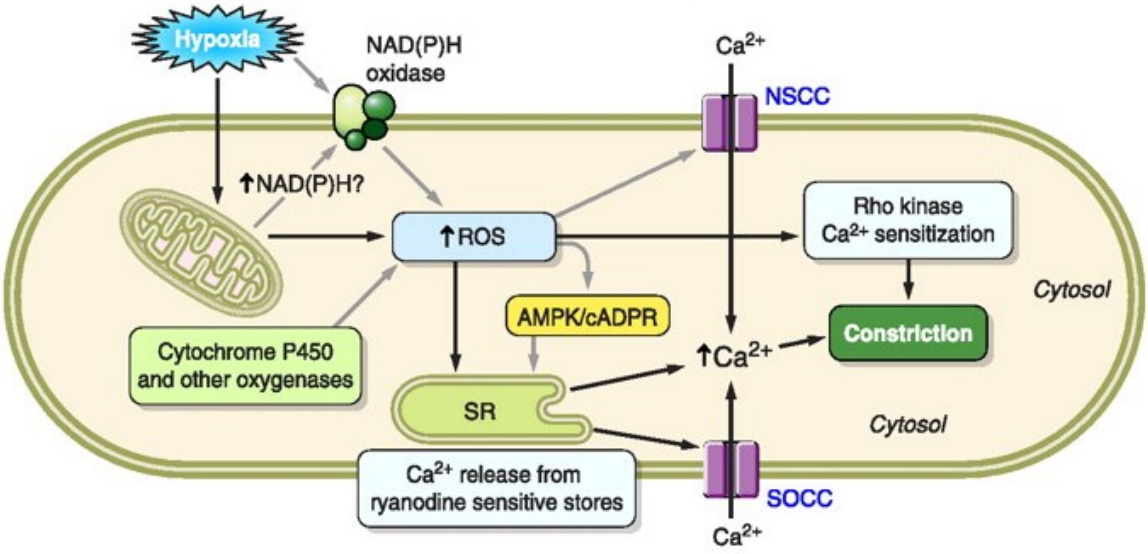

Figure 1: Proposed signalling pathways contributing to hypoxia-induced pulmonary vasoconstriction. Main and alternative pathways are indicated by black and gray arrows, respectively. SOCC, store-operated Ca^{2+}, NSCC nonselective cation channels; cADPR, cyclic ADP ribose; AMPK, AMP kinase; SR, sarcoplasmic reticulum. Adapted from (Sylvester, Shimoda et al. 2012).

1.3. Cellular mechanisms leading to Pulmonary Vascular Remodelling in PH

In physiological conditions, the thickness of the vascular wall is maintained at an optimal level by a balance between proliferation and apoptosis of the resident cell types. If this balance is disturbed in favour of proliferation, the vascular wall thickens, leading to the increased vascular resistance (Pak, Aldashev et al. 2007). This structural change is termed vascular remodelling (Kato and Staub 1966) .

Pulmonary vascular remodelling affects all three layers of the arterial wall and involves increased proliferation and migration of ECs, SMCs, fibroblasts, inflammatory cells, platelet aggregation and increased deposition of ECM components such as collagen, elastin and fibronectin (Savale, Tu et al. 2009; Tuder, Abman et al. 2009) In severe human PAH,

increased proliferation of endothelial and smooth muscle cells leads to the formation of plexiform lesions. The endothelial cells within these lesions show disorganised angiogenesis accompanied by increased expression of pro-angiogenic mediators such as α and β subunits of hypoxia-inducible factor 1 (HIF-1), vascular endothelial growth factor (VEGF) and VEGF receptors (VEGFR)(Hirose, Hosoda et al. 2000; Tuder, Chacon et al. 2001).

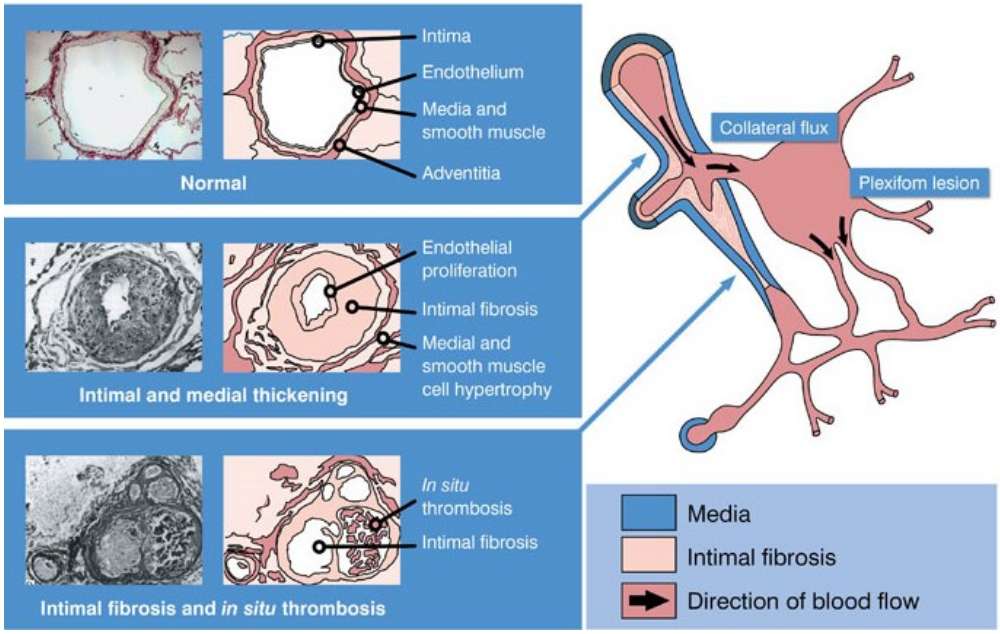

Figure 2: The most prominent histological feature of severe PAH is thickening of the three cellular layers of pulmonary arteries, due to increased cell proliferation and cell enlargement (hypertrophy). Other changes include development of plexiform lesions and *in situ* thrombosis. Adapted from (Galie, Hoeper et al. 2009)

1.3.1. Endothelial Dysfunction in Pulmonary Hypertension

It is widely considered that pulmonary endothelial damage is an early event in PH and a critical step that induces adverse structural changes in the vascular wall. The dysfunctional endothelium produces higher levels of vasoconstrictors, such as endothelin-1 and thromboxane and lower levels of vasorelaxants such as NO and prostacyclin (Budhiraja, Tuder et al. 2004). In addition to the imbalance in the production of vasodilators and vasoconstrictors, damage to the endothelium compromises endothelial barrier function and allows proliferative mediators to permeate into the underlying vascular tissues, resulting in enhanced proliferation and migration of vascular SMCs and fibroblasts (Budhiraja, Tuder et

al. 2004). Endothelial damage may be induced by hypoxia, inflammation, genetic susceptibility or response to drugs.

1.3.1.1. Imbalanced Production of Vasoconstrictors and Vasorelaxants

In a normal physiological state, the pulmonary endothelium helps maintain lung homeostasis by controlling vascular tone, barrier function, leukocyte trafficking, platelet aggregation and production of cytokines and growth factors. In PH, the pulmonary endothelium produces low levels of vasorelaxants, such as NO or prostacyclin and high levels of vasoconstrictors, such as endothelin-1 (ET-1), serotonin, or thromboxane (Budhiraja, Tuder et al. 2004). An imbalance between the levels of vasorelaxants and vasoconstrictors leads to an increase in the vascular tone and increased proliferation of vascular smooth muscle cells and fibroblasts. As showed in figure 3, nitric oxide (NO) produced by endothelial nitric oxide synthase (eNOS) protects against hypoxia-induced vasoconstriction (Perrella, Edell et al. 1992), inhibits smooth muscle proliferation (Tsihlis, Oustwani et al. 2011), leukocyte adhesion and platelet aggregation (Nong, Hoylaerts et al. 1997; Wang, Zhu et al. 1998). Another potent vasodilator, prostacyclin, synthesized from cyclooxygenase by the enzyme prostacyclin synthase in the vascular endothelium, reduces vascular tone by increasing the levels of cAMP and reducing endothelin-1 levels (Langleben, Barst et al. 1999). Apart from acting as a vasodilator, prostacyclin also inhibits platelet aggregation and smooth muscle cell proliferation (Moncada, Higgs et al. 1977; Newby, Southgate et al. 1992). Endothelin-1 is a potent endothelium-derived vasoconstrictor implicated in pulmonary vascular remodelling in PH (Stenmark, Fagan et al. 2006).

Another potent vasoconstrictor, thromboxane, has been found elevated in patient with PH (Christman, Mcpherson et al. 1992). However, the inhibition of thromboxane showed moderate effects on pulmonary haemodynamics in patients with PH (Langleben, Christman et al. 2002).

More information regarding the role of NO, endothelin-1, serotonin and other vasoconstrictors and vasorelaxants is provided in chapters 1.4 and 1.5.

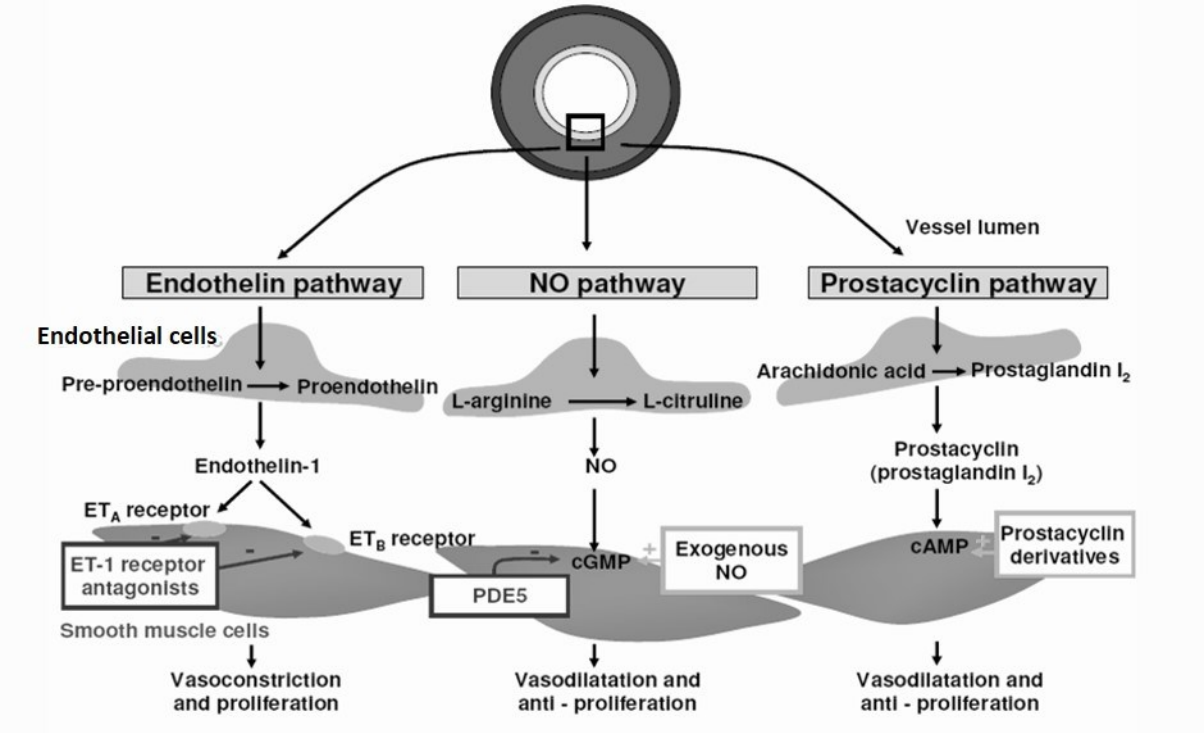

Figure 3: Pathways involved in pulmonary artery endothelial cell dysfunction. NO: nitric oxide; cAMP: cyclic 3', 5'-adenosine monophosphate; cGMP: cyclic 3', 5'-guanosine monophosphate; PDE5: phosphodiesterase type 5; ET: endothelin; ETA: endothelin A receptor; ETB: endothelin B receptor. From (Humbert, Morrell et al. 2004) with modifications.

1.3.1.2. Lung Endothelial Permeability

The endothelium regulates the exchange of fluids, nutrients, growth factors and cells between the blood and the underlying tissues. Endothelial barrier function depends on the formation of endothelial cell-cell contacts (adherens and tight junctions) and cell-matrix adhesions. Under basal conditions, the lung vascular permeability is regulated by two different pathways: the transcellular pathway and the paracellular pathway. The transcellular pathway mediates the transport of plasma macromolecules (such as albumin) by caveolae via the receptor-mediated or fluid-phase mechanism (Mehta and Malik 2006). The paracellular pathway, regulates the passage of small molecules (such as urea and glucose) through the gaps formed between the cells formed as a result of a direct interaction of adherens/tight junctions and focal adhesions with the actin cytoskeleton (Mehta and Malik 2006). The lung endothelial permeability can be increased by

inflammatory mediators, cytokines, oxidants or mechanical stress (McQuaid and Keenan 1997; van Wetering, van Buul et al. 2002). It has been shown that hypoxia-induced increase in endothelial permeability is accompanied by Rho GTPases-mediated actin polymerization and the formation of stress fibers (Wojciak-Stothard, Tsang et al. 2005). Increased levels of intracellular Ca^{2+} ions are also known to contribute to the formation of stress fibres and increased endothelial paracellular permeability (ref)

Furthermore, natriuretic peptides have been associated with endothelial permeability and PH. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are respectively synthesized and released from cardiac atrial and ventricular tissue, in response to stretch, falls in blood volume and blood pressure. A third member of the family, C-type natriuretic peptide, is released from the vascular endothelium and can regulate local blood flow in a paracrine fashion (Scotland, Foster et al. 2004). Each natriuretic peptide acts on specific receptors in the vasculature, which can trigger guanylate cyclase activities. ANP can increase endothelial cell permeability *in vitro* (Scotland, Ahluwalia et al. 2005). Hovewer, ANP can also attenuate endothelial barrier dysfunction caused by inflammatory agents such as thrombin or histamine (Kuhn 2012).

Nitric oxide and natriuretic peptides increase cGMP levels in endothelial cells. However, while Nitric oxide acts via the activation of soluble guanylate cyclase, natriuretic peptides activate particulate guanylate cyclase. Both NO and natriuretic peptides have barrier-protective effects. Recent data suggest a role of cGMP in the regulation of endothelial cell permeability (figure 4). It has been shown that cGMP at lower concentrations can inhibit phosphodiesterase 3 (PDE3) and thereby increase a local pool of cAMP, while at higher concentrations cGMP can activate PDE2, reversing the effect. Furthermore, the PDE3 inhibitors mimic the effect of low-dose ANP on permeability, while inhibitors of PDE2 prevent the increase in endothelial permeability induced by higher doses of ANP (figure 4).

Although the regulation of endothelial barrier function in the lung is not completely understood, it seems clear that a loss of endothelial barrier integrity would allow proliferative mediators to come in direct contact with smooth muscle cells and fibroblasts, leading to increased cell proliferation in the medial and adventitial vascular layers, a key feature in the pathophysiology of pulmonary hypertension (Dejana, Tournier-Lasserve et al. 2009).

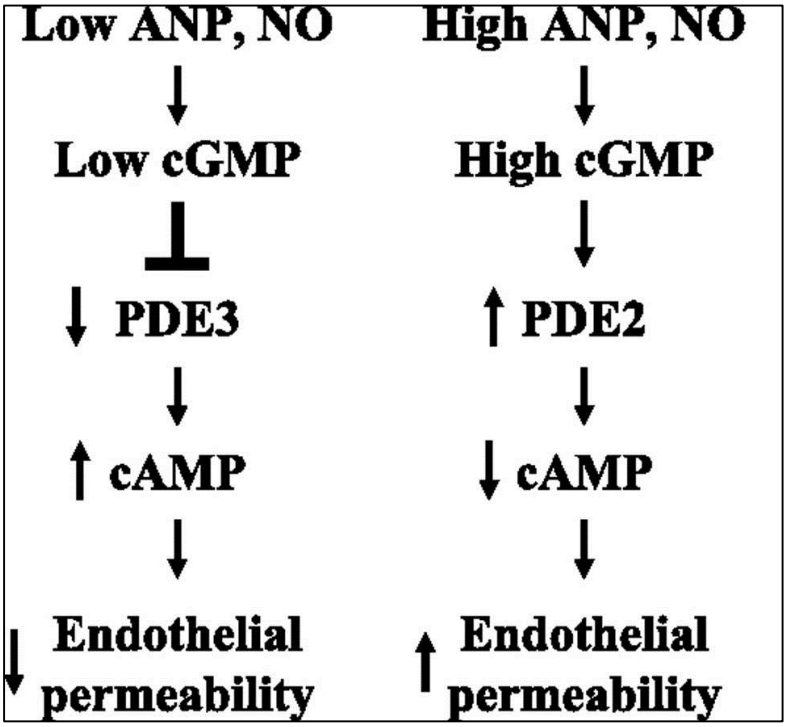

Figure 4: Proposed model of the effect of cGMP on endothelial cell permeability. cGMP generated by NO or ANP stimulation can affect endothelial permeability in two ways. cGMP can inhibit PDE3, leading to increased cAMP in the endothelial cell and decreased permeability. cGMP can also activate PDE2, leading to decreased cAMP and increased permeability. The concentration of cGMP within the cell plays a central role in whether PDE3 is inhibited (low cGMP) or PDE2 is activated (high cGMP). Adapted from (Mohamed, Oceandy et al. 2009).

1.3.1.3. Endothelial Cell Proliferation and Angiogenesis

Severe pulmonary arterial hypertension is characterised by increased proliferation of endothelial cells in the medium-sized pre-capillary arteries, leading to the formation of obliterative plexiform lesions (Tuder, Groves et al. 1994; Lee, Shroyer et al. 1998). Due to the increased levels of pro-angiogenic vascular endothelial growth factor (VEGF) and its receptor, VEGF receptor 2 (VEGFR-2), it has been postulated that plexiform lesions are formed as a result of dysregulated angiogenesis. Interestingly, plexiform lesions are absent in rodent models of mild and moderate pulmonary hypertension (Meyrick and Reid 1980). Only animal models using chronic hypoxia combined with VEGFR blocker, SU5416, have produced lesions that structurally resemble plexiform lesions seen in human PAH (Abe, Toba et al. 2010). However, these lesions develop early after treatment with SU5416 combined with hypoxia, a situation that does not fully reflect the human condition, in which a much longer period is needed (Jonigk, Golpon et al. 2011).

Although the mechanism of the abnormal endothelial proliferation is not well understood, reduced levels of vasodilatory and antiproliferative mediators such as nitric oxide (Giaid and Saleh 1995) and increased levels of pro-proliferative and vasoconstrictive mediators such as endothelin-1 (Giaid, Yanagisawa et al. 1993), may play a role.

Furthermore, mutations in growth- suppressive genes, such as transforming growth factor-beta (TGF-β), have been reported in plexiform lesions of patients with PH. Indeed, in almost 30% of plexiform lesions stop codon mutations have been shown in the transforming growth factor-beta type-2 receptor (TGF-βR2) gene (Taraseviciene-Stewart, Kasahara et al. 2001).

1.3.1.4. Endothelial Apoptosis

Apoptosis is a form of cell death characterized by cell contraction, membrane blebbing, and chromatin condensation (Kerr, Wyllie et al. 1972). When completed, apoptotic cell debris is removed by phagocytic cells present in the immediate area (Granville, Carthy et al. 1998). Dysregulation of apoptosis is associated with a number of diseases, including pulmonary hypertension, cancer, ischaemia-reperfusion lung injury, heart failure, and sepsis.

Endothelial apoptosis resulting from endothelial injury is thought to play a major role in the pathogenesis of PH (Sakao, Tatsumi et al. 2009). Lungs from animals with monocrotaline and chronic hypoxia-induced pulmonary hypertension show reduced levels of VEGF (Arcot, Lipke et al. 1993). VEGF is not only a potent pro-angiogenic factor, but also acts as a potent inhibitor of apoptosis (Alavi, Hood et al. 2003). In *vitro* studies have shown that VEGFR blockade induces endothelial apoptosis followed by selection of proliferating, apoptosis-resistant endothelial cells (Sakao, Taraseviciene-Stewart et al. 2005). The absence of apoptotic endothelial cells in mature plexiform lesions (Yeager, Halley et al. 2001) may suggest that apoptosis is an early event in the lesion formation, followed by a clonal selection of apoptosis-resistant, highly proliferative endothelial cells (Jurasz, Courtman et al. 2010). The development and significance of these lesions in the pathogenesis of PAH have been debated and are not yet fully understood (Jonigk, Golpon et al. 2011).

Additionally, the link between cell apoptosis and pulmonary hypertension has been strengthened by the finding that overexpression of VEGF and angiopoietin-1 reduces endothelial cell apoptosis and prevents the development of monocrotaline-induced PH

(Campbell, Zhao et al. 2001; Zhao, Campbell et al. 2003). Moreover, it has been shown that lungs from patients with pulmonary hypertension show increased levels of anti-apoptotic protein Bcl-2 (Levy, Maurey et al. 2007).

1.3.2. Role of Inflammation

Accumulating data show the involvement of inflammation in the regulation of vascular remodelling in PH (Rabinovitch, Guignabert et al. 2014). Pulmonary vascular lesions in human PH as well as in animal models of PH are characterized by perivascular inflammatory infiltrates comprising T- and B-lymphocytes, macrophages, dendritic cells, and mast cells (Cool, Kennedy et al. 1997; Pinto, Higuchi et al. 2004; Hall, Brogan et al. 2009).

Inflammatory cell numbers correlate with the thickness of pulmonary arterial wall and mean pulmonary arterial pressure, suggesting an important role of perivascular inflammation in pulmonary vascular remodelling (Stacher, Graham et al. 2012). The fact that inflammation precedes vascular remodeling in experimental PH suggests that altered immunity is a cause rather than a consequence of vascular disease (Tamosiuniene, Tian et al. 2011).

Cytokines and chemokines, which act as chemoattractants for circulating inflammatory cells, have been implicated in the pathophysiology of pulmonary hypertension. Abnormally elevated levels of tumour necrosis factor (TNF)-α, chemokine RANTES and interleukins (IL-1β, IL-2, IL-6, IL-8, IL-10 and IL-12) have been observed in PH (Humbert, Monti et al. 1995; Dorfmuller, Zarka et al. 2002; Soon, Holmes et al. 2010) with some cytokines showing an inverse correlation with patient survival (Soon, Holmes et al. 2010). IL-1β and TNF-α, have been shown to increase expression of extracellular matrix proteins such as fibronectin in the remodelled vessels, while IL-6 has been shown to increase proliferation of smooth muscle cells (Humbert, Morrell et al. 2004; Rabinovitch 2008). In fact, overexpression of IL-6 combined with hypoxia, was shown to trigger the development of PH in mice, whereas mice deficient of IL-6, showed ameliorated phenotype (Miyata, Sakuma et al. 1995)

The accumulation of perivascular macrophages in the lung was shown to be essential for the development of hypoxia-induced pulmonary hypertension, suggesting an important role of macrophage infiltrates in human IPAH lung (Vergadi, Chang et al. 2011). In addition, it has been postulated that compromised function of T-reg (regulatory T cell) population and

natural killer (NK) cells may predispose individuals to PAH, as it does in animal models of PH (Humbert, Morrell et al. 2004; Rabinovitch 2008).

1.3.3. Smooth Muscle Cells

In pulmonary hypertension, an entire pulmonary vascular tree undergoes histological alterations with smaller vessels (such as arterioles) primarily affected. These alterations include medial cell hypertrophy, hyperplasia and arteriolar muscularization. Hypertrophy (enlargement) of SMC cells can be seen predominantly in larger, proximal arteries, while hyperplasia (increase in cell numbers) affects mainly smaller arteries (Meyrick and Reid 1980). Increased SMC proliferation is associated with upregulation of growth factor expression, ion channel activity, and altered balance of vasoactive mediators (Rich, Dantzker et al. 1987). During development of PH, SMC phenotype changes from a contractile to a proliferative, migratory and synthetic phenotype (Thyberg, Blomgren et al. 1995; Louis and Zahradka 2010). This conversion depends, at least in part, on the release of active forms of growth factors such as transforming growth factor beta (TGF-β) or fibroblast growth factors (FGF) from the extracellular matrix (Boudreau, Turley et al. 1991).

Increase in endothelial permeability and an imbalance in endothelium-derived vasoconstrictors and vasorelaxants such as ET-1 and NO are also thought to contribute to SMC migration and proliferation (Di Luozzo, Bhargava et al. 2000; Napoli, Paolisso et al. 2013). In addition, aberrations in the TGF-β signalling are thought to contribute to SMC dysfunction in PH (Morrell, Yang et al. 2001).

The role of BMPR2 mutations and receptor signaling in pulmonary hypertension will be discussed further in chapter 1.5.4.

1.3.4. Fibroblasts

Several studies support an active role of adventitial fibroblasts in pulmonary vascular remodeling in PH (Sartore, Chiavegato et al. 2001; Jeffery and Morrell 2002; Stenmark, Davie et al. 2006) In response to vascular stress, fibroblasts show phenotypic changes characterised by increased proliferation, migration, differentiation and increased release of extracellular matrix components and growth factors that stimulate smooth muscle growth and recruitment of inflammatory and progenitor cells to the vessel wall. The key role of fibroblasts in vascular remodelling is supported by data showing that phenotypic changes in adventitial cells precede the intimal and medial remodeling (Siow, Mallawaarachchi et al. 2003; Herrmann, Samee et al. 2005). Hypoxia and other cellular stresses can increase the release of endothelial adenosine triphosphate (ATP) that acts synergistically with other growth factors released by smooth muscle cells or endothelial cells (Gerasimovskaya, Ahmad et al. 2002). Hypoxia-induced release of angiotensin II is also thought to facilitate fibroblast proliferation (Krick, Hanze et al. 2005).

Adventitial fibroblasts can differentiate into myofibroblasts expressing α-SMC actin (Zalewski, Shi et al. 2002) and this differentiation is regulated by several factors such as growth factors, cytokines, adhesion molecules and ECM molecules (Stenmark, Davie et al. 2006). Myofibroblasts trigger significant changes in the function and structure of the vessel wall by enhancing production of ECM proteins such as fibronectin and elastin (Gabbiani 2003). Among others, TGF-β is capable of inducing fibroblast-myofibroblast transition by stimulating α-SMC-actin expression and collagen production (Gebb and Jones 2003).

1.4. Key signalling mediators in PH

1.4.1. Nitric Oxide

Decrease in nitric oxide (NO) bioavailability is one of the key manifestations of pulmonary endothelial dysfunction in PH. NO is required for the maintenance of pulmonary endothelial barrier function and has vasodilatory and anti-inflammatory effects in the vasculature (Predescu, Predescu et al. 2005).

NO is synthesized in a series of biochemical reactions that transfer electrons from molecular oxygen through a number of cofactors, such as reduced nicotinamide adenine dinucleotide phosphate (NADPH), 6(R)-5,6,7,8-tetrahydrobiopterin (BH4), flavin, calmodulin and iron. These reactions are catalysed by a family of enzymes termed nitric oxide synthases (NOS), which through the oxidation of L-arginine lead to the formation of NO and L-citrulline (Palmer, Ferrige et al. 1987). Three isoforms of NOS have been found in mammals with different tissue distribution and regulation: neuronal NOS (nNOS; NOS1), which acts as neurotransmitter in the central nervous system, inducible (iNOS; NOS2) that takes part in inflammatory responses, and endothelial NOS (eNOS; NOS3), important in the regulation of vascular tone.

Synthesis of NO via NOS is dependent on the availability of substrates and cofactors such as L-arginine and tetrahydrobiopterin (BH4). NO synthesis can be enhanced by increasing nutritional intake of arginine, even though the intracellular concentrations of L-arginine greatly exceed the Km of NOS (Flam, Eichler et al. 2007). One explanation for this “arginine paradox” is that the intake of L-arginine can antagonise asymmetric dimethylarginine (ADMA), the endogenous NOS inhibitor (Albsmeier, Tsikas et al. 2002). In fact, all NOS isoforms can be competitively inhibited by the naturally occurring arginine analogues ADMA and L-NG-monomethyl arginine (L-NMMA). Apart from competing with L-arginine, ADMA and L-NMMA can also “uncouple” NOS, leading to reactive oxygen species (ROS) generation, implicated in pathogenesis of PH (Wilcox 2012). ADMA metabolism is further described in chapter 1.5.

Pulmonary eNOS expression increases during fetal life and then declines during progression to adulthood. This temporal increase is crucial for the dilation of the pulmonary vascular bed after birth. In fact, disruption of NO synthesis has been implicated in animal model of foetal PH and human congenital pulmonary hypertension (North, Moya et al. 1995;

Villamor, LeCras et al. 1997). In the adult, eNOS-derived NO acts as a vasodilator, stabilises endothelial barrier function, inhibits platelet aggregation and leukocyte adhesion and inhibits vascular smooth muscle cell proliferation (Shaul 2002).

The NO/cGMP signalling cascade plays an essential role in the vascular smooth muscle relaxation (figure 5). *In vitro* and *in vivo* studies show that NO and atrial natriuretic peptide (ANP) decrease blood pressure by causing relaxation of small arteries and arterioles (Sausbier, Schubert et al. 2000). Both NO and ANP stimulate guanylate cyclase (GC) (soluble or particulate respectively) and increase intracellular levels of cyclic guanosine monophosphate (cGMP).

cGMP activates protein kinase G (PKG) which in turn activates potassium (K^+) channels and allows K^+ to flux out of the cell, causing plasma membrane hyperpolarization. This leads to inhibition of voltage-dependent Ca^{2+} channels (VOC), reduced Ca^{2+} influx and vasorelaxation. cGMP can also lower intracellular Ca2+ levels by inhibition of receptor-operated Ca^{2+} (ROC) channels (Lincoln, Dey et al. 2001). ROC channels are activated via receptor-mediated stimulation of G-protein coupled receptors (GPCRs) directly or via the second messenger, inositol 1,4,5-trisphosphate (IP3) (Qiao, He et al. 2014). PKG can also lower intracellular Ca^{2+} levels by encouraging sequestration of Ca^{2+} and inhibiting its release from sarcoplasmic reticulum (SR). PKG increases Ca^{2+} sequestration by phosphorylation of the SR protein, phospholamban (Qiao, He et al. 2014) and by activation of the SR Ca^{2+}-ATPase (SERCA) (Lincoln, Dey et al. 2001). Ca^{2+} release from the SR is decreased by PKG-mediated phosphorylation of IP3 receptor (Xu, Kong et al. 2007).

Lowering of intracellular Ca^{2+} levels reduce Ca^{2+}/calmodulin-dependent phosphorylation of myosin light chain (MLC), leading to inhibition of actomyosin contractility and vasorelaxation. In addition, PKG can decrease MLC phosphorylation by activating myosin light chain phosphatase (MLCP). This happens via the PKG-mediated phosphorylation of RhoA which in turn inhibits phosphorylation of MLCP by RhoA effector, Rho kinase (Patel and Rattan 2007).

eNOS expression and activity show inverse correlation with the severity of morphological changes in PH lungs (Giaid and Saleh 1995). eNOS knockout mice show increased right ventricular hypertrophy (RVH) under normoxic and hypoxic conditions (Steudel, Ichinose et al. 1997). Conversely, inhaled NO reduces right ventricular hypertrophy in animal models of

pulmonary hypertension (Roberts, Roberts et al. 1995). NO is a downstream effector of vascular endothelial growth factor (VEGF) and is required for the VEGF-induced effects on endothelial proliferation and angiogenesis (Bernatchez, Soker et al. 1999). In a regulatory feedback response, NO can also up-regulate VEGF expression in cells (Ziche, Morbidelli et al. 1997). The pro-angiogenic effects of endothelial NO can be beneficial during lung development and endothelial wound repair but increase in eNOS expression has also been implicated in plexiform lesion formation (Budhiraja, Tuder et al. 2004).

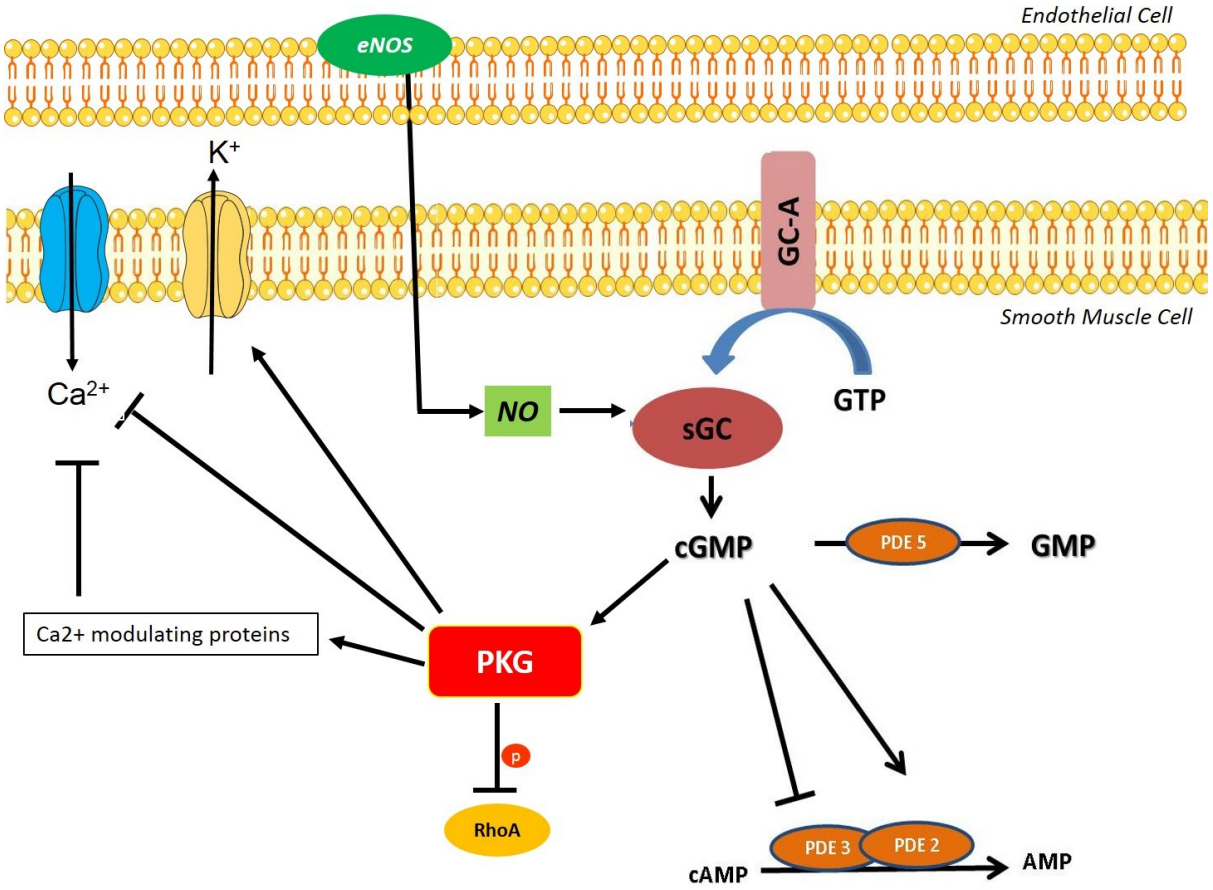

Figure 5: Nitric oxide pathway and cyclic guanosine monophosphate pathways in smooth muscle cell vasorelaxation. NO synthesized by endothelial nitric oxide synthase (eNOS) stimulates soluble guanylate cyclase (sGC) in smooth muscle cells and increases the levels of intracellular cyclic guanosine monophosphate (cGMP). The particulate form of guanylyl cyclase (GC-A) is stimulated by atrial natriuretic peptide (ANP). cGMP targets include: phosphodiesterases 2 and 3 (PDE2 and 3), and protein kinase G (PKG). PKG induces vasorelaxation by interacting with Ca^{2+} modulating proteins in plasma membrane and sarcoplasmic reticulum and lowering intracellular Ca^{2+} levels (for further details please see text page 32). In addition, PKG can phosphorylate and inactivate RhoA leading to activation of myosin light chain phosphatase (MLCP), reduction in myosin light chain phosphorylation and vasorelaxation.

1.4.2. Guanylate Cyclase

The guanylyl cyclase activity can be found in both particulate (pGC) and soluble (sGC) fractions of all mammalian cells (Lucas, Pitari et al. 2000). Soluble guanylate cyclase (sGC) is the main downstream effector of NO, which synthesises the second messenger, cyclic guanosine monophosphate (cGMP) (figure 6).

Alterations in cGMP synthesis and metabolism have been implicated in a number of cardiovascular diseases, including systemic arterial hypertension and PH, coronary artery disease, peripheral vascular disease and atherosclerosis (Tsai and Kass 2009; Stasch, Pacher et al. 2011).

sGC is a heterodimer composed of α1β1 and α2β2 isoforms. The α1β1 isoform is recognised as the most abundant in the vasculature and responsible for smooth muscle relaxation (Hobbs 1997; Russwurm and Koesling 2004), while α2β2 is thought to play a key role in synaptic transmission (Russwurm, Wittau et al. 2001). sGC is widely expressed in all mammalian cells and mediates several physiological functions, such as inhibition of platelet aggregation, immunomodulation and vasodilation/smooth muscle relaxation (Collier and Vallance 1989). In particular, sGC-α1 is essential for the pulmonary vasodilator response to inhaled NO in chronic hypoxia-induced pulmonary hypertension (Vermeersch, Buys et al. 2007).

Pharmacological agents that could target sGC-cGMP signaling directly can be divided into two main categories: sGC stimulators and sGC activators. As shown in figure 6 stimulators (such as Bay41-2272), can sensitise sGC to low levels of NO, maintaining the enzyme in its active configuration. Their action is dependent on the presence of the ferrous heme (Fe II); in addition they can increase sGC activity in the absence of NO (Evgenov, Pacher et al. 2006). In contrast, sGC activators (Bay58-2667) preferentially activate sGC in the oxidized state (Fe III) (Dumitrascu, Weissmann et al. 2006; Tamargo, Duarte et al. 2010). Both types of compounds have been shown to attenuate structural remodelling of pulmonary arteries in experimental models of hypoxia-induced pulmonary hypertension (Li, Zhou et al. 1999; Li, Laubach et al. 2001).

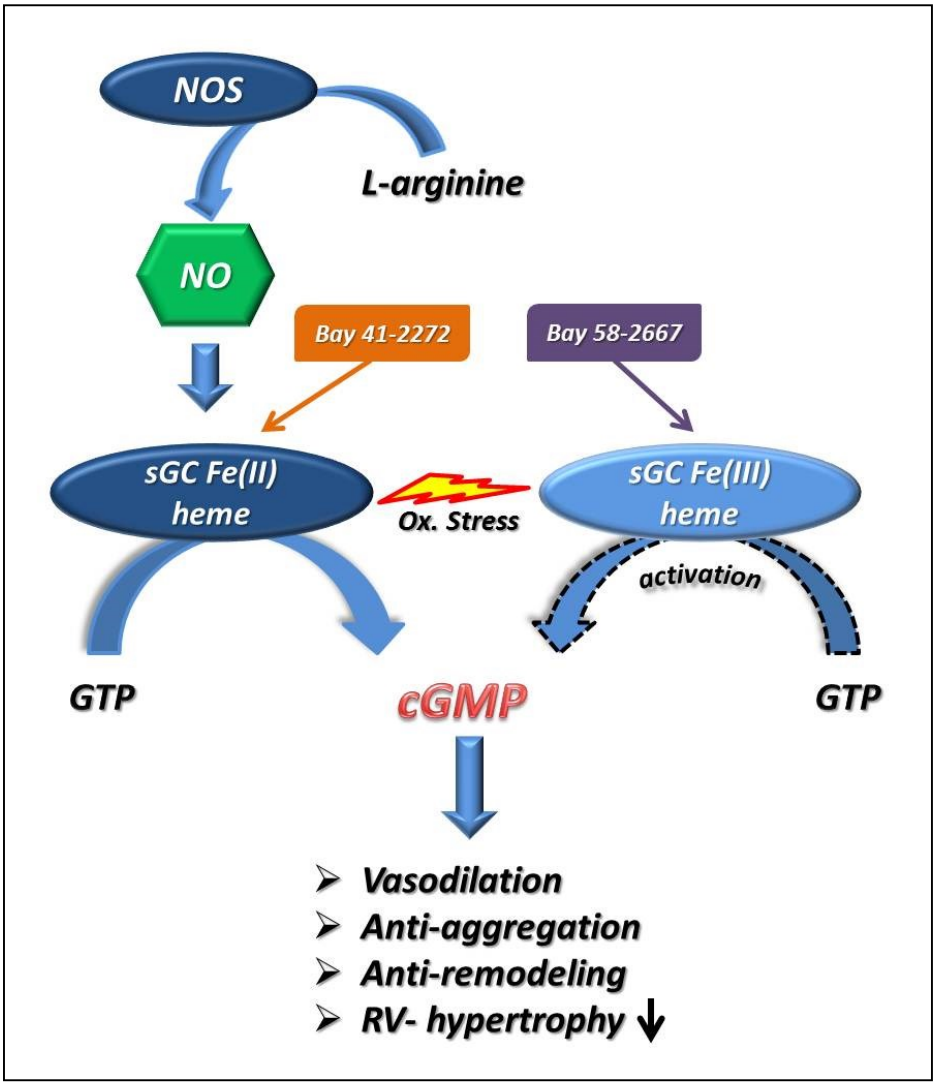

Figure 6: The sGC stimulator Bay41-2272 and the activator Bay58-2667 increase cGMP production. Bay41-2272 is a NO-dependent stimulator acting on the physiological form of sGC containing the Fe (II) heme of sGC (left). Conversely, Bay58-2667 is a NO-independent activator acting on the Fe (III) heme of sGC (right). Increased levels of cGMP start vasodilatation and anti-aggregation resulting in anti-remodeling of the vascular wall and unloading of the right ventricle. GTP (guanosine triphosphate), ox. Stress, (oxidative stress), RV (right ventricle). Adapted from (Baliga, MacAllister et al. 2011)

1.5. The DDAH/ADMA pathway

1.5.1. Methylarginines

Methylation of the amino acids histidine, arginine and lysine in proteins is an essential form of post-translational modification used by cells to expand their functional set of proteins (Teerlink 2005).

Methylarginines, asymmetric dimethylarginine (ADMA) and its isoforms NG-monomethyl-L-arginine (L-NMMA or MMA) and symmetric dimethyl-L-arginine (SDMA), are endogenous amino acids that circulate in plasma (0.4-1 µM) (Lajer, Tarnow et al. 2008). Methylarginines are generated by proteolytic degradation of proteins methylated by protein arginine N-methyltransferase enzymes (PRMTs) (figure 7). PRMTs are broadly distributed in the body and mainly two types of PRMTs (Type 1 and 2) catalyse the methylation of arginine residues. Type 1, which catalyses the formation of ADMA by adding a single methyl group, and type 2 that can methylate both of the guanosine-nitrogen on the arginine residues (Bedford and Richard 2005). As showed in figure 7, PRMTs methylate protein arginine residues. PRMTs use S-adenosylmethionine (SAM), which is synthesised from methionine and ATP, as the methyl group donor. Subsequently, the transfer of its methyl group converts SAM into S-adenosylhomocysteine (SAH). When methylated proteins are hydrolysed, free methylarginines ADMA, L-NMMA and SDMA are released (Wada, Inoue et al. 2002). L-arginine and methylarginines are transported into the cell through the same y^+ cationic amino acid transporter and so the excess of methylarginines can limit the pool of substrate available to NOS (Pope, Karuppiah et al. 2009). Only ADMA and L-NMMA can compete with L-arginine for NOS binding and inhibit NO production, while SDMA does not act as NOS inhibitor (Teerlink 2005). ADMA and L-NMMA are metabolised to L-citrulline and dimethylamine by enzymes dimethylarginine dimethylaminohydrolases 1 and 2 (DDAH 1 and DDAH 2) (Vallance and Leiper 2004), while SDMA is usually removed by renal excretion (figure 7)(Cooke and Ghebremariam 2011).

Interestingly, *in vitro* experiments have shown that exposure of endothelial cells to pathological concentrations of ADMA can drastically reduce NO synthesis and increase superoxide generation (Boger, Bode-Boger et al. 2000).

Therefore, by inhibiting eNOS, ADMA can indirectly increase blood pressure and cause vasoconstriction. Other studies showed that a long-term exposure to ADMA can enhance

atherogenesis and produce sustained hypertensive damage to the vessels and kidneys in animal models of pulmonary hypertension (Naruse, Shimizu et al. 1994).

The human body generates nearly 300 µmol/day of ADMA but only around 50 µmol/day is excreted in urine (Achan, Broadhead et al. 2003). Accordingly, data from humans studies showed that the local intra-arterial infusion of ADMA can significantly reduce forearm blood flow (Calver, Collier et al. 1993), while the intravenous infusion of ADMA can increase the mean blood pressure by 6% and systemic vascular resistance by 24% reducing the heart rate and vascular responsiveness (Achan, Broadhead et al. 2003). ADMA plasma levels are raised in a number of pathologies including pulmonary hypertension, hypercholesterolemia, type II diabetes, hypertension, congestive heart failure, renal disease and cancer (Cooke 2004).

In addition, it has been shown that ADMA inhibits VEGF-induced endothelial angiogenesis and chemotaxis *in vitro* and *in vivo* as a result of reduced NO/cGMP signalling, inhibition of vasodilator-stimulated phosphoprotein (VASP) phosphorylation and Rac1 activity, important players in cell polarization and focal adhesion turnover (Wojciak-Stothard, Torondel et al. 2009). Due to the correlation with adverse cardiovascular outcomes, ADMA has been recognized as an independent cardiovascular risk factor.

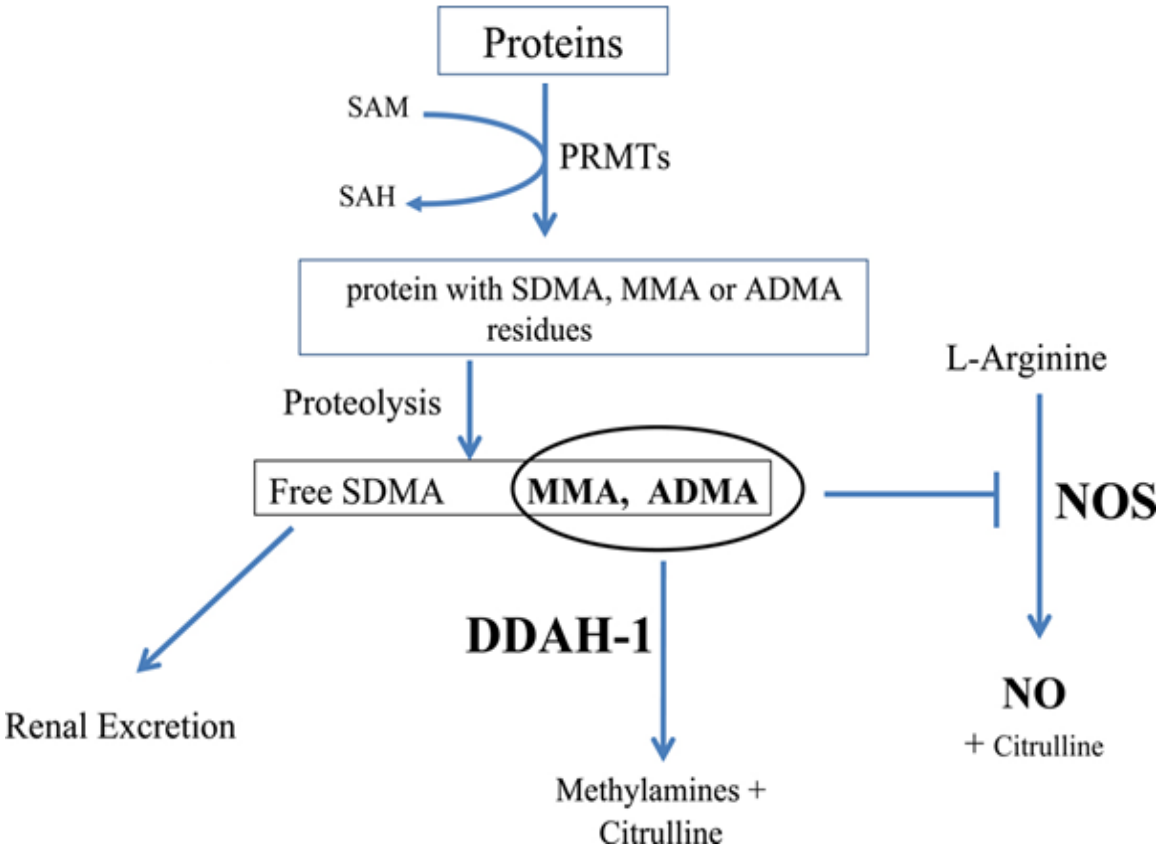

Figure 7: Synthesis and degradation of methylarginines. Protein arginine N-methyltransferase enzymes (PRMTs) transfer a methyl group from S-adenosyl-L-methionine (SAM) to each guanidino nitrogen of an arginine residue. This reaction produces a protein with methylarginines residue and S-adenosyl-L-homocysteine (SAH). Hydrolysis of the methylated proteins releases SDMA, MMA or NMMA and ADMA. MMA and ADMA are competitive inhibitors of endothelial NOS. Approximately 20% of all methylarginines are removed by urine excretion, while the remaining 80% is metabolised by DDAH. SDMA: symmetric dimethyl-L-arginine, ADMA: asymmetric dimethylarginine L-NMMA or MMA: NG-monomethyl-L-arginine. Adapted from (Cooke and Ghebremariam 2011).

1.5.2. DDAH enzymes

In PH, increased plasma and lung tissue levels of ADMA are associated with reduced expression and activity of DDAH (Leiper, Murray-Rust et al. 2002). Two isoforms of DDAH enzymes, DDAH1 and DDAH2, are respectively encoded by genes on chromosome 1p22 and 6p21.3. 38. Structurally, the two cytosolic proteins display 62% homology at the amino acid level and 63% at the nucleotide level; DDAH1 is widely expressed in the lung, liver and kidney (major sites for methylarginines metabolism) at sites of NOS expression (Nijveldt, Teerlink et al. 2003). DDAH-1 expression has also been detected in the pancreas, aorta, peritoneal neutrophils and macrophages in fetal and adult tissues (Tran, Fox et al. 2000). Mouse homozygous gene deletion of DDAH1 is lethal *in utero* and DDAH1 +/− mice show increased right ventricular and pulmonary pressure (Leiper, Nandi et al. 2007).

The DDAH-2 enzyme is abundant in fetal tissues, vascular endothelium, and tissues that play a role in immune responses (spleen, thymus, peripheral leukocytes, lymph nodes, and bone marrow) (Palm, Onozato et al. 2007). Both DDAH1 and DDAH2 have been implicated in the regulation of NO production in the vascular endothelium. Furthermore, the fact that DDAH2 is highly expressed in immune cells and DDAH-2 gene is located near the MHC III region of the chromosome 6p21.3, suggests a potential role of DDAH2 in development of autoimmune diseases.

While both DDAH1 and DDAH2 have been implicated in the pathogenesis of PH, their individual contributions are not well understood. Studies of DDAH gene silencing/deletion in rodents lead to the conclusion that plasma levels of ADMA are predominantly regulated by DDAH1, while DDAH-2 plays a lesser role (Palm, Onozato et al. 2007). For example, in transgenic mice overexpressing the human DDAH1 (hDDAH1), circulating levels of ADMA were 30–50% lower compared to wild-type (WT) mice. The hDDAH1 transgenic mice also exhibited a reduction in systolic blood pressure and systemic vascular resistance (Schwedhelm, von Leitner et al. 2009). Under pathophysiological conditions, DDAH1 overexpression reduces development of graft coronary artery disease (Tanaka, Sydow et al. 2005), improves endothelial regeneration (Konishi, Sydow et al. 2007) and reduces myocardial reperfusion injury (Stuhlinger, Conci et al. 2007), supporting the hypothesis that circulating ADMA concentrations are directly influenced by DDAH activity in cardiovascular tissues. In addition, studies reported that the ADMA/DDAH pathway is also implicated in the

regulation of the activity of Rho GTPases, which can be controlled by phosphorylation/dephosphorylation cycle, dependent upon the level of NO (Loirand, Guilluy et al. 2006).

Although many of the effects of DDAH appear to be mediated by NO signalling, recent data show that DDAH enzymes can also activate important processes in a NO-independent way (Zhang, Hu et al. 2011). Indeed, this study found evidence of DDAH1/Ras interaction, suggesting a novel pathway where Ras acts as a downstream effector of DDAH1 and regulates endothelial function via Akt phosphorylation (protein kinase B) (Zhang, Hu et al. 2011).

1.5.3. Rho signalling

Rho GTPases are Ras homologues proteins (hence the name ''Ras homologous'') that regulate organisation of the actin cytoskeleton via the cell surface receptors; in humans, more than 25 Rho GTPases have been identified with 80-90% homology among them (Hall 1998). Apart from the actin cytoskeleton organisation, Rho proteins have several cellular functions such as regulation of cell motility, cell survival, cell cycle progression, membrane trafficking and transcriptional regulation (Etienne-Manneville and Hall 2002). Rho GTPases can be activated by several stimuli including vasoactive molecules (ET-1, VEGF, histamine) or by mechanical stimuli such as shear stress, pressure and hypoxia (figure 8) (Wojciak-Stothard, Tsang et al. 2005). Rho proteins can switch between an active, guanosine 5c-triphosphate (GTP) bound, and an inactive, guanosine 5c diphosphate (GDP) bound conformation (Jaffe and Hall 2005). In their active conformation, Rho proteins interact with downstream effectors, such as Rho Kinase (ROCK), to stimulate cellular responses.

Several studies established the importance of RhoA and its downstream mediator, ROCK, in pulmonary arterial contractility and remodelling (Loirand and Pacaud 2010; Connolly and Aaronson 2011). The importance of RhoA/ROCK pathway has been highlighted in smooth muscle cells, where both contractile and proliferative phenotypes may depend on RhoA/ROCK signalling (Mack, Somlyo et al. 2001). In addition, due its ability to increase phosphorylation of myosin light chain (MLC), the RhoA/ROCK pathway is crucial in the regulation of actomyosin contractility and cell motility (Connolly and Aaronson 2011).

In endothelial cells, well characterised members of the Rho GTPases family, RhoA, and Rac1 play opposing roles in the regulation of endothelial barrier function. Activation of RhoA in

vascular endothelial cells increases actomyosin contractility that pulls the intercellular junctions apart, compromising endothelial barrier function while Rac1 can neutralize the actions of RhoA by increasing cell-to-cell adhesion (Wojciak-Stothard, Torondel et al. 2009). A coordinated activation of Rho GTPases RhoA, Rac1 and Cdc42 is required for cell movement. In particular, Rac1 and Cdc42 control the formation of membrane protrusions at the cell front, lamellipodia and filopodia respectively, while RhoA is required for retraction of the rear end of the cell (Ridley 2001).

Studies have demonstrated that protein kinase G (PKG)-mediated phosphorylation of RhoA on serine 188 can inhibit its activation at the cell membrane (Ellerbroek, Wennerberg et al. 2003). ADMA can activate RhoA by preventing NO/PKG-mediated phosphorylation, leading to a firm cell-substratum adhesion and inhibition of endothelial cell motility (Ridley 2001). ADMA has also been shown to abrogate VEGF-induced endothelial angiogenesis by inhibiting the activity of Rac1 in a NO/cGMP/PKG-dependent way (Wojciak-Stothard, Torondel et al. 2009).

Recently, it has been shown that RhoB, a protein homologous to RhoA, is required for the hypoxia-induced changes in endothelial barrier function and pulmonary vascular smooth muscle cell migration and proliferation (Wojciak-Stothard and Wilkins 2012). Indeed, RhoB gene knockout attenuated development of chronic hypoxia-induced PH in mice, probably due the inhibition of Hypoxia-Inducible Factor-1 (HIF-1) and platelet-derived growth factor (PDGF) signalling (Wojciak-Stothard and Wilkins 2012). Interestingly, while RhoA can be phosphorylated and inactivated by PKG, RhoB lacks of Ser188 phosphorylation site and therefore cannot be directly modified by NO/PKG signaling.

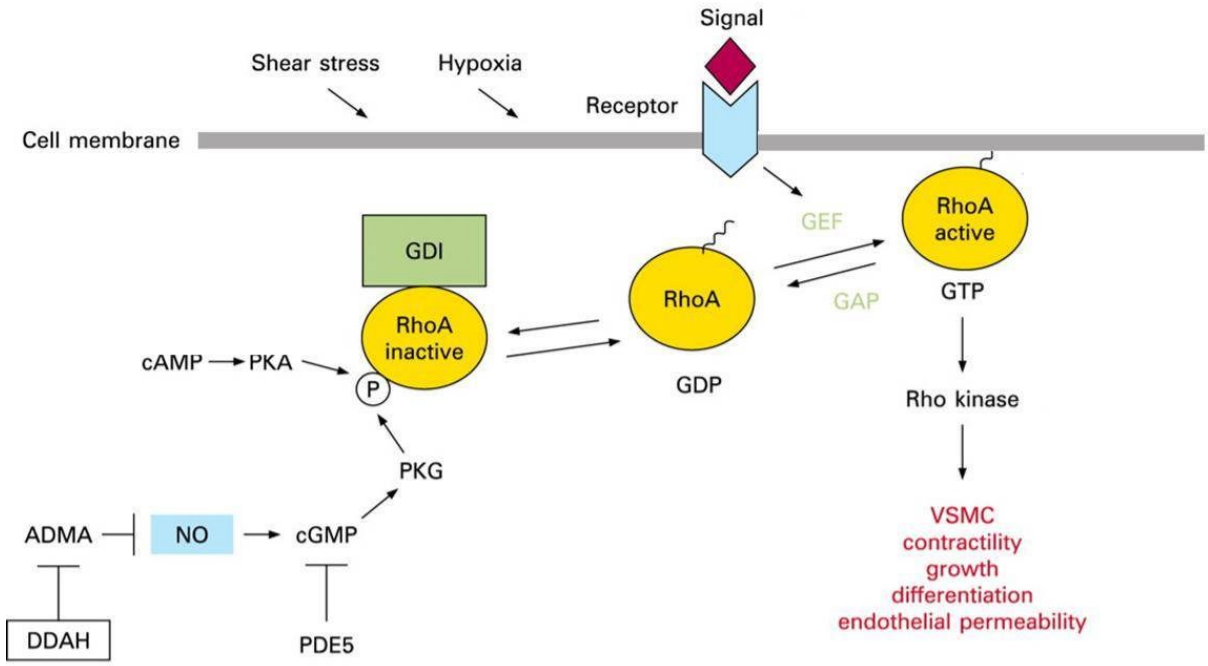

Figure 8: Pathways affecting RhoA activity in pulmonary vasculature. RhoA can switch between an active guanosine triphosphate (GTP) bound state, and an inactive guanosine diphosphate (GDP) bound state. To exert its effects, RhoA has to be anchored to the cell membrane by an isoprenoid tail. Phosphorylation of RhoA by protein kinase A (PKA) or protein kinase G (PKG) stimulates the association of RhoA with guanine dissociation inhibitor (GDI), which renders the protein inactive. The inhibition of Rho kinase can prevent endothelial dysfunction, smooth muscle cell (SMC) contractility, growth and differentiation. ADMA, asymmetric methylarginine; DDAH, dimethylarginine dimethylaminohydrolases; GAPs, GTPase activating proteins; GEFs, guanosine nucleotide exchange factors; VSMC, vascular smooth muscle cells. Adapted from (Wojciak-Stothard 2008)

1.5.4. BMP signaling

BMPs are the largest group of cytokines within the TGF-β superfamily. They were originally identified as molecules regulating growth and differentiation of bone and cartilage (Reddi 1994). The TGF-β superfamily is composed of mediators such as TGF-β isoforms (TGF-β3), BMPs, activins and growth and differentiation factors (GDF). Genetic studies have shown that 10%–20% of patients with IPAH, and 70% of patients with hereditary PH, are heterozygous for a mutation in BMPR2 (Lane, Machado et al. 2000).

The TGF-β superfamily has several roles in a variety of cellular processes such as cell proliferation, immunity, differentiation and inflammation. Fifteen BMPs have been identified so far. BMPs act via transmembrane serine/threonine kinase receptors and the binding to complexes containing type I and type II receptors. Type I receptors (50-55 kD) require type II receptors for the ligand binding, while type II receptors (70-80 kD) can bind ligands in the absence of type I receptors, but they require type I receptors for their signaling (Gilboa, Nohe et al. 2000). The ligand/receptor interaction results in signaling through SMAD-dependent or -independent pathway (Sieber, Kopf et al. 2009) (figure 9). Meanwhile in the SMAD-dependent pathway, BMP/type I receptor interaction activate SMAD 1/5/8 protein complex and induce the translocation of this signaling complex to the nucleus via the interaction with SMAD4, in the SMAD-independent pathway, MAP kinase (MAPKs), phosphatidylinositol 3-kinase/AKT, and protein kinase C are involved (Lowery and de Caestecker 2010).

The type II receptor (BMPR2) is expressed ubiquitously and after the ligand binding, BMPR2 phosphorylates the intracellular glycine-serine rich domain on of the co-receptor BMPR1 (figure 9. The phosphorylation allows the conformational changes that are essential for the subsequent signal transduction (Morrell 2006). BMPR2 seems to play a critical role in PH; for example, mutation in BMPR2 can have different effects depending on the cell type. For example, in PASMCs from PH patients BMPR2 mutations promote cell proliferation (Davies and Morrell 2008). In endothelial cells, loss of BMPR2 function results in increased apoptosis (Teichert-Kuliszewska, Kutryk et al. 2006); an early event in the pathogenesis of PH. Furthermore, activation of endothelial BMPR2 by BMP2 or BMP4 can stimulate eNOS phosphorylation and activation. The activation of eNOS by BMP2 and BMP4 interaction with BMPR2 has been shown to be largely dependent on protein kinase A (PKA) activity

(Gangopahyay, Oran et al. 2011). Consistent with the hypothesized central role of this signaling pathway in PH, a recent study associated the loss of BMPR2 function with abnormal DNA repair in PH, suggesting an association between EC dysfunction and genomic instability (Li, Vattulainen et al. 2014).

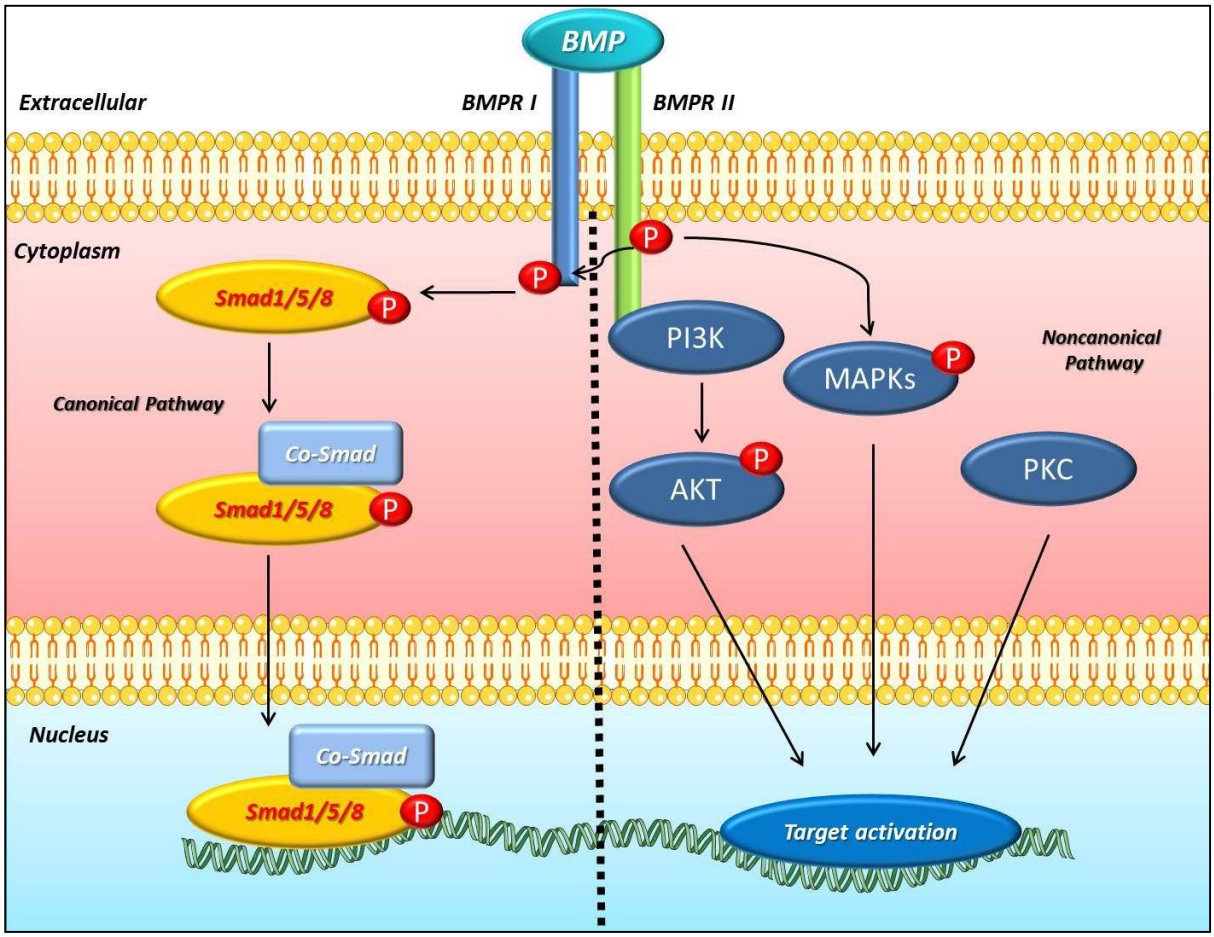

Figure 9: Schematic diagram of canonical and noncanonical BMP pathways. The dashed line separates canonical TGF-β signaling (on the left) from some of the noncanonical pathways activated by BMPR2. In the Smad-mediated pathway, the activation of the BMP receptor complex by BMP ligand interaction leads to phosphorylation of R-Smads 1, 5, and 8, which enables the interaction between R-Smads and Co-Smad proteins resulting in the nuclear transportation of the heteromeric complexes and activation of gene transcription. In the Smad-independent pathway, several intracellular pathways are activated, such as PKC (protein kinase C), MAPK pathway and AKT (protein kinase B) after phosphorylation by PI3K (phosphoinositide 3-kinase), resulting in gene target activation.

1.5.5. Other mediators

1.5.5.1. Hypoxia-inducible factors

Hypoxia-inducible factors 1 have been implicated in the development of PH. The β subunit of HIF-1 (HIF-1β) is constitutively expressed, while the α-subunit (HIF-1α) is usually not detectable under normoxic conditions. Several years after the discovery of HIF-1α, a closely related protein was identified based on its sequence similarity and subsequently named HIF-2α (Shimoda and Semenza 2011). Like HIF-1α, HIF-2α dimerizes with HIF-1β; however, unlike HIF-1α, which is found in all nucleated cells, HIF-2α protein expression is restricted to the vascular endothelium and type II pneumocytes (Shimoda and Semenza 2011). HIF-1α and HIF-1α undergo ubiquitination (hence degradation) after hydroxylation of two proline residues (Semenza 2005). Under hypoxic conditions, hydroxylation becomes inadequate and HIF-1α or HIF-2α accumulates. Thus, HIF-1α or HIF-2α via hydroxylation controls the activation of the HIF-1 transcription complex.

Several studies have documented the important role for HIF in the development of hypoxia-induced PH (Shimoda and Semenza 2011). For example, either HIF-1α or HIF-2α heterozygous knockout mice show attenuated chronic hypoxia-induced increase in pulmonary arterial pressure and right ventricular hypertrophy (Yu, Shimoda et al. 1999; Brusselmans, Compernolle et al. 2003). Pharmacologic inhibitors of HIF activity reduce vascular remodeling in hypoxic-induced PH mice (Abud, Maylor et al. 2012).

The mechanism by which HIF mediates vascular remodeling during chronic hypoxia needs further investigation, but it is likely to involve both Ca2+ and pH homeostasis (Iyer, Kotch et al. 1998). HIF also regulates expression of factors involved in the pathogenesis of PH, such as ET-1 and VEGF (Tuder, Chacon et al. 2001; Prabhakar and Semenza 2012).

1.5.5.2. Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) promotes proliferation, angiogenesis and survival of cultured vascular endothelial cells. Although endothelial cells are the primary target of VEGF, mitogenic effects in other, non-endothelial cell types have also been reported (Ferrara, Gerber et al. 2003) (Liu, Cox et al. 1995; Nor, Christensen et al. 1999).

In vivo, VEGF induces vascular leakage and plays a central role in the regulation of angiogenesis and vasculogenesis (Le Cras, Markham et al. 2002). VEGF binds to two tyrosine-kinase receptors, VEGFR-1 and VEGFR-2, which are expressed almost exclusively in endothelial cells. Normally, ECs do not secrete VEGF; however, in PH ECs have been shown to express VEGF as a protective response (Budhiraja, Tuder et al. 2004). Consistent with the postulated protective role of VEGF signaling, VEGF receptor blockade in combination with hypoxia, induces severe PH in rats while VEGF overexpression protects against the disease in rats (Le Cras, Markham et al. 2002). However, opinions regarding the role of VEGF in PH are divided, as it can also contribute to the pathogenesis of the disease by promoting plasminogen and collagenase activation, monocyte infiltration and plexiform lesion formation (Unemori, Ferrara et al. 1992).

1.5.5.3. Endothelin-1

Endothelin-1 (ET-1) is a potent peptide vasoconstrictor secreted by ECs. There are three isoforms of endothelin (ET 1-3), but ET-1 is the most widely expressed and studied.

Apart from vasoconstriction, ET-1 can induce proliferation and migration of vascular cells (Shao, Park et al. 2011). In PASMCs, ET-1 binds two receptor (ETA and ETB), triggering the signaling pathway that involves inhibition of activity of potassium channels (K^+), increased levels of calcium (Ca^{2+}) and activation of Rho downstream mediators, such as ROCK. PASMCs highly express ETA and ETB receptors that can activate contraction, proliferation and migration, meanwhile in PAECs only ETB receptor is expressed (Shimoda, Sham et al. 2002).

While the activation of ETA can cause vasoconstriction, ETB activation can mediate either vasoconstriction by its effects on smooth muscle cells, or vasodilation through the action on endothelial cells. Interestingly, mRNA expression of both ETA and ETB is increased in experimental models of PH (Li, Chen et al. 1994; Frasch, Marshall et al. 1999), while in severe human PAH only ETB mRNA is increased (Bauer, Wilkens et al. 2002). Thus, it seems

that the effects of ET-1 depend on the balance of ETA and ETB receptors on endothelial and smooth muscle cells. Although ET receptor inhibitors are used in clinical practice (Hong, Coe et al. 2014), it is unclear if the improvement of survival in patients treated with ET-1 inhibitors is due to reduced vasoconstriction or remodelling.

1.5.5.4. Serotonin (5-HT)

Serotonin (5-HT, 5-hydroxytryptamine) has long been recognized as one of the most potent naturally occurring pulmonary vasoconstrictors (Egermayer, Town et al. 1999). It is synthesised by tryptophan hydroxylase 1 (Tph1) in the pulmonary endothelium (Mlczoch 1984). 5-HT was first implicated in the pathogenesis of PAH after an outbreak of the disease in Switzerland in the 1960's among patients using aminorex fumarate, an appetite suppressant that inhibits serotonin uptake by platelets (Lepine, Mordasini et al. 1982).

More than fifteen serotonin receptors (SERT or 5-HTT) have been identified so far, all of them capable of initiating signalling cascade involving generation of reactive oxygen species, activation of MAPKs and ROCK, and induction of genes involved in cell growth regulation (Maclean and Dempsie 2010). Studies have shown functional interaction between serotonin and BMP signalling (Long, MacLean et al. 2006). The serotonin receptor inhibition was shown to block proliferative responses in cultured pulmonary vascular cells (Welsh, Harnett et al. 2004).

1.6. MicroRNAs involved in PH

MicroRNAs (or MiRNAs, or miR) are small non-coding RNA molecules around 22 nucleotides long that target the 3'-untranslated region (UTR) of mRNA to negatively regulate gene expression (Bartel 2004). As illustrated in figure 10, the transcription of miRNAs is started by RNA polymerase II, which synthesises a molecule over 1kb in size called primary miRNA (pri-miRNA) from which several miRNAs can be produced. The pri-miRNA is then processed by the RNase III enzyme Drosha, forming a 50-60 nucleotides stem loop molecule called precursor miRNA (pre-miRNA). Afterwards, pre-miRNAs are transported out of the nucleus into the cytoplasm by Exportin-5 where they undergo cleavage by the endonuclease Dicer. The cleavage by Dicer creates a miRNA duplex and after the binding with argonaute protein (Ago2), the mature miRNA is formed. This mature miRNA is then incorporated into the RNA-induced silencing complex (RISC) which can bind the 3-UTR of the target mRNA sequence and regulate gene expression (Bartel 2004).

Several studies have now demonstrated an involvement of miRNAs in the pathogenesis of PH. The first report by Caruso et al, showed dysregulation of miRNAs (miR-22, miR-30, miR-322,miR-451,miR-21) in monocrotaline (MCT) and chronic hypoxia models of PH (Caruso, MacLean et al. 2010). MiRNAs disregulation (in particular miRNA-204) was found also in human pulmonary arteries from PH patients (Courboulin, Paulin et al. 2011), highlighting the role of miRNAs in the pathophysiology of PH.

Among all the miRNAs involved with the pathophysiology of PH, miR-21 has been investigated in several cell types and *in vivo* models (Parikh, Jin et al. 2012). Hypoxia can up-regulate miR-21 expression in human PASMCs (Sarkar, Gou et al. 2010) and human PAECs (Parikh, Jin et al. 2012) and in mouse lungs exposed to hypoxia (Yang, Banerjee et al. 2012). In addition, BMP signaling also has a role in modulating miR-21; in particular BMP4 via SMAD proteins can stimulate Drosha complex and promote maturation of miR-21, which in turn, can downregulate expression of target genes (Davis, Hilyard et al. 2008). However, the role of miR-21 in the pathogenesis of PH is not fully understood, with various laboratories showing conflicting data. For example, tissues from animal models of cardiac disease and patients with heart failure showed an up-regulation of miR-21 in cardiac fibroblasts (Thum, Gross et al. 2008), while another study reported that a reduction in miR-21 did not influence cardiac hypertrophy and fibrosis in mice (Patrick, Montgomery et al. 2010). For example, the

knock down of miR-21 in hypoxic mice reduced the right ventricular systolic pressure (RVSP) and a lowered muscularization of pulmonary arteries (Yang, Banerjee et al. 2012), while another study observed an exaggerated PH phenotype in mice lacking of miR-21 with an increased RVSP and increased pulmonary arterial muscularization (Parikh, Jin et al. 2012).
The dissimilarity among these studies could be due to differences in species and/or distinct methods used to control miR-21 levels or even differences in gender. Although the effects of miRNAs are not fully understood, these findings highlight the potential role of miRNAs in the pathogenesis of PH.

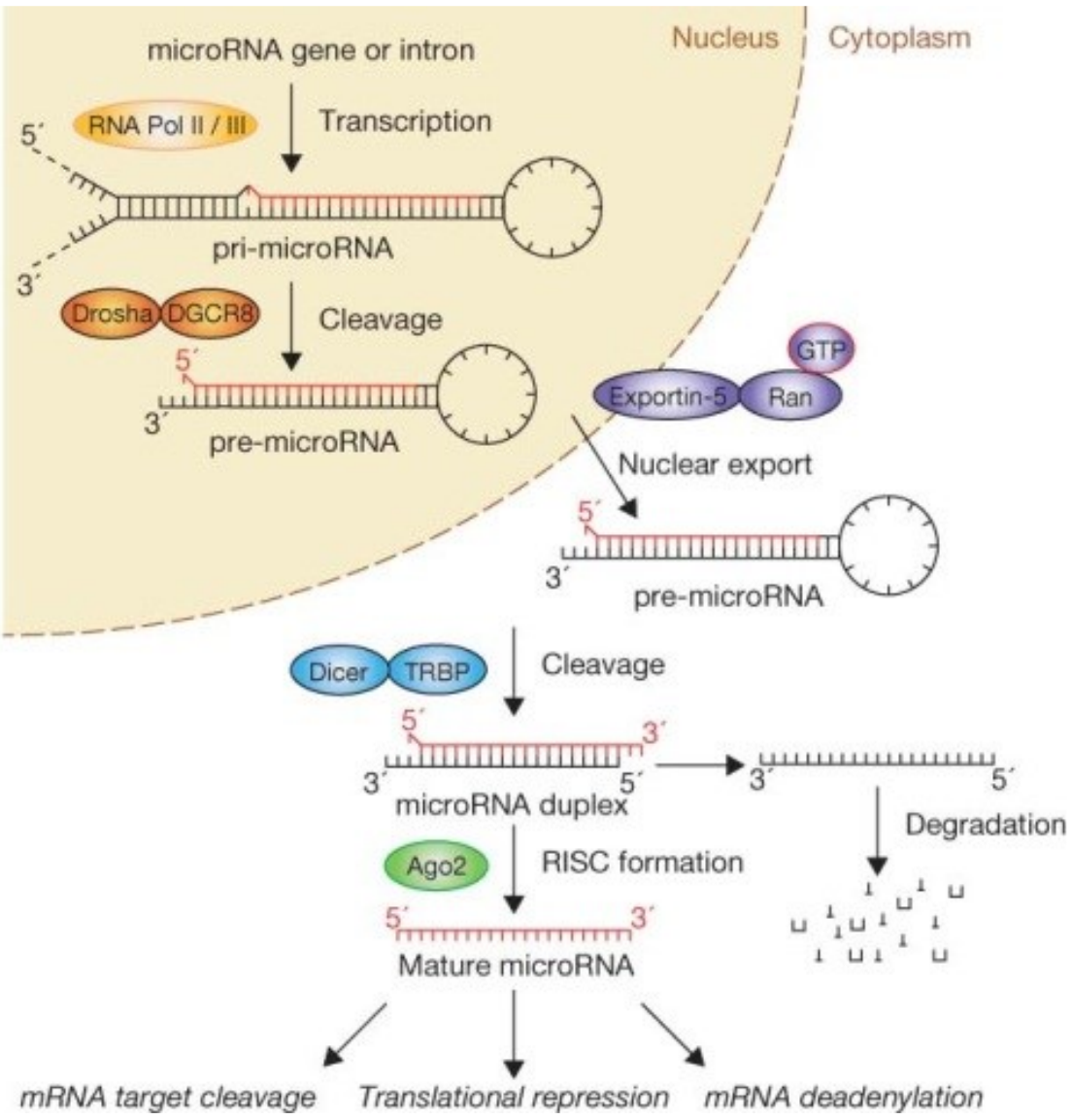

Figure 10: The miRNA processing pathway. The maturation comprises the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and the subsequent cleavage of the pri-miRNA by the Drosha–DGCR8 complex within the nucleus. The resulting pre-miRNA is afterward exported from the nucleus by Exportin-5in the cytoplasm where the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA to its mature length. The mature miRNA is bonded with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it leads RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation. Adapted from (Winter, Jung et al. 2009).

1.7. Animal models of PH

1.7.1. Chronic Hypoxia model

Hypoxia is frequently used to induce PH in a wide variety of animal species. The most commonly used species are rats and mice, where this model produces predictable and reproducible results within a selected animal strain. In rats, muscularization of small arteries starts quickly, due to medial SMCs proliferation (Meyrick and Perkett 1989). After 2-3 weeks of hypoxia, rats and mice develop moderate PH with increased pulmonary artery pressure and vascular remodeling. Inflammation has been implicated in the hypoxia-induced remodeling process, at least in some strains of rodents. It has been reported that hypoxia induced an early and persistent lung-specific inflammatory response manifested by mononuclear cells infiltrates (Burke, Frid et al. 2009).

Microarray analysis of the lung tissue demonstrates differences in gene expression induced by hypoxia between the species (Bull, Coldren et al. 2007). Chronic hypoxic exposure in the rat increased expression of genes involved in endothelial cell proliferation and decreased expression of genes associated with apoptosis. Furthermore, genes involved in proliferation and inflammation, such as phosphoinositide-3 kinase, were upregulated in rat lungs but not in mouse lungs. In chronic hypoxia-induced PH, animals develop right ventricular hypertrophy and show pulmonary vascular remodelling but there are no indications of plexogenic lesions, characteristic of severe PAH in humans (Yi, Kim et al. 2000). Although chronic hypoxia is not sufficient in inducing plexiform lesions, it is regarded as an important contributing factor.

1.7.2. Monocrotaline injury

Monocrotaline is a toxic pyrrolizidine alkaloid, which is activated *in vivo* by oxidases in the liver. The reactive compound MCTP leads to vascular injury, particularly in rats, after a single subcutaneous or intraperitoneal injection. Although monocrotaline induces PH in both rats and mice, rats remain the preferred species for this model. The response to monocrotaline is variable among species and strains of animals because of differences in the hepatic metabolism of this compound by cytochrome P-450. While the exact mechanism needs to be clarified, it has been speculated that monocrotaline induces endothelial damage that triggers pulmonary vascular remodelling (Jasmin, Lucas et al. 2001). Reports have also

suggested that adventitial inflammation, in particular macrophage accumulation, contributes to the pulmonary smooth muscle hypertrophy in this model (Wilson, Segall et al. 1989). Indeed, it has been suggested that adventitial inflammation may have more dramatic effects on the pathogenesis of PH than the ECs (Wilson, Segall et al. 1989). This model is considered to be an acute toxic model of damage to the lung vasculature and other organs (heart, kidney and liver). Similar to the chronic hypoxia- induced model of PH, monocrotaline does not induce obstructive intimal lesions in the peripheral vasculature of the lung.

1.7.3. Sugen 5416 and Hypoxia

Taraseviciene-Stewart developed a model of severe PH for a greater understanding of the mechanisms involved in the endothelial cell hyper-proliferation, which characterizes the plexogenic lesions of human PH (Taraseviciene-Stewart, Kasahara et al. 2001). The compound Sugen 5416 (SU-5416), a VEGF receptor inhibitor, causes mild PH and pulmonary vascular remodelling in normoxic rats, while in chronic hypoxic rats causes severe PH associated with arterial endothelial cell proliferation. The SU-5416-injected animals exhibit increased pulmonary vascular SMC proliferation and endothelial cells apoptosis. The authors speculate that the synergy between chronic hypoxia and SU-5416 vasoconstriction may stimulate the proliferation of apoptosis-resistant endothelial cells. In contrast to other animal models of the disease, in this model, PH can persist and progress besides the end of the hypoxic stimulus. SU-5416 injection, and concomitant exposure to hypoxia for 2 weeks, decrease the mRNA expression of the anti-apoptotic proteins (such as Bcl-2, Bcl-X), as compared with chronic hypoxic lung (Burke, Frid et al. 2009). Remarkably, in contrast to MCT, VEGF receptor blockade affects only the lung and no other organs. Another unexpected feature of this model is the absence of perivascular infiltration of monocytes/macrophages. This finding is somehow surprising since chronic hypoxia causes infiltration of mononuclear cells in the pulmonary arterial wall (Dumitrascu, Weissmann et al. 2006).

1.7.4. Additional animal models

1.7.4.1. Genetic Model of BMPR2 mutations

As previously mentioned, BMPR2 signaling plays a critical role in the pathogenesis of idiopathic PH and familial PH (Lane, Machado et al. 2000). Due the importance of BMPR2 pathway in PH, the mouse heterozygous for the BMPR2 allele (BMPR2 +/−) model was studied in order to provide an insight into the disease mechanisms (Beppu, Ichinose et al. 2004). BMPR2 +/− mice showed moderately elevated mean pulmonary artery pressure and pulmonary vascular resistance under basal conditions (Beppu, Ichinose et al. 2004), even though other reports demonstrated no significant difference in RVSP between BMPR2 +/− and wild type mice (Beppu, Ichinose et al. 2004). Furthermore, it has also been shown that mice with short hairpin RNA (shRNA)-mediated, constitutive knockdown of BMPR2, did not exhibit increases in PVR (Liu, Wang et al. 2007). Mice carrying a specific mutation in the BMPR2 tail domain developed PH with significant vascular remodeling and inflammation (West, Harral et al. 2008). Interestingly, only some mice with lung endothelium-specific deletion of the BMPR2 gene had elevated RVSP and RV hypertrophy (Hong, Lee et al. 2008). In spite of its limitations, the genetic model of BMPR2 mutations can be used to improve our understanding of the effects of gene mutations in PH.

1.7.4.2. The S100A4-overexpressing mice

The S100A4 protein is highly expressed in metastatic mouse mammary adenocarcinoma cells, conferring a metastatic phenotype that correlates with advanced stages of human tumors. The S100A4-overexpressing mouse, a cancer animal model (Ebralidze, Tulchinsky et al. 1989), has been also used to study PH (Ambartsumian, Klingelhofer et al. 1998).

Although in the lungs of healthy individuals low levels of S100A4 expression were detected, a marked expression of S100A4 has been observed in plexiform lesions. These findings suggest that S100A4 may be not involved in the development of PH but may have a role in the development of severe vascular lesions in the end-stage PH.

In addition, this model shows a marked inflammatory response, supporting the view that inflammation triggers the development of plexiform lesions in PH. However, increased S100A4 expression has also been noted in hypoxic models of PH, which do not show plexiform lesions (Kwapiszewska, Wilhelm et al. 2005). Hypoxic S100A4-overexpressing mice

show increased RV hypertrophy, but unlike in chronic hypoxia model, the RV hypertrophy does not regress on return to normoxic conditions (Merklinger, Wagner et al. 2005). In summary, this model shows a marked inflammatory response and development of plexogenic arteriopathy, suggesting once again that the inflammatory insult plays a key role in development of PH.

1.7.4.3. IL-6 overexpression in mice

Studies have shown increased levels of IL-6 in serum and lungs of patients with PH. Steiner et al. studied the role of IL-6 in the pathogenesis of PH using lung-specific, IL-6-overexpressing mice under normoxic and chronic hypoxic conditions (Steiner, Syrkina et al. 2009). These mice showed increased right ventricular systolic pressure, right ventricular hypertrophy and pulmonary vasculopathic changes, all exacerbated by hypoxia. The occlusive vascular lesions in this model were composed of ECs and T lymphocytes, probably due the activation of VEGF and pro-proliferative factors, such as mitogen-activated protein kinase (MAPK) (Steiner, Syrkina et al. 2009). Additionally, studies have also linked IL-6 expression to reductions in TGF-β signaling (Brock, Trenkmann et al. 2009) via inhibition of BMPR2/STAT3-microRNA 17/92 pathway (Brock, Trenkmann et al. 2009). The authors speculated that STAT3 plays an important role in the development of PH, since it has been shown that the activation of STAT3 can activate cell survival in PAECs from patients with PH. Conversely, hypoxic IL-6 −/− mice showed reduced inflammatory cell recruitment in the lungs compared with hypoxic wild-type mice, supporting the hypothesis that IL-6 has a pivotal role in hypoxia-induced pulmonary vascular remodeling (Savale, Tu et al. 2009).

1.8. Treatments for PH

1.8.1. Current treatments for PH

Patients with PH often receive diuretics, an anti-coagulant drug, Warfarin and oxygen, all treatments that are referred to as a conventional therapy. While diuretics provide a relief without affecting the course of the disease, Warfarin may improve survival, although this contribution is difficult to estimate. In the last 30 years, new treatments have been introduced in the clinical practice to directly target the pathology of PH. The three main categories of drugs have been approved for PH therapies are: prostanoid analogues (Epoprostenol, Beraprost), endothelin receptor antagonists (Bosentan, Macitentan) and phosphodiesterase-5 inhibitors (PDE-5) (Sildenafil, Tadalafil).

The first prostanoid analogue used in PH was Epoprostenol. Epoprostenol was the first treatment targeted directly at PAH pathology, and has a proven survival advantage (Rubin, Mendoza et al. 1990). In fact, the intravenous (IV) injection of epoprostenol improved the pulmonary haemodynamic and enhanced survival (Rubin, Mendoza et al. 1990). However, it has short half-life (< 5 min) and the route of administration requires constant monitoring. The adverse effects, in common with all the prostanoids, are headache and gastrointestinal upset, but the main problem remains the route of delivery (Agarwal and Gomberg-Maitland 2011). These problems have led to the development of more stable analogues with more favourable means of administration and pharmacokinetic profiles, iloprost, trepostinil and beraprost are all used in the clinical management of PH patients. For example Beraprost, an orally administrated prostanoid analogue, shows a modest efficacy in PH patients (Galie, Humbert et al. 2002). Recent studies show that orally active prostanoid analogues were effective as a single therapy but did not display significant benefits when used in combination with endothelin receptor antagonists or PDE-5 (de Jesus Perez, Rosenzweig et al. 2012) (Jing, Parikh et al. 2013).

Endothelin receptor antagonists such as bosentan or ambrisentan have been shown to improve pulmonary haemodynamics, exercise capacity and reduce PAH symptoms (Williamson et al., 2000; Channick et al., 2001; Barst et al., 2004; Galie et al., 2005a); Although these drugs showed clinical benefit, in particular with PH associated connective tissue disease (Denton et al., 2008), a significant survival effect has not been observed. However, macitentan, a novel and highly potent antagonist of ET-A/B receptors, used during

the SHERAPHIN trial, showed reduced risk of morbidity/mortality event by 30% (3mg dose) to 45% (10mg dose) compared with placebo control (Pulido, Adzerikho et al. 2013).

Phosphodiesterases (PDEs) are the enzymes that facilitate the breakdown of the second messengers, cAMP and/or cGMP (Bender and Beavo 2006). 11 PDE families have been characterised, with each type consisting of several isoforms and splice variants. PDE inhibitors have been a major focus in drug development, particularly for cardiovascular disease. In the vasculature, PDE inhibitors exert several favourable effects including vasodilatation, inhibition of smooth muscle proliferation and prevention of platelet aggregation (Wharton, Strange et al. 2005). In particular, the blockade of PDE5, which metabolizes cGMP exclusively, reduces systemic and pulmonary artery pressure under physiological conditions in animals and humans (Madhani, Okorie et al. 2006).

Indeed, the first PDE-5 inhibitor approved for PH therapy was Sildenafil (Galie, Ghofrani et al. 2005) even though there were doubts regarding its impact on long term survival and haemodynamics. Tadalafil, another PDE-5 inhibitor, has also been examined as treatment and received approval for use in patients with PH in 2009 (Galie, Brundage et al. 2009).

Although PDE5 inhibitors represent a therapeutic advance, their effects on PAP are limited (only ~ 5 mm Hg reduction in PAP) (Baliga, MacAllister et al. 2011). A significant number of PH patients do not respond to sildenafil treatment. Additionally, in patients that respond well to sildenafil, there is often a dose-dependent systemic hypotension that can limit the beneficial effects of the drug. Nowadays, there is no evidence to suggest that modern PDE5 inhibitors have a greater effect than sildenafil, or that sildenafil-resistant patients will respond to other PDE5 inhibitors.

1.8.2. Emerging Treatments for PH

There are more than 30 drugs at different stages of development for treatment of PH. Although some represent variations of current drugs used in PH, the majority are novel drugs. These include receptor tyrosine kinase inhibitors (Imatinib, Sorafenib)(Ghofrani, Seeger et al. 2005; Moreno-Vinasco, Gomberg-Maitland et al. 2008), agents that enhance the coupling of eNOS (Cicletanine) (Waxman, Lawler et al. 2008), guanylate cyclase activators (Riociguat)(Ghofrani, Hoeper et al. 2010), prostacyclin receptor agonists (Selexipag) (Simonneau, Torbicki et al. 2012), vasoactive intestinal peptides (Petkov, Mosgoeller et al. 2003), serotonin inhibitors (Terguride) (Kekewska, Gornemann et al. 2012) and drugs targeting epigenetic activities (Zhao, Chen et al. 2012).

In addition, augmenting cGMP levels is thought to have a therapeutic value in PH, either in animal models or in patients with the disease (Baliga, MacAllister et al. 2011). Soluble guanylate cyclise (sGC) appears a good target in PH as its expression and activity are increased in conditions of decreased NO bioavailability (Black, Sanchez et al. 2001) and genetic deletion of this enzyme results in an exaggerated response to hypoxia-induced PH (Vermeersch, Buys et al. 2007).

Two different classes of sGC agonists have been developed. First class includes sGC 'stimulators' which stimulate the native Fe^{2+}-sGC and synergize with NO (Stasch, Dembowsky et al. 2002). Second class includes the sGC 'activators', which activate the Fe^{3+} or heme-free form of the enzyme and are additive with NO (Belik 2009). Both classes of drugs have favourable effects in experimental models of PH (Chester, Tourneux et al. 2009; Weissmann, Hackemack et al. 2009).

Riociguat is the first sGC stimulator that has entered clinical development. Clinical trials demonstrated that it improves pulmonary vascular haemodynamics and increases exercise ability in PH patients. Additionally, riociguat reduces mean pulmonary arterial pressure in and pulmonary vascular resistance in PH patients. Overall, these promising results suggest that sGC stimulators are a valuable new approach for treatment of PH (Stasch and Evgenov 2013).

Recently, experiments using animal models demonstrated the potential of microRNA-based therapies in PH.

MicroRNAs have a critical role in regulating gene expression in the cardiovascular system (Small and Olson 2011) and in particular two miRNAs have been found to regulate BMPR2 expression (Brock, Samillan et al. 2012; Pullamsetti, Doebele et al. 2012). It has been demonstrated that injection of inhibitors of miRNA-17 or miRNA-20 can improve haemodynamics and reduce cardiovascular remodelling in both hypoxia- and MCT-induced PH. Other potential therapies involving stem or progenitor cells have been proposed to enhance endothelial repair (Zhao, Courtman et al. 2005).

1.9. Main Hypothesis and Project Aims

Hypoxia causes pulmonary vasoconstriction and pulmonary hypertension, but the mechanisms involved are not well understood. In the current study, I hypothesized that increased ADMA metabolism would protect against hypoxia-induced endothelial and smooth muscle dysfunction *in vitro* and *in vivo* and would prevent pulmonary vascular remodelling in chronic hypoxia-induced PH.

The aims of the project:

1) To study the role of ADMA/DDAH in hypoxia-induced responses in human pulmonary artery endothelial and smooth muscle cells and to study the effect of DDAH over expression on hypoxia-induced cell responses *in vitro*.

2) To study the effect of increased DDAH expression on pulmonary vascular responses *in vivo*, in chronic hypoxia-induced mouse model of PH. Contributions of DDAH to hypoxia-induced pulmonary responses and pulmonary vascular remodelling will be characterised in transgenic mice overexpressing DDAH1 or DDAH2.

3) To identify mechanism responsible for the loss of DDAH activity in hypoxic pulmonary vasculature and to characterise main signalling mediators involved in DDAH-induced responses to hypoxia.

2. Materials and Methods

2.1. Cell Culture

Human Pulmonary Artery Endothelial Cells (HPAECs) and Human Pulmonary Artery Smooth Muscle Cells (HPASMCs) were purchased from Promo Cell (C-12241; C-12521). HPAECs were cultured in Endothelial Cell Basal Medium 2 (Promo Cell, C-22211) in tissue culture dishes coated with 10 mg/L bovine serum fibronectin (Sigma Aldrich F1141-5MG) in PBS, while HPASMCs were cultured in Smooth Muscle Cell Growth Medium (Promo cell, C-22062). The cells were purchased at passage 2 and used for experiments between passages 4-10.

The cells were left in a humidified incubator in normoxic conditions (PO_2 90-100 mmHg; 5% CO_2, 19% O_2, 37ºC) or exposed to hypoxia (PO_2 14-30 mmHg, 5% CO_2, 2% O2, 37ºC) for 1-48 h. These conditions were previously shown to affect pulmonary vascular cell phenotype, protein expression, and angiogenic potential *in vitro* (Wojciak-Stothard 2012; Tsang, Leiper et al. 2013).

Prior to hypoxic exposure, the cells were infected with adenoviruses to express mutant DDAH proteins. Alternatively, the cells were transfected with miR overexpression constructs or with miR inhibitors, locked nucleic acids (LNAs). In some experiments, the cells were incubated with 100 µM of ADMA, SDMA or L-257 (DDAHs inhibitor) for 0-24h. Following the treatment, the cells were used for studies of DDAH expression, cell proliferation, apoptosis or endothelial permeability.

2.2. RNA extraction and cDNA Synthesis

RNA was extracted using PureLink RNA Mini Kit (Invitrogen, 12183018A) according to manufacturers' instructions. In order to remove possible DNA contamination, all the samples were treated with DNase I (Amplification grade; AMPD1, Sigma-Aldrich) and the amount of RNA was quantified by absorption at 260 nm using NanoDrop® (ND-1000; NanoDrop Technologies, Willington, DE, USA). The first strand of complementary DNA (cDNA) was synthesised by reverse transcription (RT)-polymerase chain reaction (PCR). 2 µg of total RNA in 10 µl of diethylpyrocarbonate (DEPC)-treated RNase/DNase-free water (Invitrogen, 750024) were mixed with 4 µl 5x cDNA synthesis buffer, 1 µl 0.1M DTT, 1 µl RNase OUT™, 1 µl ThermoScript™ reverse transcriptase with 1 µl of 50 ng/µl of random hexamers, and 2 µl of 10 mM dNTP mix (Invitrogen ThermoScript™ RT-PCR System, 11146-

016). The 20 µl mixture was subsequently placed into a thermal cycler (Peltier PTC-200) with profiles as follows: 25˚C for 10 min, 50 ˚C for 50 min and 85 ˚C for 5 min. 1 µl of RNA-H (Invitrogen ThermoScript™ RT-PCR System) was then added and the solutions were incubated for 20 min at 37 ˚C in order to digest the remaining RNA. Finally, the samples were diluted in 100 µl of DEPC-treated RNase/DNase-free water to obtain a 20 ng/µl cDNA solution. As a negative control, the RNA treated with DNase I with omission of the RT step, was used in all experiments.

2.3. qRT-PCR

Following exposure to normoxia/hypoxia, quantitative real-time PCR was prepared with 100 ng of cDNA using 10 µl of TaqMan MasterMix™ 2X (Applied Biosystems 4304437), 1 µl of 20X primer-probe and DEPC water to reach 20 µl final volume. The qPCR was carried out using a HT-7500 thermo-cycler (Applied Biosystems) with the following thermal profile: 95˚C for 10 min for denaturation and 95 ˚C for 15 seconds and 60 ˚C for 1 min (the underlined repeated for each cycle, for 40 cycles). TaqMan Primers/probes set for DDAH1 (Hs00201707_m1), DDAH2 (Hs00967863_g1), VEGF (Hs00900055_m1), miR21 (Hs04231424_s1) and mir128 (Hs04231494_s1) were purchased from Applied Biosystems. For each sample, the level of expression of transcript was determined relative to the expression of the housekeeping gene (2-ΔCt) TATA box binding protein (TBP)(Applied Biosystems, 4351372) or GAPDH (Applied Biosystems, 4331182).

To assess mRNA stability, RNA synthesis was terminated by the addition of 5 mg/L of actinomycin D.

Actinomycin D is an antineoplastic antibiotic that inhibits blocks the movement of RNA polymerase interfering with RNA synthesis. The cells were harvested at various time points (1-4 h) after the actinomycin D addition, and the RNA was isolated using the PureLink RNA Mini Kit protocol (Invitrogen, 12183018A). In some experiments, the cells were incubated as previously published (Wojciak-Stothard, Abdul-Salam et al. 2014) with the 10µmol/L of NFκB inhibitor, BAY 1170-85 (Sigma Aldrich) for 24h before the measurement of miR-21 and DDAH1 mRNA.

2.4. Western Blot Analysis

To study changes in DDAH protein expression, homogenised lung tissues, HPAECs and HPASMCs were lysed in RIPA lysis buffer (Sigma Aldrich, R0278) with protease inhibitor (1:100 in RIPA buffer; Sigma Aldrich R8346) on ice and harvested using a cell scraper. Samples were centrifuged at 13000 rpm at 4°C for 5 minutes and protein concentration was measured using a Pierce BCA Protein Assay Kit (Thermo Scientific). Equal amounts of proteins were denatured in lithium dodecyl sulphate (LDS) loading buffer and reducing agent (both from Invitrogen) by heating to 70°C for 10 min. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 12% gel 1.5 mm) at 120V for approximately 2 hours in migration buffer (25 mM Tris base, 190 mM glycine 0.1% SDS, pH 8.8). The spectra multicolor broad range protein ladder (10 to 260kDa; Thermo Scientific 26623) was run on each gel to show migration of marker proteins for estimation of protein molecular weight. Proteins were electrophonically transferred to hydrophobic polyvinylidene difluoride (PVDF) membranes (GE Healthcare Amersham, RPN2020F) using the wet transfer method (BioRad). The gel and the PVDF membrane were sandwiched in between sponges and filter papers in a transfer cassette soaked with transfer buffer (20% methanol, in 1xTris-glycine-SDS) and was placed in a transfer tank (BioRad) filled with transfer buffer and run at 100V for 1 h and 30 min with an ice pack to avoid overheating. The membranes were then washed with phosphate buffered saline (PBS) and 0.1% Tween-20 (Sigma Aldrich, 94158) (PBS-T) and blocked with PBS-T 5% BSA (blocking solution) (from Sigma Aldrich). Consequently, the membranes were either incubated for 1 h at room temperature (RT) or overnight (ON) at 4°C with primary antibodies in blocking solution. The membranes were incubated with goat anti-DDAH 1 or goat anti-DDAH 2 antibodies (generated in Leiper's Laboratory, dilution 1:3000) (Leiper, Maria et al. 1999), mouse monoclonal anti-PCNA antibody (fl-261 Santa Cruz Biotechnology; dilution 1:1000), rabbit anti-pSer188 RhoA antibody (sc-32954 Santa Cruz Biotechnology; dilution 1:1000), mouse monoclonal anti-RhoA (sc-418 Santa Cruz Biotechnology, dilution 1:1000) and rabbit anti-RhoB (sc-180 Santa Cruz Biotechnology; dilution 1:1000) for 1 h 30'. Mouse monoclonal anti- β-Tubulin antibody (Sigma, T7816-2ML; dilution 1:5000) was used as a loading control. After washing 3 times with PBS-T, 10 min each, membranes were incubated for 1h at RT with respective, species-specific horseradish peroxidase (HRP)-conjugated secondary

antibodies (HRP-conjugated sheep anti-mouse IgG Sigma Aldrich A9044, HRP-conjugated goat anti-rabbit IgG Sigma Aldrich A0545, HRP-conjugated rabbit anti-goat IgG Sigma Aldrich A5420) and then washed 3 times in PBS-T, 15 mins/each wash.

Afterwards, bands were visualized using Luminata Crescendo Western HRP Substrate (Millipore, WBLUR) in ChemiDoc™ (BioRad, 170-8070) system. The membranes were then re-probed for β-tubulin expression to confirm equal protein loading. Semi-quantitative analysis of protein expression was performed using densitometric measurement by Quantity One Software (BioRad) and proteins of interest were normalised to the loading control β-tubulin. The antibodies used and the optimal incubation conditions are summarised in Table 2.

Primary Antibody	Species of source	Primary antibody dilution used & condition	Secondary antibody dilution used & condition	Optimal protein loading (µg)
DDAH1	Goat pAb	1:3000 1h 30' RT	1:9000 1h RT	20
DDAH2	Goat pAb	1:1000 1h 30' RT	1:9000 1h RT	50
PCNA	Mouse mAb	1:2000 1h 30' RT	1:10000 1h RT	20
RhoA	Mouse mAb	1:1000 ON 4°C	1:5000 1h RT	30
RhoB	Rabbit pAb	1:1000 ON 4°C	1:5000 1h RT	30
pSer188 RhoA	Rabbit pAb	1:1000 1h 30' RT	1:10000 1h RT	20
β-Tubulin	Mouse mAb	1: 5000 1h 30' RT	1:10000 1h RT	Re-probing

Table 2: Primary and secondary antibodies used in Western blotting. All the secondary antibodies were HRP-conjugated and used for the respective species-specific primary antibodies.

2.5. Construction and Purification of Recombinant Adenoviruses

2.5.1. Adenoviruses construction

Adenoviral vectors for DDAH1, DDAH2 and the inactive mutants of DDAH1 (ΔDDAH1-GFP) and DDAH2 (ΔDDAH2-GFP) were constructed as described in (Wojciak-Stothard, Torondel et al. 2007). Briefly, cDNA encoding DDAH1or DDAH2 were subcloned into the transfer vector pAdTrack-CMV. The transfer vector was then co-transformed with pAdEasy-1 DNA plasmid into E. coli BJ5183 strain. The transformed colonies were selected for kanamycin resistance, identified by restriction digest using Pac I (New England BioLabs R0547S), purified, linearized and transfected into 293 human embryonic kidney cells (Microbix Biosystems Ltd) that express E1 genes to allow purification of adenoviral particles. The purification was carried

out using AdEasy Virus Purification™ (Agilent Technologies, 240243) kit instructions. Infected viral lysate was resuspended in 20ml of DMEM media containing 20µl of benzonase nuclease mixed and incubated at 37 ºC for 30 min. A 50ml syringe was set up in a retort stand above the conical flask and connected to the tube set supplied that consist of a three way tap attached to a feed tube. Air was removed from the syringe and the benzonase nuclease-treated supernatant drawn up into the syringe through the feed tube. A 0.45 µm filter was attached to the syringe outlet and the volume of supernatant was filtered into a fresh container. The Sartobind™ filter unit (membrane absorbers) was washed by syringing 8ml of sterile PBS through it. A 50ml syringe was filled with the prepared supernatant and passed through the Sartobind filter unit (10ml/min), followed by an equal volume of 1X washing buffer. In order to eluate the adenovirus from the Sartobind filter unit, 5ml of elution buffer were passed through the filter unit (1min/ml) and collected in a 15ml falcon tube (BD, Bioscience). The eluate was centrifuged for 15 min at 3000g and the buffer exchanged with 4ml of storage buffer (25mM NaCl, 20mM TrisHCl, 2.5% Glycerol). The virus was recovered by pipetting gently up and down and the titre determinate using the Adeno-X- titre kit by ClonoTech. Adenoviral vectors inducing expression of green fluorescent protein (GFP) were taken as controls. Inactive DDAH mutants have Cys249Ser mutation in their active site, which completely inactivates the enzyme but does not affect the protein folding. DDAH1, DDAH2 and DDAH mutants (ΔDDAHs) were co-expressed with GFP, allowing easy identification of over expressing cells (Wojciak-Stothard, Torondel et al. 2007).

2.5.2. Propagation of adenoviruses

Human embryonic kidney cells (HEK 293) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL Life Technology) supplemented with 10% Fetal Bovine Serum (FBS) and plated into a 6 well plate (SARSTEDT) with 5 X 10^5 cells/well and growth until ~80% confluent. Cells were infected in 1ml serum-free DMEM containing viruses at the multiplicity of infection (MOI) 1:100. After 4h, 1 ml of 4% FBS DMEM was added to each well to give a final concentration of 2% FBS DMEM. HEKs were cultured until full cytopathic effect (CPE) of the virus was visible. The medium containing lysed cells was harvested and subjected to three freeze-thaw cycles using liquid nitrogen and water bath set at 37 ºC. The resultant lysate was then used to infect a T75 (SARSTEDT) of 80% confluent HEK 293 cells. The procedure was repeated as for a 6 well plate and the resultant lysates from the T75

were used to infect four T175 flasks (SARSTEDT) of HEK 293. Once the cells started rounding up, the cells were harvested and pelleted by centrifugation at 1500rpm for 15min. The supernatants were discarded and the pellets resuspended in 5ml of fresh DMEM. The lysates were then subjected to ulterior freeze-thaw cycles and purified using the AdEasy Virus Purification Kit (Agilent technology UK Ltd).

2.5.3. AdEasy virus purification

AdEasy Virus purification was carried out following the kit instructions. Infected viral lysate was resuspended in 20ml of DMEM media containing 20µl of benzonase nuclease mixed and incubated at 37 ºC for 30 min. A 50ml syringe was set up in a retort stand above the conical flask and connected to the tube set supplied that consist of a three way tap attached to a feed tube. Air was removed from the syringe and the benzonase nuclease-treated supernatant drawn up into the syringe through the feed tube. A 0.45 µm filter was attached to the syringe outlet and the volume of supernatant was filtered into a fresh container. The Sartobind™ filter unit (membrane absorbers) was washed by syringing 8ml of sterile PBS through it. A 50ml syringe was filled with the prepared supernatant and passed through the Sartobind filter unit (10ml/min), followed by an equal volume of 1X washing buffer. In order to eluate the adenovirus from the Sartobind filter unit, 5ml of elution buffer were passed through the filter unit (1min/ml) and collected in a 15ml falcon tube (BD, Bioscience). The eluate was centrifuged for 15 min at 3000g and the buffer exchanged with 4ml of storage buffer (25mM NaCl, 20mM TrisHCl, 2.5% Glycerol). The virus was recovered by pipetting gently up and down and the titre determinate using the Adeno-X- titre kit by ClonoTech.

2.5.4. Adenovirus Titration

HEK 293 cells were plated at 10 x 10^3 cells/well in a 96 well plate (SARSTEDT) and infected with serial dilutions of the adenovirus from 10-5 to 10-10 for 7 days in 100µl of DMEM containing 2% FBS at 37 ºC / 5%CO2. At day 8, the number of wells showing cytopathic effect (CPE) /fluorescent staining was counted for each dilution. The titre was calculated using 50% tissue culture infective dose equation:

(TCID50) = Highest dilution giving 100% CPE + 0.5-(total wells with CPE).

2.6. Manipulation of DDAH gene expression and activity in cultured cells

2.6.1. Plasmid and lock nucleic acids cell transfection

To investigate the effect of miR-21 and miR-128 on DDAH1 expression, the cells were seeded into 6-well plates (500,000 cells per well) in 2 ml of growth medium and cultured to 80-90% confluence. HPAECs and HPASMCs were transfected with pCMV-miR21 (p-miRNA21), pCMV-miR128 (p-miR-128) and the negative control, pCMV-EGFP (p-EGFP) expression vectors from Origene, using electroporation system (Nucleofector I; AMAXA, M03). Briefly, the culture dishes were pre-incubated/equilibrated with 2ml of supplemented culture media in a humidified 37°C/5% CO_2 incubator. Trypsinised cells were centrifuged in culture media containing 10% serum at 100g for 10 minutes at RT. The cells were re-suspended in 5ml of serum-free medium, counted and the required number of cells (500 000 cells/single transfection) was centrifuged again. Following centrifugation, the resulting pellet was resuspended in 100µl of Nucleofector™ Solution and combined with 1-5µg of plasmid and 2µg of pmaxGFP™ (Amaxa) as transfection control. Consequently, the cell/DNA suspension was transferred into a close cap cuvette and the appropriate Nucleofector™ cell program was used (M03 for HPAECs and A33 for HPASMCs). Following electroporation, the suspension was finally added to the equilibrated media and left undisturbed for 24 h (Amaxa; VPI-1001, VPI-1004; Lonza).

Has-miR-21 or Has-miR-128 lock nucleic acids (LNA21, LNA128), able to inhibit their respective miRNA, were purchased from Exiqon and were used at a final concentration of 50nmol/L. After 4 h following transfection in Amaxa Nucleofector, the cells were left in normoxia or were exposed to hypoxia for 24 h.

2.6.2. DDAH1 3'UTR Luciferase constructs

The 3' UTRs of DDAH1 gene was cloned by GenScript into the 3' UTR of the Firefly luciferase gene (using XbaI sites) in the luciferase pGL3-CMV plasmid (Promega, E1741). The full-length DDAH1 3'UTR (~3000 nucleotides) was cut with *EcoI-FseI* restriction enzyme (NEB; R3101S-R0588S) to obtain two fragments of different size, a short fragment of ~400 nucleotides and a long fragment of ~2600nt. These two fragments were run on 0.8% gel and extracted using QIAquick Gel Extraction Kit (Qiagen, 28704). Successively, 100 ng of total DNA (75 ng insert + 25 ng empty vector) were used in a standard ligation reaction using T4 DNA Ligase

(NEB; M0210S). 2µl of ligation reaction were transformed in E. Coli competent cells DH5alpha (Invitrogen; 11319-019) and plated on ampicillin agar plates. The following day, 10 colonies were selected and analysed by PCR using specific primers. The primer sequences for the short fragment and the long fragment were as follows: 400bp, forward: TGCCCACTCCTGTTGTTTT, reverse: AAGGAGCTGACTGGGTTGAA; 2600bp, forward: TCAAAGAGGCGAACTGTGTG, reverse: GGTGTTGGAGCAAGATGGAT. The PCR reaction was carried out on ice using for each colony: 28µl of sterile water, 5µl of 10X PCR buffer (Invitrogen), 3µl of 25mM $MgCl_2$, 10mM dNTPs, 1µl of 20µM forward primers, 1µl of 20µM revers primer and 1µl of Taq polymerase (Invitrogen, 11306-016). To each PCR tube, a small amount of colony, diluted in 30µl of SOC medium (Invitrogen, 15544-034) and 5µl were added to the PCR reaction. The colony PCR was carried out using a Peltier PTC-200 with the following thermal profile: 95°C for 5 min, 95°C for 1 min, 54° for 3 min, 72°C for 1 min and 72°C for 5 min (the underlined was repeated in 35 cycles). Thus, the PCR-positive colonies were first growth in 5ml of SOC medium for 4 hours at and successively in 100ml of SOC medium overnight in a 37°C/5% CO2 incubator. The 2600 and 400bp constructs were then purified with the plasmid maxi kit (Qiagen, 12965) following the manufacturer's instructions.

2.6.3. Luciferase Assay System

Genetic reporters are used commonly in cell biology to study gene expression and other cellular events coupled to gene expression, such as mRNA processing. Firefly luciferase was used as a reporter because the reporter activity is available immediately upon translation and the assay is very sensitive; indeed its light production has the highest quantum efficiency known for any chemiluminescent reaction (Gould and Subramani 1988).

The two luciferase constructs (2600bp and 400bp) described in 2.6.2 were separately transfected into cells using electroporation system as described in paragraph 2.6.1. After 24 hours, the cells seeded in 96-well plates were exposed to normoxia/hypoxia and lysed using 20µl of 1X lysis buffer from Promega. Cells were left for 10 minutes with lysis buffer and then 100µl of Luciferase Assay Reagent™ (Promega, E1500) was dispensed in each well. The total 120µl suspensions were transferred in a white 96-well plate (Corning™ 3912) and luminescence was measured in the Glomax™ luminometer (Promega, E6501). The luminometer was set to perform a 2-second measurement delay followed by 10-second measurement read for luciferase activity (peak emission wavelength 560 nm). Renilla

luciferase plasmid (peak emission wavelength 480 nm) (Promega, E2810) served as a transfection control in all the experiments.

2.6.4. Network-Based Analysis

The analysis of interactions between DDAH1 gene sequence and miRNAs was performed with the use of consolidated databases of miRNAs such as TargetScan, Diana and miRBASE. TargetScan 5.2 (http://www.targetscan.org/vert_50/) predicts biological targets of miRNAs by searching for the presence of conserved 8monomer and 7monomer (8mer, 7mer respectively) sites that match the seed region of each miRNA, meanwhile DIANA (http://diana.cslab.ece.ntua.gr/) is specifically focused on a positive and a negative set of miRNA Recognition Elements (MREs) located in the 3'-UTR. MiRBASE (http://www.mirbase.org/) is a searchable database of published miRNA sequences and annotation. All the results were confirmed at least on 2 algorithms used for miRNA target prediction.

2.7. Methylarginines and DDAH Assays

2.7.1. Methylarginines and cGMP Measurement

HPAECs, HPASMCs and plasma from mice were used for quantification of methylarginines. Cell media were harvested and briefly centrifuged to remove unattached cells. Following the addition of an internal standard 2,3,3,4,4,5,5-D7-asymmetric dimethylarginine (D7-ADMA, 10 µmol/L; NovaChem DLM-7476-5) and methanol protein precipitation, the supernatants were dried for 1 hour using a vacuum centrifuge and the resultant pellets were re-suspended in mobile phase (0.1% Formic Acid in H_2O). Quantification of ADMA and L-NMMA was performed using a reversed-phase high-performance liquid chromatography (HPLC) system with an ASI-100 auto sampler, model P680 gradient pump (model RF-2000). Briefly, samples were cleaned-up by solid-phase extraction on polymeric cation-exchange extraction columns using D7-ADMA as an internal standard. After derivatisation with ortho-phthaldialdehyde reagent containing 3-mercaptoproprionic acid, analytes were separated by isocratic HPLC with fluorescence detection. The chromatography was performed on a C18 column (3.9 X 150mm; 5µm particle size; 100Å pore size, Waters) and the mobile phase A consisted of 50mM potassium phosphate buffer pH 6.5, containing 8.7% acetonitrile. Mobile phase B was 50% acetonitrile in water. Separation was performed under isocratic

conditions with 100% mobile phase A at 30°C. After elution, the column was flushed with mobile phase B for 22 min in order to remove retained compounds. An injection of 20µl was used for each sample and the fluorescence was measured at excitation wavelengths of 340 nm and emission of 455 nm. Peaks were quantified on the basis of peak area Analytical recovery was 98–102%, and the inter-assay coefficient of variation was <3%. (Teerlink, Nijveldt et al. 2002).

For cGMP measurement, at day 0 lung tissue samples were homogenised in cold 6% (weight/volume) trichloroacetic acid. After centrifugation, supernatants were washed four times with water-saturated diethyl ether. The aqueous extracts were then lyophilized and redissolved in the assay buffer. cGMP levels were measured with cGMP Enzyme immunoassay Biotrak (EIA) System (GE Healthcare, UK) according to the manufacturer's instructions. Briefly, frozen lungs from mice were homogenised in cold 6% trichloroacetic acid at 4 °C and centrifuged at 2000 x g for 15 minutes at 4°C. The supernatant was recovered and washed 4 times with 5 volumes of water saturated diethyl ether. Consequently, the aqueous extract was dried under a stream of nitrogen at 60 °C. At day 1, the standard curve was prepared and 100µl of each dilute standard was added into the appropriate wells, at the same time 100µl of the samples were pipetted into specific wells. Subsequently, 100µl of diluted antibody was added into wells and incubated at 4 °C overnight. At day 2, cGMP conjugate was pipetted into wells and incubated for 3 hours at 4 °C. After, wells were washed 4 times with wash buffer and 200µl of enzyme substrate were added into all wells. After 30 minutes incubation the blue color developed in the well was read at 630nm using the Glomax luminometer (Promega). The result was calculated using the average optical density (OD) for each set of replicate wells.

2.7.2. DDAH Activity Assay

After incubation in normoxic/hypoxic conditions, the cells grown in 6-well dishes were scraped into 0.5 ml of RIPA buffer (50 mmol/L Tris HCl pH 8, 150 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) and centrifuged at 10000 rpm for 10 minutes to pellet cellular debris.

Lung samples were homogenized in ice-cold sodium phosphate buffer (pH 6.5) and the samples were centrifuged at 10,000g for 10 minutes to obtain a supernatant. Crude lysates were incubated for 30 min at 37°C on a rotor shaker with 500 µmol/L of DDAH substrate,

2,3,3,4,4,5,5-D7-asymmetric dimethylarginine (D7-ADMA). DDAH enzyme activity was determined by measuring the formation of D7-citrulline from D7-ADMA. Quantification of was performed using a HPLC system with an ASI-100 auto sampler, model P680 gradient pump as described in 2.7.1. 50µg of the lysates or lung homogenates were incubated at 37°C for 1h. One unit of enzyme was determined as the amount of enzyme that catalysed the formation of 1 mole of D7-citrulline from D7-ADMA per hour at 37°C. In all the experiment the enzyme activity measured at 4°C was considered as background and subtracted from the final result. Data were reported as the peak area ratio of D7-ADMA to D7-citrulline.

2.8. Cell Function Assays

2.8.1. Endothelial Cell permeability

The effects of hypoxia and DDAH over expression on transendothelial permeability were studied using HPAECs grown in Transwell-Clear chambers (Corning Life Sciences). HPAECs were seeded in transwells (3 µm pore size; Corning Life Sciences, 3452) in complete medium at cell density of 100,000 cells/well and grown till confluence. HPAECs were used for experiments 24 h after infection with AdDDAH1 and/or ADMA addition (100 µM). Fluorescein isothiocyanate (FITC)-conjugated dextran (Sigma, 46947 molecular weight 40 kDa; 1g/L) was added to the upper chamber of Transwells. Passage of FITC-dextran across the endothelial layer following 1 hour incubation was determined using a Glomax luminometer (Promega) programmed to read fluorescence at excitation wavelength of 485 nm and emission at 510 nm.

2.8.2. Cell proliferation

5-bromo-2'-deoxyurine (BrdU) assay (Millipore, 2750) incorporation was used to assess DNA synthesis. BrdU is a thymidine analogue that is incorporated into new synthesised DNA during the S phase of the cell cycle.

HPAEC and HPASMC were seeded into 96 well plates (3000 cells per well) in 100µl/well of appropriate cell culture media. In each experiment, some of the wells were set aside as controls without cells (blank) and controls without BrdU reagent. 20µl of BrdU (Millipore, 2752a) were added into the medium 24h before the end of experiment, in order to achieve a better sensitivity/noise ratio. After 24 hours exposure to normoxic or hypoxic conditions,

cells were fixed using Millipore Fixing Solution (Millipore, 2752b) for 30 minutes and then washed 3 times with 1X Millipore Washing Buffer (Millipore, 2752h). Subsequently, the cells were incubated for 1h with 100µl/well of pre-diluted Millipore Anti-BrdU Monoclonal Antibody (2752c) at room temperature followed by 3 additional washings. Afterwards, cells were incubated with 1:2000 diluted Millipore Goat anti-mouse IgG peroxidase conjugate (Millipore, 2752e) for 30 minutes at room temperature, as recommended by the manufacturer. The cells were then washed 3 x in Millipore Washing Buffer and once with DEPC-water (Sigma Aldrich, 95284). After that, 100µl/well of TMB Peroxidase Substrate (Millipore, 2752g) were added to each well and incubated in the dark for 30 minutes, followed by the addition of 100µl/well of Millipore Acid Stop Solution (Millipore, 2752d). Finally, the colorimetric quantification was carried out using 96-well plate reader (Glomax, Promega) with a dual wavelength setting of 450/550nm. The level of absorbance at 450nm is considered directly proportional to the number of living cells in culture. In all the experiments, the background and blank values were subtracted from the sample values.

In some experiments, HPAEC were treated with protein kinase G (PKG) inhibitor Rp-8-Br-PET-cGMPS (R&D; 0.5 µmol/L) 24h prior to permeability measurement.

2.8.3. Matrigel tube formation assay

Angiogenic capacity of cultured cells was studied using the Matrigel™ tube formation assay. Matrigel is a solubilised preparation of extracellular matrix protein from Engelbreth-Holm-Swarm mouse sarcoma cells and is widely used to test the *in vitro* angiogenic capacity of endothelial cells (Arnaoutova and Kleinman 2010).

Matrigel (Corning™, 354230) was thawed on ice for 4 hours and after 25 µl of ice-cold Matrigel was added into each well in a 96 well plate and left at 37°C for 30 minutes to allow polymerization.

Cells, less than 80% confluent, were detached with 0.05% Trypsin-EDTA and resuspended in Endothelial Cell Basal Medium 2 (Promo Cell, C-22211) serum-deprived after centrifugation at 1500 rpm for 5 min and seeded at 5000 cells/well in triplicate onto Matrigel™. Plates were then incubated in a humidified incubator under normoxic or hypoxic conditions. After 24-48 hours, cells were fixed in 4% formaldehyde (Sigma Aldrich, 252549) in PBS and then used for microscopic examination. For each well, 3 phase-contrast images were captured

using Olympus IX70 with Peltier CCD camera. The total tubule length was calculated in blind using Image J software.

2.8.4. Apoptosis Assay

The analysis of cell viability was carried out using the propidium Iodide (PI) staining, followed by flow cytometry. PI easily penetrates membranes of non-viable cells, while live cells are not stained. PI binds to double stranded DNA by intercalating between base pairs and its fluorescence can be measured at excitation 488 nm and emission at 617 nm. HPAECs, HPASMCs ($1X10^6$cell/well) and the respective media (10ml) were collected in flow cytometric tubes (Sarstedt, 55.1578) and briefly centrifuged at 1500rpm for 10 min. The resulting pellet was gently resuspended in 150µl of PBS and 150µl of 2X PI lysis buffer (0.1% sodium citrate, 0.1% 100 x Triton and 0.5% PI). After incubation at room temperature for 10 minutes in the dark, the samples were analysed by flow cytometry using FACSCalibur (BD Biosciences) followed by data analysis with CellQuestPro software (BD Biosciences). Arsenic trioxide (As2O3), an apoptosis enhancer, was used as a positive control.

2.8.5. Immunofluorescence and Confocal Microscopy

HPAECs and HPASMCs (50,000 cells/well) were cultured on 24-well plastic coverslips (Nunc, 174950) until confluence and subjected to normoxia/hypoxia or infected with adenoviruses. After 24-48 hours, cells were fixed with 4% formaldehyde solution in PBS for 20 minutes at room temperature and permeabilised for 3 minutes with 0.1% Triton X-100 in PBS. Cells were then incubated in 0.5% BSA in PBS for 45 minutes to block non-specific antibody binding and incubated with mouse monoclonal anti-VE-cadherin antibody (Santa Cruz Biotechnology) at 1:100 dilution for 1 hour. The coverslips were then washed 3 times in PBS and incubated with Cy5 goat anti-mouse antibody (Zymed, 81-6516) at 1 µg /ml and 1µg/ml TRITC-phalloidin (Sigma Aldrich, P1951) to stain actin filaments for 15 minutes. Coverslips were washed 3x in PBS and mounted in Vectashield mountant containing nuclear stain DAPI (Vector Laboratories, H-1200). Images of cells were taken under the confocal laser scanning fluorescence microscopy (Leica TCS SP5).

2.9. *In Vivo* Studies

2.9.1. Chronic Hypoxia Studies *In Vivo*.

All studies were conducted in accordance with UK Home Office Animals (Scientific Procedures) Act 1986 and institutional guidelines. Twelve- to 15-week-old C57BL male mice (~20 g; Charles River, Margate, UK) and DDAH1tg male mice (~20 g; Jackson Laboratory, Maine, USA) were either housed in normal air or placed in a specially constructed environmental chamber and exposed to hypoxia in a normobaric hypoxic chamber (FIO_2 10%) for 2 weeks (n=6–8/group).

At 2 weeks, animals were weighed and anesthetized and the development of pulmonary hypertension was confirmed measuring RV systolic pressure (RVSP) in the anesthetized animals (Hypnorm 0.25 mL/kg; Midazolam 25 mg/kg by intraperitoneal injection) via direct cardiac puncture using a closed-chest technique in the spontaneously breathing. The animals were then euthanized, the hearts were removed, and the individual ventricular chambers were weighed. Right ventricular hypertrophy was assessed as the ratio of right ventricle /septum with left ventricle + septum. The right lungs were snap-frozen in liquid nitrogen and stored at −80°C for biochemical measurements. The left lungs were fixed by inflation with 10% formalin, embedded in paraffin, and sectioned for histology. Transverse lung sections were stained with van Gieson's elastic method or α smooth muscle-actin antibody from Sigma Aldrich. Vascular muscularization was defined as the proportion of fully and partially muscularised vessels (with immunoreactivity for α smooth muscle-actin as evidence for muscularization) on total number of vessels stained with elastin. Two separate lung sections from each animal were used for quantification, and counting was performed by two investigators blinded to genotypes.

2.9.2. Treatment of Mice with LNA21

In order to study the effects of miR-21 inhibition *in vivo*, C57BL/6 male mice (~20 g; Charles River, Margate, UK) were given intraperitoneal (IP) injection of 100 µl of vehicle or 100 µl locked nucleic acid anti-miR-21 probes (LNA21) in PBS at 10 mg/kg once every other day for a total of 8 times based on (Elmen, Lindow et al. 2008). At day 1, after the first administration of the probes, the mice were exposed to hypoxia for two weeks (n=8/group). Following the hypoxic exposure, the mice were phenotyped as described in 2.9.1. In all the

in vivo experiments, two separate sections from each animal were quantified, with counting performed by two investigators blinded to genotypes.

2.10. Human study

Tissue samples were obtained with the informed consent of patients and the approval of the Brompton Harefield & NHLI and Hammersmith Hospitals Research Ethics committees. Surgical samples of lung tissue were acquired from treatment-naïve PAH patients at lung transplantation (n=12). Control tissues (n=12) comprised uninvolved regions of lobectomy specimens from patients undergoing surgery for bronchial carcinoma. Patient characteristics are shown in Table 1. Tissues were transported on ice, and samples for protein extraction were snap-frozen between 0.5 and 4.0 hours before being stored at −80°C until further analysis; transport times for both control and PAH samples were similar. Tissue samples (excluding large vessels and airways) suitable for RNA analysis were collected from peripheral regions of the lung. RNA was extracted from tissue samples using the Trizol method (Invitrogen). 1ml of Trizol was used to extract RNA per 100 mg tissue and homogenised using in-tube pestles (VWR, Leicestershire, UK). RNA was quantified by measurement of absorption at 260 nm in a spectrophotometer to ensure that equal amounts of RNA were used in each RT-PCR reaction. RNA concentration was calculated as Abs 260nm x dilution factor x 40 / 1000. 2 µg RNA in up to 10µl RNase/DNase-free water (Invitrogen) were added to 4µl 5x cDNA synthesis buffer, 1µl 0.1M DTT, 1µl RNaseOUT™, and 1µl ThermoScript™ reverse transcriptase (Invitrogen) with 50 ng random hexamers, and 2µl 10 mmol/L dNTP mix. RT-PCR conditions were as follows: 25˚C for 10 min, followed by 50 min at 50˚C, terminated at 85˚C for 5 mi

Table 3: Patient characteristics

Diagnosis	Sex	Age (years)	Disease duration (months)	mPAP mmHg	Vasodilator therapy
IPAH patients					
IPAH	F	41	18	61	naive
IPAH	F	28	42	52	PGI_2 tested
IPAH	M	28	14	50	naive
IPAH	F	51	96	60	naive
IPAH	M	48	N/A	70	naive
IPAH	F	10	N/A	64	naive
IPAH	M	42	40	69	naive
IPAH	F	38	N/A	N/A	N/A
IPAH	F	41	N/A	54	naive
IPAH	F	41	60	58	PGI2 tested
IPAH	F	25	N/A	N/A	N/A
IPAH	F	28	N/A	N/A	N/A
Control subjects					
Ca	F	50			
Ca	F	61			
Ca	M	63			
Ca	M	56			
Ca	M	59			
Ca	M	52			
Ca	M	27			
Ca	M	67			
Ca	F	79			
Ca	F	78			
Ca	M	55			
Ca	F	45			

2.11. Data presentation and statistical analysis

Results are presented in bar charts or scatter plots where appropriate, and error bars indicate means with standard deviation error of the mean (SEM), while “n” refers to the number of samples per group. All numerical data were tested for normal distribution using Pearson normality test (*P*); specifically, normally distributed data were analysed by unpaired t-test. One-way ANOVA was used for data of more than 2 groups, with Bonferroni post-hoc analysis performed if $P < 0.05$. Two-way ANOVA was applied for comparing data of multiple groups with multiple experimental conditions followed by Bonferroni post-hoc analysis performed if $P < 0.05$. Statistical significance was regarded as $P < 0.05$ for all presented data. All graphs and calculation were performed with GraphPad Prism6 (GraphPad Software Inc).

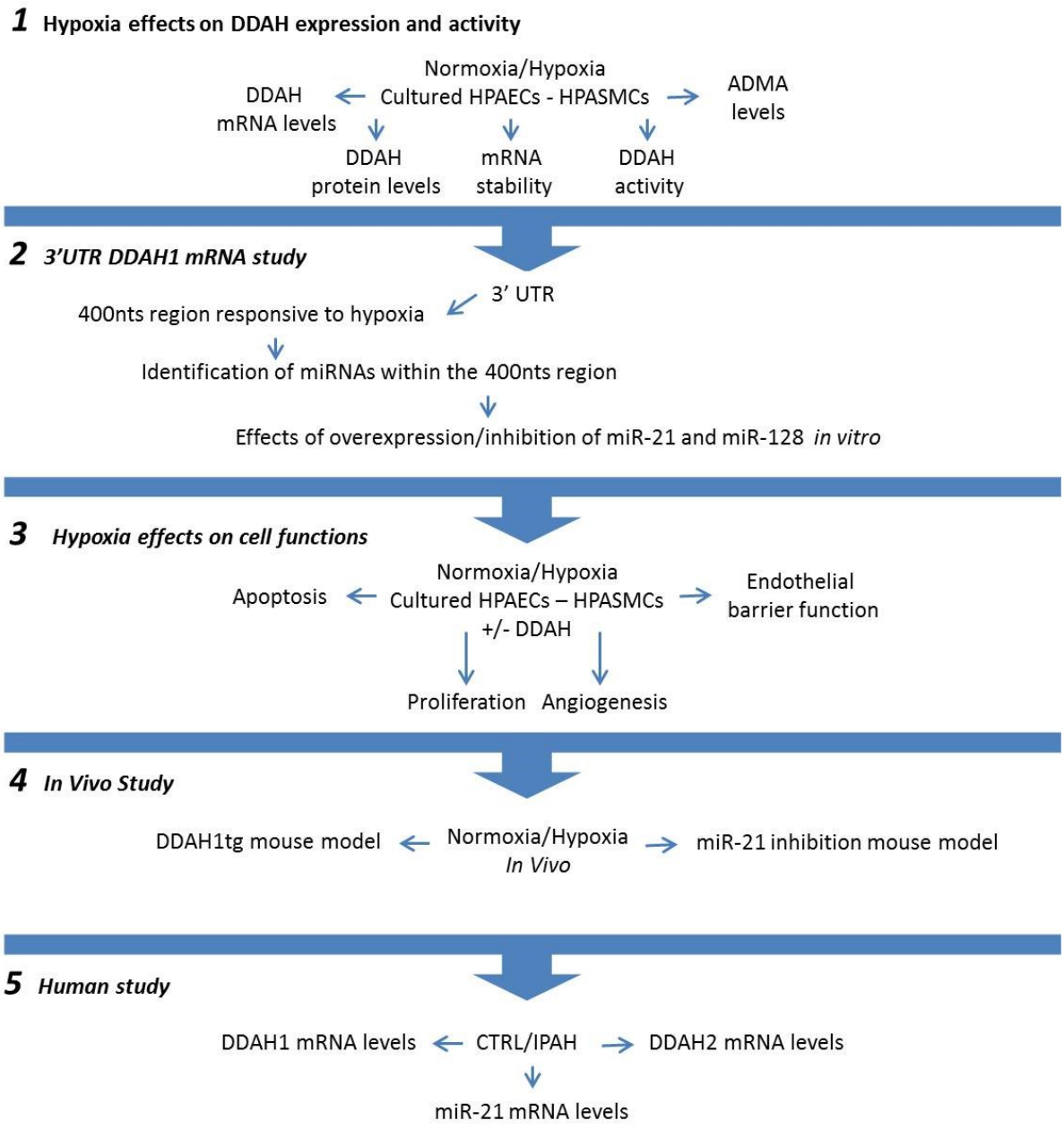

Diagram: Schematic diagram of experimental design process.

3. The Effects of Hypoxia on DDAH Expression and ADMA Metabolism in Pulmonary Vascular Cells

3.1. Introduction

Dysregulation of ADMA metabolism has been implicated in the development and progression of hypoxia- associated chronic respiratory diseases (Millatt, Whitley et al. 2003). The effects of hypoxia on pulmonary vascular cells and the underlying molecular mechanisms are not completely understood. Hypoxia can be considered as a cause for vascular changes or as a consequence of chronic respiratory diseases, in either case hypoxia plays a pivotal role in the regulation of the ADMA/NO pathway.

Elevated concentrations of intracellular ADMA may result from increased methylation of arginine residues in proteins or decreased metabolism of ADMA. ADMA and L-NMMA are actively metabolized to L-citrulline and methylamines by the action of DDAH enzymes (Ogawa, Kimoto et al. 1989).

Two human DDAH isoforms (DDAH 1 and 2) have been identified (Leiper, Maria et al. 1999) but their individual contributions to ADMA metabolism in vascular cells are not completely understood.

In this chapter we studied the effects of hypoxia on DDAH1 and DDAH2 gene and protein expression and DDAH activity in human pulmonary artery endothelial cells (HPAECs) and human pulmonary artery smooth muscle cells (HPASMCs).

3.2. Results

3.2.1. Hypoxia Decreases DDAH mRNA and Protein Expression *in vitro*

Chronic hypoxia reduces NO signalling and DDAH expression in the lung (Dayoub, Achan et al. 2003; Millatt, Whitley et al. 2003; Smith, Birdsey et al. 2003) but mechanisms are not well understood. The purpose of the experiments described in this section was to investigate the effects of hypoxia on DDAH1 and DDAH2 mRNA, protein expression and activity in cultured human pulmonary artery endothelial cells (HPAECs) and human pulmonary artery smooth muscle cells (HPASMCs).

Hypoxia (1-48 hours) led to a 3-fold decrease in DDAH1 and DDAH2 mRNA levels in HPAECs, with a maximal reduction at 2 hours of hypoxic exposure (figure 11 A and B). DDAH1 mRNA levels were unaffected by hypoxia in HPASMCs, while DDAH2 mRNA was reduced by 2-fold following 4 hours hypoxia (figure 11 C, D). Consistent with the changes in the mRNA levels, DDAH1 and DDAH2 protein levels were both significantly reduced by hypoxia in HPAECs (figure 12 A and B), whereas in HPASMCs only DDAH2 protein levels were reduced (figure 12 C and D).

In summary, these results show that hypoxia selectively downregulates DDAH1 expression in pulmonary endothelial cells, while DDAH2 expression is reduced in both pulmonary endothelial and smooth muscle cells.

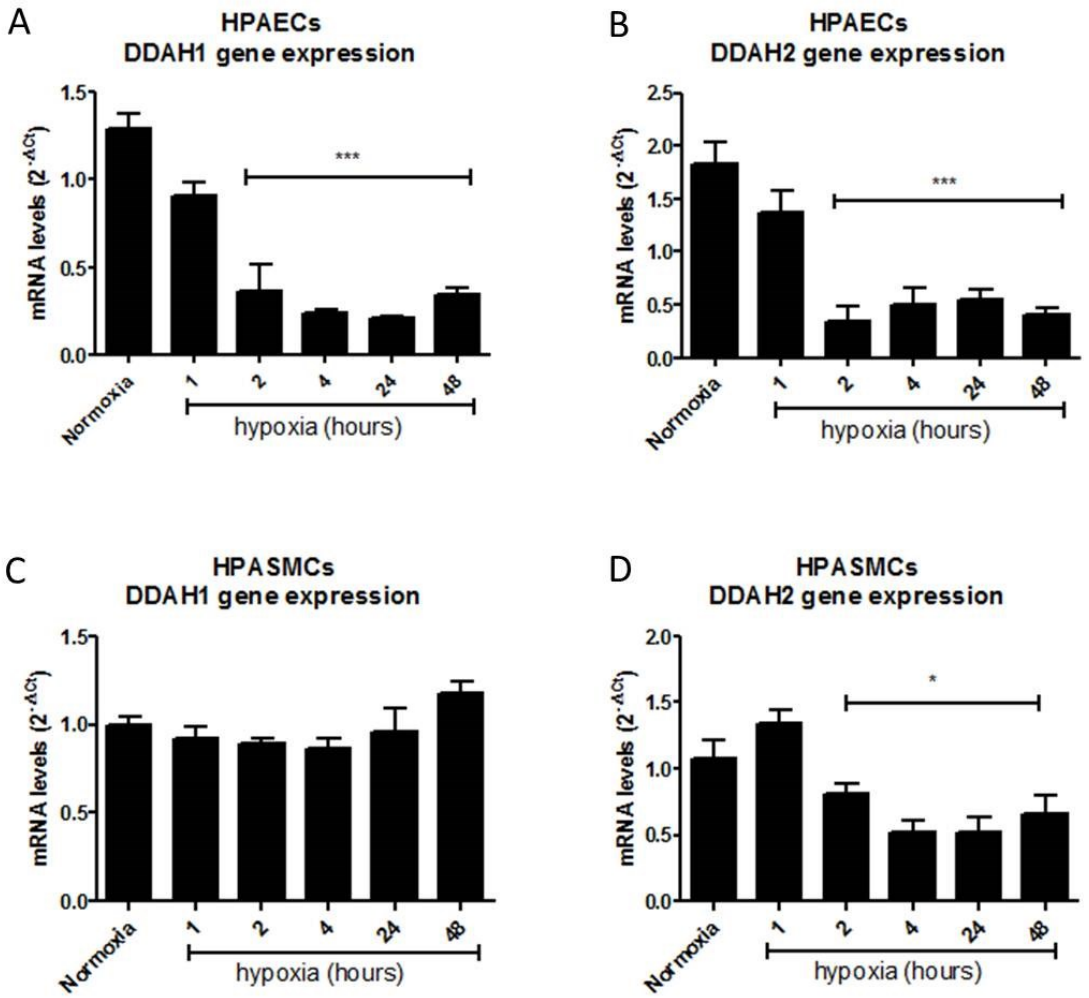

Figure 11: The effect of hypoxia (1-48hr) on DDAH1 and DDAH2 mRNA expression in HPAECs and HPASMCs. (A) and (B) show gene expression of DDAH1 and DDAH2 in HPAECs while (C) and (D) show gene expression of DDAH1 and DDAH2 in HPASMCs, as indicated. Results are normalized to the expression levels of the housekeeping gene TATA Binding Protein (TBP). ***P<0.0001; *P<0.05 (comparison with normoxic controls); n= 8.

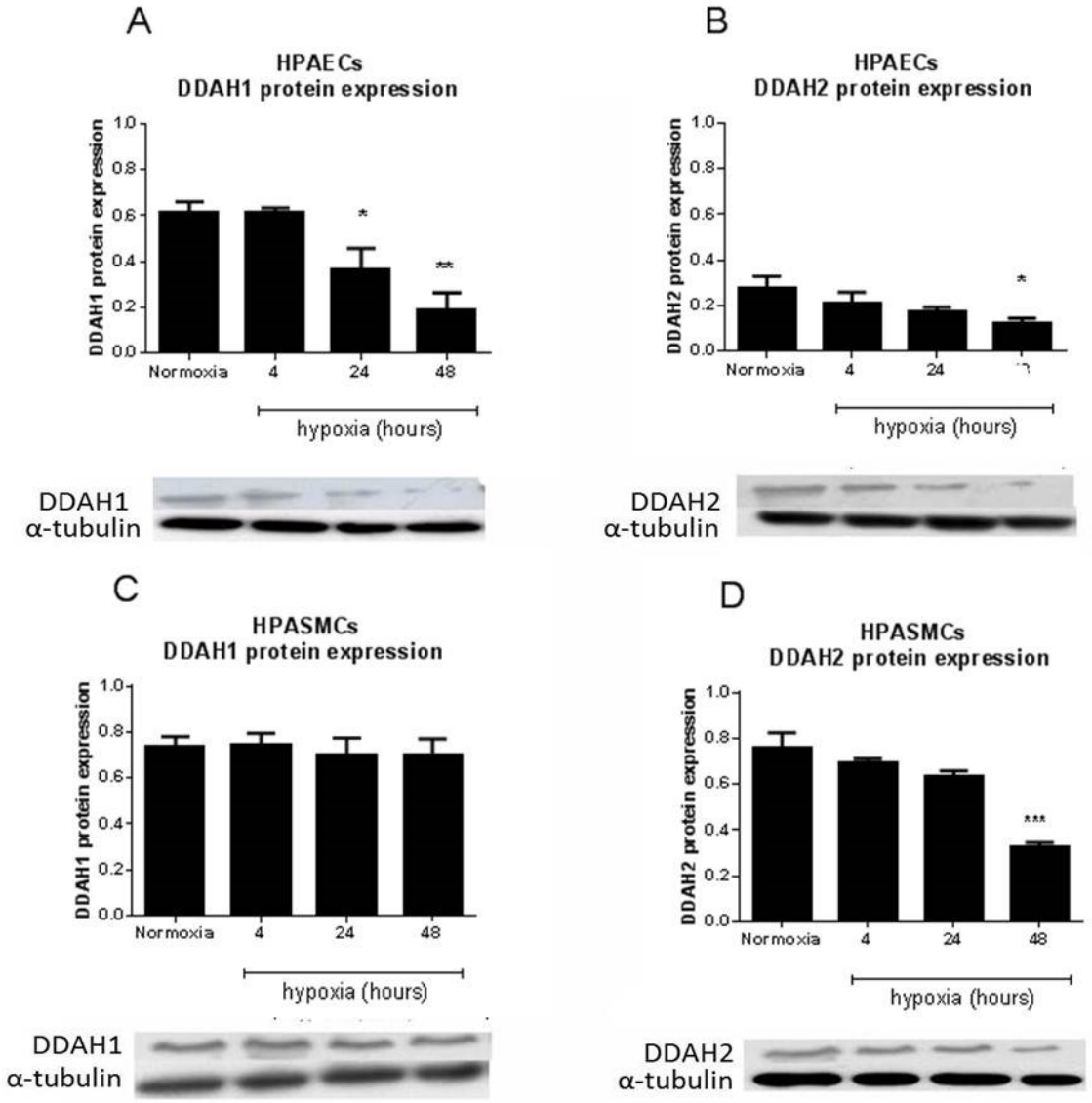

Figure 12: The effects of hypoxia on DDAH1 and DDAH2 protein expression in HPAECs and HPASMCs. (A) DDAH1 and (B) DDAH2 protein expression in HPAECs; (C) DDAH1 and (D) DDAH2 protein expression in HPASMCs. Representative examples of western blots are shown underneath the graphs. Comparison with normoxic controls; ***P<0.0001; **P<0.001; *P<0.05; n=8.

3.2.2. Hypoxia decreases ADMA Metabolism via inhibition of DDAH1 activity in HPAECs.

Changes in DDAH activity have a major impact on ADMA metabolism and thus ADMA levels (Sibal, Agarwal et al. 2010). In order to study the metabolism of methylarginines during hypoxia, ADMA, L-NMMA and SDMA were extracted from culture media of normoxic or hypoxic HPAECs or HPASMCs and their levels were measured using liquid chromatography mass spectrometry (LC-MS), as described in materials and methods 2.7.1. The levels of methylarginines in the media of hypoxic cells were compared with normoxic controls.

As shown in figure 13 A-D, L-NMMA and SDMA levels were not significantly affected by hypoxia in either HPAECs or HPASMCs.

Consistent with the reduced DDAH expression in HPAECs, the concentration of ADMA in HPAEC culture media increased by 2 and 3-fold respectively after 24 and 48 hours of hypoxic exposure (figure 14 A). In contrast, ADMA levels in HPASMC culture media were unaffected by hypoxia (figure 14 B). Taken together, our experiments suggest that hypoxia selectively affects ADMA metabolism in endothelial cells.

DDAH activity was measured directly using labelled D^7 ADMA as a substrate for DDAH enzymes and measuring the levels of D^7 L-citrulline, which is a product of ADMA metabolism. As can be seen in figure 14 C, DDAH activity showed a 5-fold decrease ($P< 0.0001$) following hypoxic exposure in HPAECs, while DDAH activity in HPASMCs was unaffected (figure 14 D).

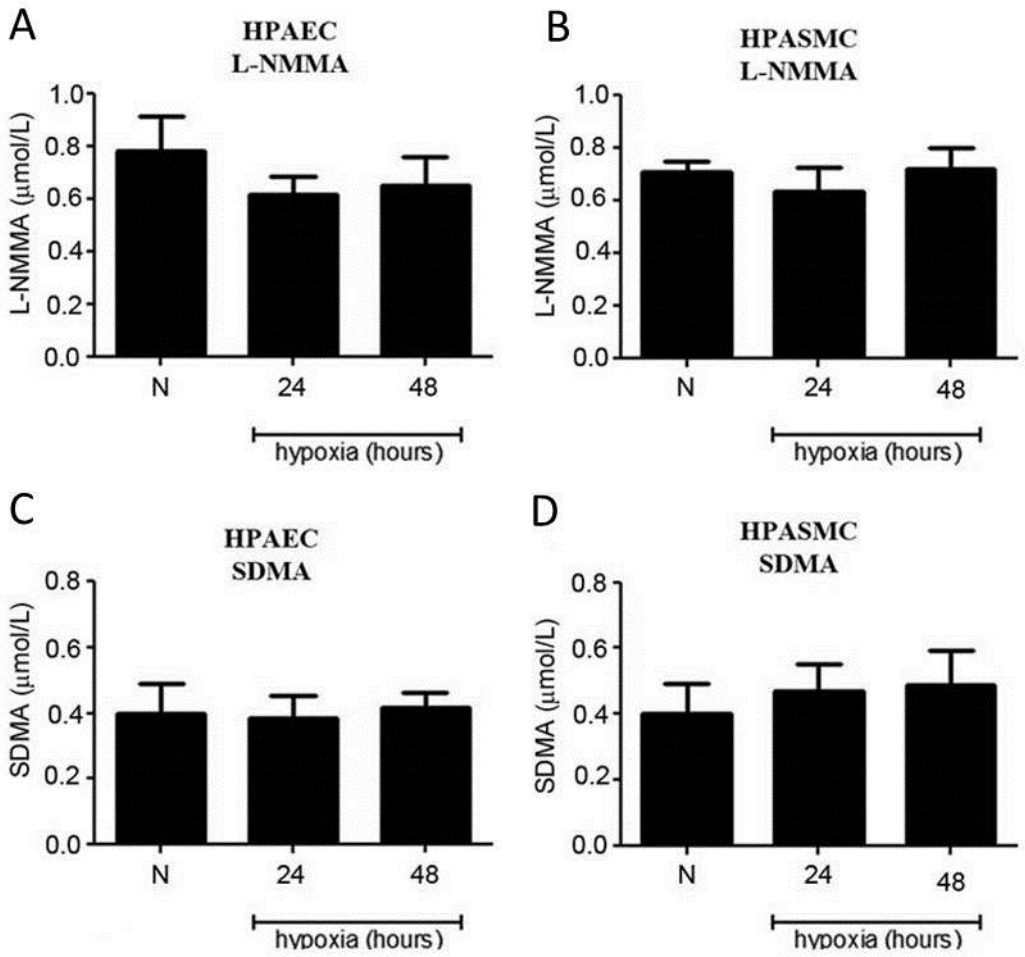

Figure 13: Methylarginine levels in HPAECs and HPASMCs following hypoxic exposure. L-NMMA and SDMA levels were measured using liquid chromatography mass spectrometry (LC-MS). L-NMMA levels (a, b) and SDMA levels (c, d) in normoxic and hypoxic HPAECs and HPASMCs are shown, as indicated. Comparison with normoxic controls; n=8.

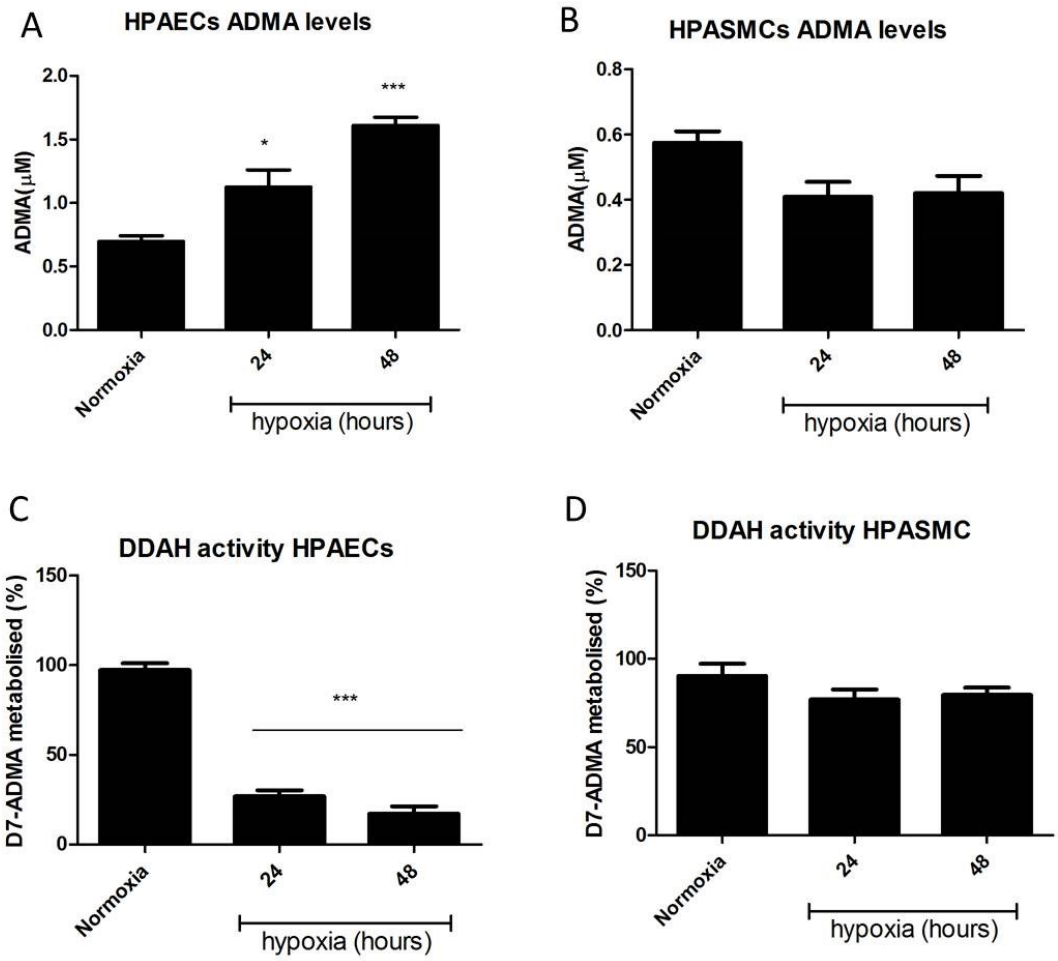

Figure 14: Hypoxia-induced changes in DDAH activity. ADMAs levels were measured using liquid chromatography mass spectrometry (LC-MS). The levels of ADMA in cell media increased 3-fold in HPAECs during 48 hours of hypoxic exposure (A), while no significant changes were observed in HPASCMs (B). DDAH activity was measured directly, by using labelled D7 ADMA as a substrate for DDAH enzymes and measuring the levels of D7 L-citrulline, which is a product of ADMA metabolism. A 2.5-fold decrease in DDAH activity was observed in hypoxic HPAECs (C), while HPASMCs did not show significant changes in DDAH activity (D). ***P<0.0001; *P<0.05; n=6

To further verify the role of DDAH expression in hypoxia-induced ADMA metabolism, DDAH1, DDAH2 and inactive DDAH (ΔDDAH) mutants were over expressed in HPAECs using adenoviral gene transfer. ΔDDAH1 and ΔDDAH2 have a Cys249Ser mutation in their active site, which inactivates the enzyme without affecting protein folding (Wojciak-Stothard, Torondel et al. 2007). DDAH overexpression was confirmed by Western blot and EGFP was used as adenoviral control in all the experiments. The multiplicity of infection (MOI) and expression time were established experimentally to induce a physiological (~3-fold) increase in DDAH expression (figure 15 C and D).

DDAH1 overexpression reduced ADMA levels by 1.3-fold (P<0.001) in HPAECs exposed to 24 hour hypoxia, while DDAH2 or inactive DDAH mutants had no effect (figure 15A, B).

In contrast, ADMA levels in HPASMCs were not significantly affected by overexpression of DDAH1. Although a decreasing tendency in ADMA levels was observed, it is possible that due to the low basal levels of ADMA in HPASMCs, the changes induced by DDAH1 were too small to render significance.

Taken together, these results may help clarify the ambiguity regarding the role DDAH enzymes in ADMA metabolism in pulmonary vascular cells. The fact that hypoxia-induced increase in ADMA levels was prevented by overexpression of DDAH1 and not DDAH2, argues for the predominant role of DDAH1 in ADMA metabolism.

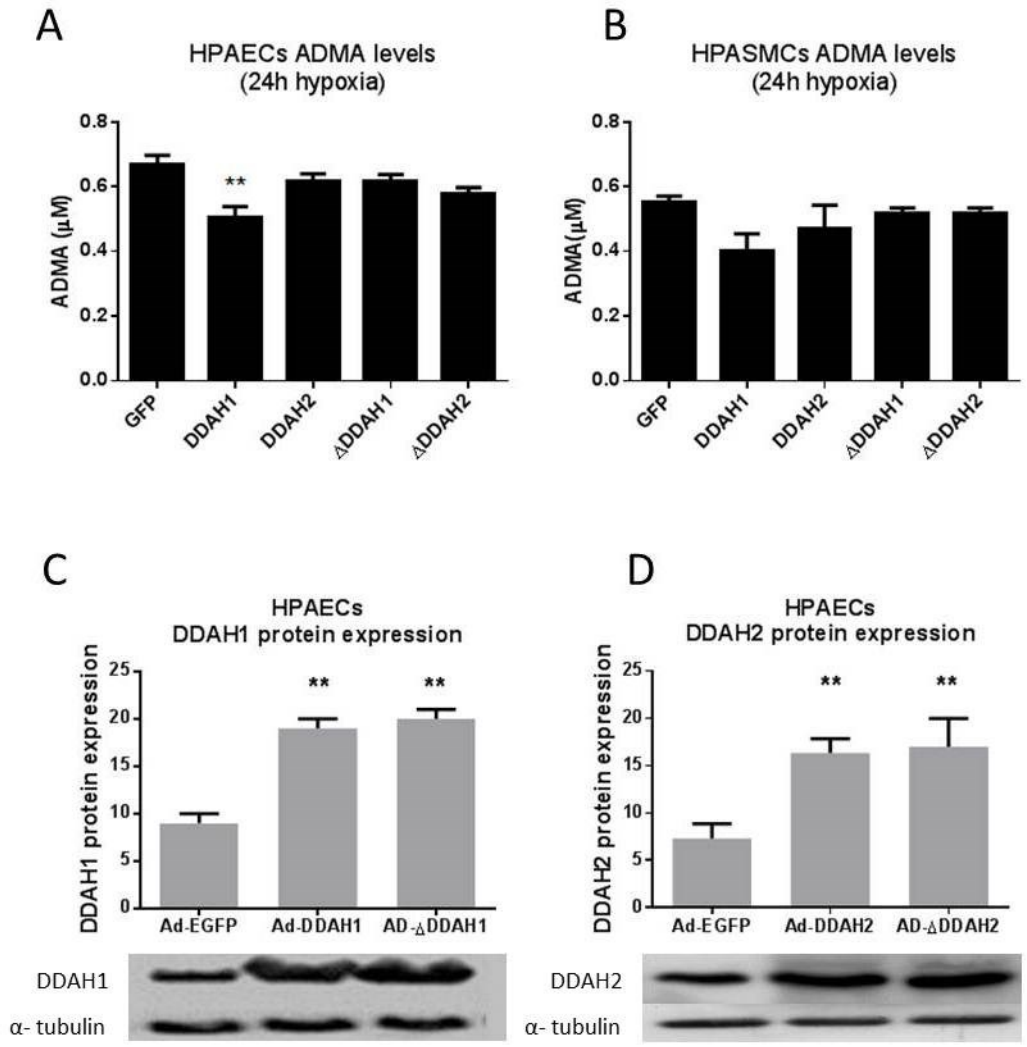

Figure 15: ADMA levels in culture media of (A) HPAECs and (B) HPASMCs over expressing AdGFP (control), AdDDAH1, AdDDAH2, AdΔDDAH1 and AdΔDDAH2, as indicated. The graphs in (C) and (D) show DDAH1 and DDAH2 expression levels following infection of HPAECs with AdDDAH1-2 and inactive mutants, AdΔDDAH1 and AdΔDDAH2 with a representative Western blot below the graph. **P<0.001, comparison with adenoviral controls (AdGFP): n= 6.

3.2.3. Hypoxia decreases DDAH mRNA stability in HPAECs

I have established that hypoxia downregulates DDAH1 and DDAH2 mRNA and protein expression in HPAECs and that only DDAH1 is involved in the regulation of hypoxia-induced changes in ADMA metabolism in cultured pulmonary vascular cells. In the next series of experiments, I investigated the mechanism of hypoxia-induced downregulation of DDAH mRNA.

Changes in DDAH mRNA levels may result from changes in mRNA stability; indeed hypoxia has been shown to affect mRNA synthesis and stability (Levy, Chung et al. 1998) (Paulding and Czyzyk-Krzeska 2000).

In order to study mRNA stability, the half-life ($t_{1/2}$) of DDAH1 and DDAH2 mRNA was measured in HPAECs and HPASMCs cultured in hypoxic and normoxic conditions. After the addition of mRNA synthesis inhibitor, actinomycin D to the cells, mRNA levels were studied by qRT-PCR, as described in material and methods 2.3.

The half-life of DDAH1 mRNA in HPAECs was reduced during hypoxia when compared to normoxic conditions (figure 16 A), (N $t_{1/2}$ = 1.5h ± 5 versus H $t_{1/2}$= 1h ± 5 min; $P < 0.001$), while no significant reduction was observed in HPASMCs (N $t_{1/2}$= 1.35h ± 4 min; H $t_{1/2}$= 1.1h ± 7 min) (figure 16 B). Consistent with changes in mRNA levels, DDAH2 mRNA half-life was reduced by hypoxia in both HPAECs (N $t_{1/2}$= 2h ± 7 min; H $t_{1/2}$ = 0.7 h ± 5min) and HPASMCs (N $t_{1/2}$= 0.8h ± 5 min; H $t_{1/2}$= 0.2h ± 8 min) (figure 16 C, D). The half-life of VEGF mRNA (serving as a positive control) increased, as expected (Levy, Chung et al. 1998), from 0.4h ± 2 min in normoxia to 2h ± 3 min in hypoxia.

In summary, these experiments demonstrated for the first time that the decrease in DDAH mRNA and protein expression in hypoxic pulmonary vascular cells could be caused by a decrease in DDAH mRNA stability.

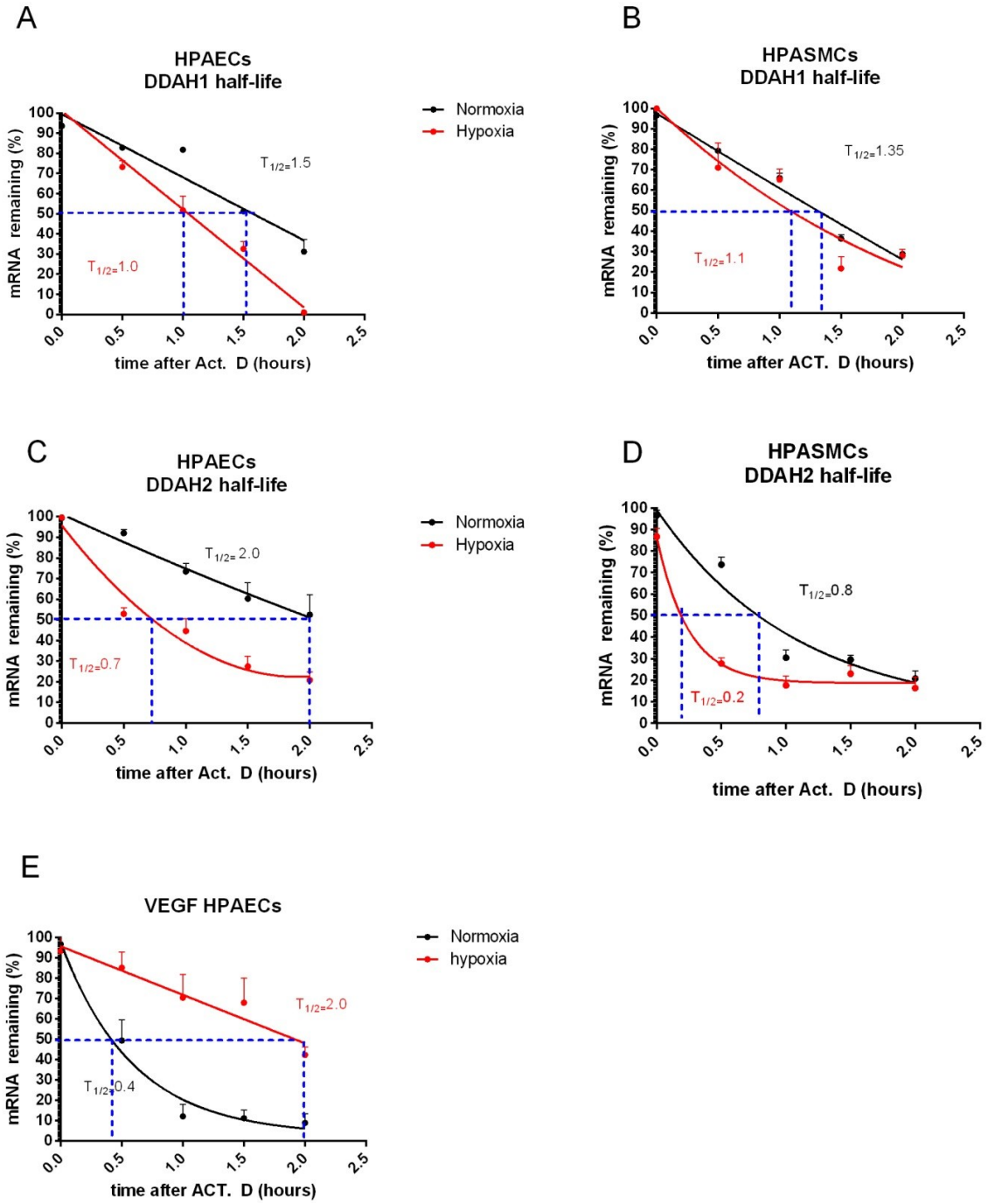

Figure 16: Hypoxia plays a key role in the regulation of DDAH mRNA stability. HPAECs and HPASMCs were pre-incubated with actinomycin D (5.0 mg/ml) and exposed to hypoxia (2% O2) for different time intervals (0 – 2.5 hours). The cells were harvested at designated time points, and total RNA was isolated and reverse transcribed. The fold change in DDAH mRNA expression was measured by real-time PCR and is represented as percentage decrease in mRNA levels compared to the untreated samples. Results are representative of the three independent experiments with all readings repeated in triplicate. The half-life of DDAH1 mRNA was reduced during hypoxia when compared to normoxic conditions in HPAECs (Figure A), (Nt1/2 = 98 ± 5 min; H t1/2 = 60 ± 5 min) but not in HPASMCs (B) (Nt1/2 = 79 ± 4 min; H t1/2 = 65 ± 5 min). DDAH2 half-life was reduced by hypoxia in both HPAECs (C) (Nt1/2 = 120 ± 7 min; H t1/2 = 60 ± 5min) and HPASMCs (D) (Nt1/2 = 70 ± 5 min; H t1/2 = 25 ± 8 min); (E) VEGF was used as positive control for hypoxic conditions (Nt1/2 = 0.4 ± 3 min; H t1/2 = 2 ± 1 min).

3.3. Discussion

In this chapter we have described the effects of hypoxia on gene and protein expression of DDAH isoforms and ADMA metabolism in pulmonary vascular cells. The key findings are summarised in the following points:

1. Hypoxia downregulates mRNA and protein expression of DDAH1 and DDAH2 in endothelial cells.
2. In smooth muscle cells, only DDAH2 mRNA and protein expression is downregulated by hypoxia.
3. Hypoxia decreases ADMA metabolism via inhibition of DDAH1 activity in endothelial cells.
4. DDAH1 overexpression reduces ADMA levels in endothelial cells.
5. Hypoxia decreases DDAH1 and DDAH2 half-life in endothelial cells but only DDAH2 half-life is reduced by hypoxia in smooth muscle cells.

Dysregulation of the ADMA/NO pathway plays a pivotal role in the development of hypoxia-associated respiratory diseases (Liu, Wang et al. 1998; Pullamsetti, Savai et al. 2011), but the underlying molecular mechanisms remain unclear.

Both DDAH isoforms are expressed in the vasculature; early studies demonstrated that the pharmacological inhibition of DDAH increases cellular concentrations of ADMA, contributing to NO deficiency and vascular contraction (MacAllister, Parry et al. 1996).

Contrasting findings have been reported regarding the role of DDAH1 and DDAH2 in the metabolism of ADMA. In fact, it has been shown that DDAH1 is required for metabolizing ADMA and L-NMMA *in vivo*, while DDAH2 had no detectable role for degrading ADMA and L-NMMA (Hu, Atzler et al. 2011). Genetic mutation studies showed detrimental effects of altered DDAH function in human diseases (Ding, Wu et al. 2010). Indeed, a loss of function mutation in DDAH1 gene promoter identified in the Chinese Han population is associated with a reduced DDAH1 mRNA expression, raised plasma ADMA and significantly increased risk heart disease (Ding, Wu et al. 2010). Furthermore, a study of 1300 middle-aged Finnish men identified a mutation in DDAH1 gene linked to elevated plasma ADMA levels, increased

risk of developing hypertension and coronary heart disease (5-fold and 50-fold increase, respectively)(Valkonen, Paiva et al. 2001).

Han et al observed that after 48 h of hypoxia exposure (1% O_2) human lung microvascular endothelial cells showed a decrease of DDAH1 expression that led to an increase in ADMA levels and consequently, a decrease in NO production, while DDAH2 was undetectable in the cell type used (Han, Pope et al. 2009). Moreover, *in vivo* experiment performed using hypoxic rats (1 week exposure) showed that DDAH1 protein was strongly expressed in the lungs of normoxia-exposed animals and that its expression was significantly decreased in the lungs of hypoxia-exposed animals (Millatt, Whitley et al. 2003).

Conversely, an *in vitro* study by Pope et al supported the role of DDAH2 as a major enzyme in ADMA metabolism (Pope, Karrupiah et al. 2009). In this study performed using bovine aortic endothelial cells (BAECs), the authors observed that the overexpression of DDAH2 can improve endothelium-dependent vasorelaxation and increases NO synthesis, whereas small interfering RNA knockdown of DDAH2 reduces NO synthesis (Pope, Karrupiah et al. 2009). Additionally, it has been reported that DDAH2 overexpression can prevent endothelial dysfunction and cardiovascular diseases associated with hyperhomocysteinemia (Liu, Guo et al. 2012).

In order to clarify the contrasting findings regarding the role of DDAH1 and DDAH2 in the metabolism of methylarginines, ADMA levels and DDAH activity were quantified in pulmonary vascular cells. We showed that hypoxia increases ADMA levels by reducing DDAH1 expression in human pulmonary artery endothelial cells but not in human pulmonary artery smooth muscle cells. In agreement with the hypothesized major role of DDAH1 in control of ADMA metabolism (Hu, Xu et al. 2009), overexpression of DDAH1 but not DDAH2 had a major impact on ADMA levels in hypoxic endothelial cells. This differential effect is somewhat surprising because both DDAH isoforms are capable of metabolising ADMA in cultured normoxic cells. It is possible that the activity of DDAH2 is suppressed in hypoxic conditions and the mechanism of this response will require further studies.

To further investigate the mechanism of hypoxia-induced effects on DDAH1 and DDAH2 expression, the half-life ($t_{1/2}$) of DDAH1 and DDAH2 mRNA was measured in normoxic and hypoxic cells in which transcription had been blocked by the action of actinomycin D, using qRT-PCR (Levy, Chung et al. 1998).

It is estimated that 1.5% of the human genome is transcriptionally responsive to hypoxia (Denko, Fontana et al. 2003). The best studied transcription factor induced in hypoxic cells is HIF-1, which controls more than 100 downstream genes involved in diverse cellular processes (Manalo, Rowan et al. 2005). Hypoxia is also able to regulate the stability of several mRNAs using different mechanisms. For example, it has been reported that hypoxia can downregulate IL-7 mRNA expression *in vitro* in a calcium-dependent manner (Lu, Clark et al. 2009), and VEGF *in vitro* (Shweiki, Itin et al. 1992; Goldberg and Schneider 1994; Levy, Levy et al. 1995; Levy, Levy et al. 1996) and *in vivo* (Sharma, Wunsch et al. 1992; Aiello, Avery et al. 1994). The key control point for the hypoxic induction of the VEGF gene is the regulation of the steady-state level of mRNA (Levy, Levy et al. 1995), which is determined by the relative rates of mRNA synthesis and decay. Post-transcriptional mechanisms of regulation have previously been suggested for erythropoietin mRNA (Goldberg, Gaut et al. 1991; Rondon, MacMillan et al. 1991) and demonstrated for tyrosine hydroxylase (Czyzykrzeska, Dominski et al. 1994), two other hypoxia-inducible genes.

In the present study we examined post-transcriptional regulation of DDAH1 and DDAH2 mRNA expression under both normoxic and hypoxic conditions showing for the first time that hypoxia causes DDAH1 and DDAH2 mRNA instability in endothelial cells. The mechanism of this endothelium-specific regulation will be discussed in detail in the next chapter.

4. Regulation of DDAH mRNA Stability during Hypoxia

4.1. Introduction

Messenger RNA (mRNA) is transcribed as a complementary copy of DNA, which can subsequently be translated into an amino acid chain. Synthesised in the nucleus by RNA polymerases, the mRNA travels to the cytoplasm for translation where can eventually be degraded by nucleases. When these two events occur at a constant rate, they give rise to a steady-state mRNA population for each unique transcript (Ross 1995). Since the mRNA is constantly being degraded in the cytoplasm, it has to be synthesized by the cell at a much higher rate than is needed for the maintenance of a steady amount (Wilusz, Wormington et al. 2001). Messenger RNA steady amount often changes in response to a stimulus, thereby rapidly increasing or decreasing mRNA abundance to satisfy the cell needs for specific proteins. Several RNA-binding proteins and noncoding RNAs can bind to mRNA and control its stability (Storz, Opdyke et al. 2004; Glisovic, Bachorik et al. 2008). Three major pathways are involved in the regulation of mRNA stability, with the deadenylation-dependent mRNA decay considered the main pathway in mammalian cells (Goldstrohm and Wickens 2008). The process is driven by a combination of three deadenylases that perform the poly(A) tail removal, which results in the degradation of the mRNA. Another way of regulating mRNA stability involves the family AU-rich elements (AREs) located within the 3'UTR of many mRNAs (Shaw and Kamen 1986). AREs are sequence elements of 50–150nt that are rich in adenosine and uridine bases and located in the 3'-UTRs of many mRNAs that have a short half-life. ARE sequences have been identified by their capacity to cause mRNA degradation by a mechanism dependent on the shortening of the poly(A) tail (Shaw and Kamen 1986; Wilson and Brewer 1999).

MicroRNAs (microRNAs) also regulate mRNA stability by interacting with their target consensus sequence on the 3'-UTRs. These 21–25-nt RNAs comprise about 3% of all human genes and regulate gene expression by controlling translation and mRNA degradation (Huntzinger and Izaurralde 2011). The 3'-UTR contains microRNA response elements (MRE) and the binding between microRNAs and MRE sequences within the 3'-UTR, can cause translational repression or degradation of the mRNA transcripts (Jackson 1993).

Hypoxia alters the expression of numerous miRs in various cell types (Huang, Ding et al. 2009; Ali, Mah et al. 2012; Mace, Collins et al. 2013). We have studied the DDAH 3'-UTR in order to identify the mechanism controlling DDAH mRNA stability under hypoxic conditions.

4.2. Results

4.2.1. The DDAH1 3' Untranslated Region Regulates DDAH1 Gene Expression in HPAECs

In order to identify the mechanisms controlling the DDAH1 mRNA stability, the 3'-UTR of DDAH1 was sub-cloned as a whole (≈3000nts) or as two shorter fragments of ≈2600nt and ≈400nt downstream the luciferase gene as described in 2.6.2 (figure 17).

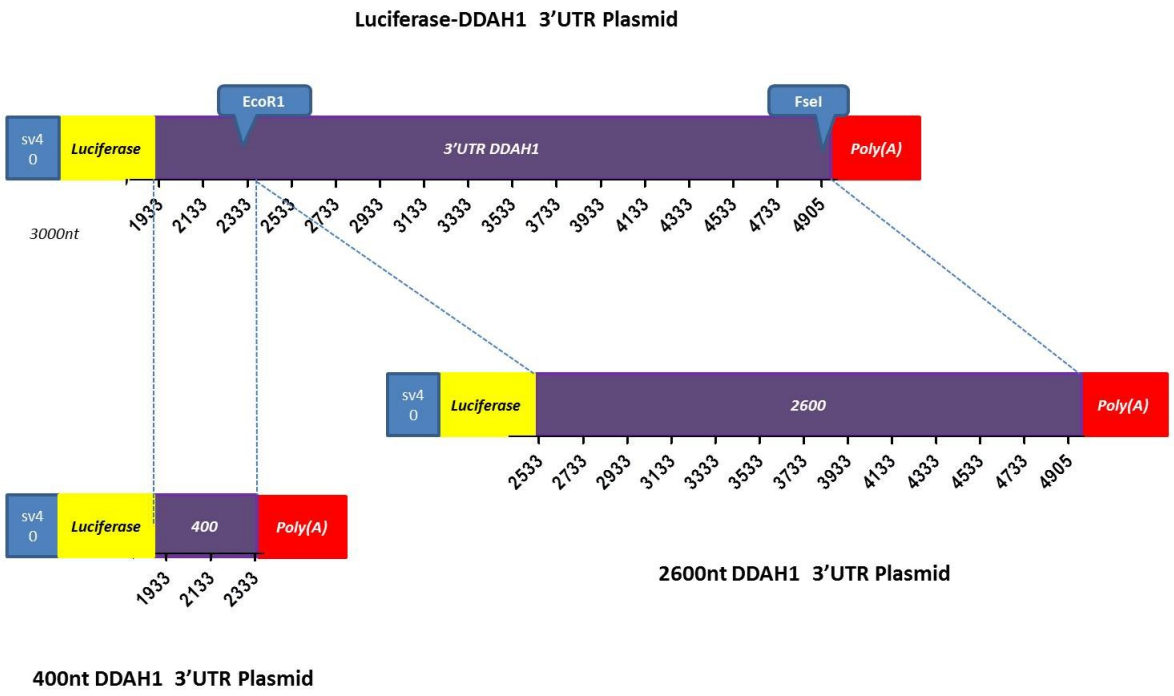

Figure 17: Luciferase Reporter Assay System with 3'UTR of DDAH1 mRNA. Regulated by the SV40 promoter, the luciferase reporter vector contains the 3'UTR of DDAH1 mRNA downstream of the firefly luciferase gene (Luciferase in yellow) and the poly A tail. Restriction enzymes EcoRI and FseI were used to reduce the length of the 3'UTR from 3000 nt to approximately 400 nt and 2600 nt

To determine the role of the DDAH1 3'UTR in the regulation of its mRNA, HPAECs and HPASMCs were transfected with luciferase gene reporter constructs containing the whole 3000nt DDAH1 3'UTR, the 2600nt and 400nt fragments of DDAH1 3'UTR. No significant changes in luciferase activity were detected in hypoxic HPASMCs transfected with the whole DDAH1 3'-UTR (figure 18 A). By contrast, hypoxic exposure of HPAECs transfected with the whole DDAH1 3'-UTR construct resulted in a 2-fold reduction in luciferase activity (***P< 0.0001) (figure 18 B), indicating that hypoxia-induced regulation of DDAH 3'UTR may be cell-type specific. The same effect was observed when the cells were transfected with the reporter construct containing 400nt fragment of DDAH1 3'UTR (figure 18 C), (2-fold reduction in luciferase activity, ***P< 0.0001, comparison with normoxic controls), while the 2600nt construct luciferase activity was not affected by hypoxia. These results indicated that the first 400 nucleotides of the DDAH1 3'UTR are essential for the downregulation of DDAH1 mRNA in endothelial cells.

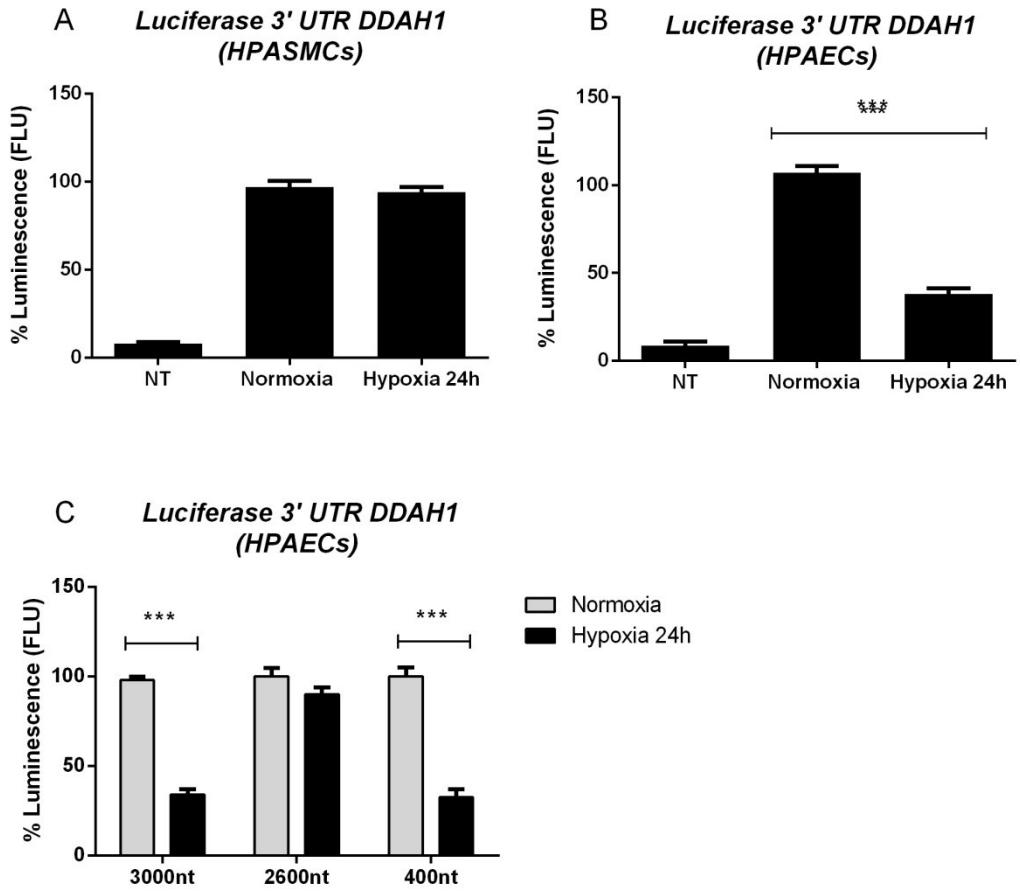

Figure 18: The DDAH1 3'-UTR is regulated by hypoxia. (A) HPASMCs and (B) HPAECs were transfected with Luciferase 3'UTR DDAH1 reporter plasmid. In C, HPAECs were transfected with luciferase reporter construct containing 3000 nt, 2600 nt and 400nt fragments of DDAH1 3'UTR. Decrease in luciferase activity in hypoxic HPAECs transfected with 400 nt construct indicates that the regulation occurs on the first 400nt of DDAH1 3'UTR. Each graph represents values from 4 independent experiments performed in quadruplicates. Renilla Luciferase was used as control in all the experiments; ***P<0.0001 comparison with normoxic control as indicated; n=4

4.2.2. MiR-21 Directly Targets the 3'-UTR of DDAH1 and Represses its Expression in HPAECs

In order to identify potential microRNA candidates (miRs) that can bind to the first 400nts 3'-UTR of the DDAH1 sequence and downregulate DDAH1 mRNA, three commonly utilised algorithms (DIANA, Target Scan and microRNA) were used. A total of 13 miRs were predicted to target DDAH1 3'-UTR (figure 19). Among the 13 predicted miR candidates, only 2 showed links to hypoxia signalling and pulmonary hypertension, miR-21 and miR-128 (Yang, Banerjee et al. 2012; Rhodes, Wharton et al. 2013) and therefore were chosen for further analysis.

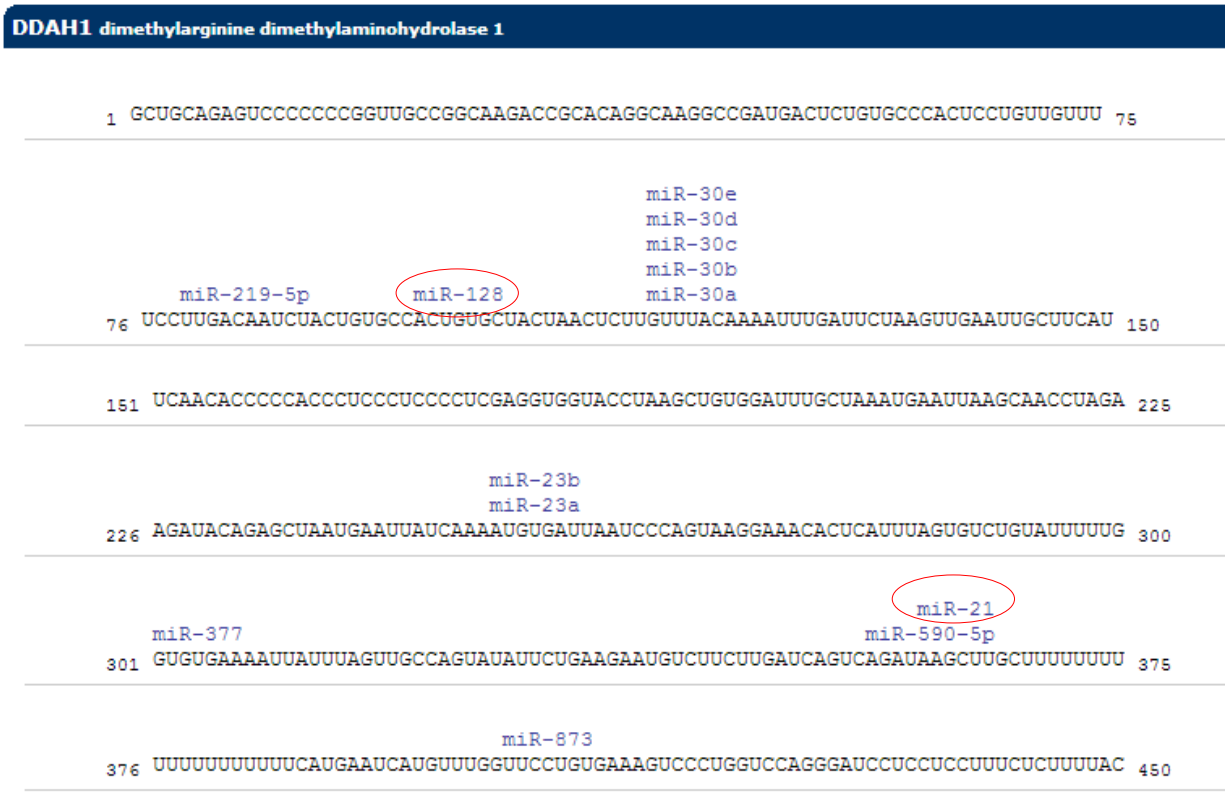

Figure 19: Predicted microRNA target sites on the 400 nt fragment of 3'UTR of DDAH1 mRNA. MiR target sites were identified with programs MicroRNA, Target Scan and DIANA lab software. MiR-21 was aligned to position 344–364 on the 3'UTR of DDAH1 mRNA with a PhastCons score of 0.5085, while miR-128 was aligned to position 83-106 with a PhastCons score of 0.7177. The sequence is adapted from Microrna.org website (http://www.microrna.org/microrna/getMrna.do?gene=23576&utr =20215&organism=9606).

To verify the role of miR-21 and miR-128 in the regulation of DDAH1 3'UTR and DDAH1 mRNA levels, HPAECs and HPASMCs were transfected with the microRNA precursors (~33 nt) pre-miR-128 and pre-miR-21 expression plasmids. Subsequently, mRNA expression levels of DDAH1 and DDAH2 were analysed. As shown in figure 20 A, the overexpression of miR-21 reduced DDAH1 mRNA in HPAECs, and HPASMCs, while miR-128 did not show any significant effects on DDAH1 expression in either endothelial or smooth muscle cells (figure 20 A and C). In addition, to assure that the inhibition observed was due to site-specific microRNA-3'UTR interaction, DDAH2 mRNA expression was also investigated. As expected, due the lack of consensus sequences for these microRNAs on DDAH2 3'-UTR, the overexpression of miR-128 or miR-21 did not have any significant effect on mRNA levels of DDAH2 (figure 20 B and D).

To further study the mechanism of endothelium-specific inhibition of DDAH1 in hypoxia, miR-21 expression levels were measured in normoxic and in hypoxic (24 hours) HPAECs and HPASMCs. As shown in figure 20 E, a 3-fold increase in endogenous miR-21 was observed in hypoxic HPAECs, while in hypoxic HPASMCs the increase was too small to render significance.

The endothelial-specific effects of miR-21 on DDAH1 expression were also confirmed by measuring DDAH1 protein levels. MiR-128 overexpression did not affect DDAH1 protein levels, while miR-21 overexpression significantly reduced DDAH1 protein levels in HPAECs. The role of miR-21 in the regulation of DDAH1 expression was further verified with the use of miR-21 knockdown probes.

Locked nucleic acid (LNA, Exiqon) anti-microRNA probes bind to specific microRNAs forming heteroduplexes, thereby sequestering and preventing the microRNA from binding to the 3' UTR of its target mRNAs (Jensen, Lamy et al. 2011). As shown in figure 21 B, co-transfection of LNA21 with the 400nt DDAH1 3'UTR luciferase plasmid prevented hypoxia-induced degradation of DDAH1 3'-UTR, while co-transfection of LNA128 had no effect.

Taken together, these results show that the hypoxia-induced, endothelium-specific increase in miR-21 is required for the downregulation of DDAH1 expression. MiR-21 inhibits DDAH1 expression by regulating the 3'-UTR of DDAH1 mRNA and this downregulation can be prevented by miR-21 inhibitors.

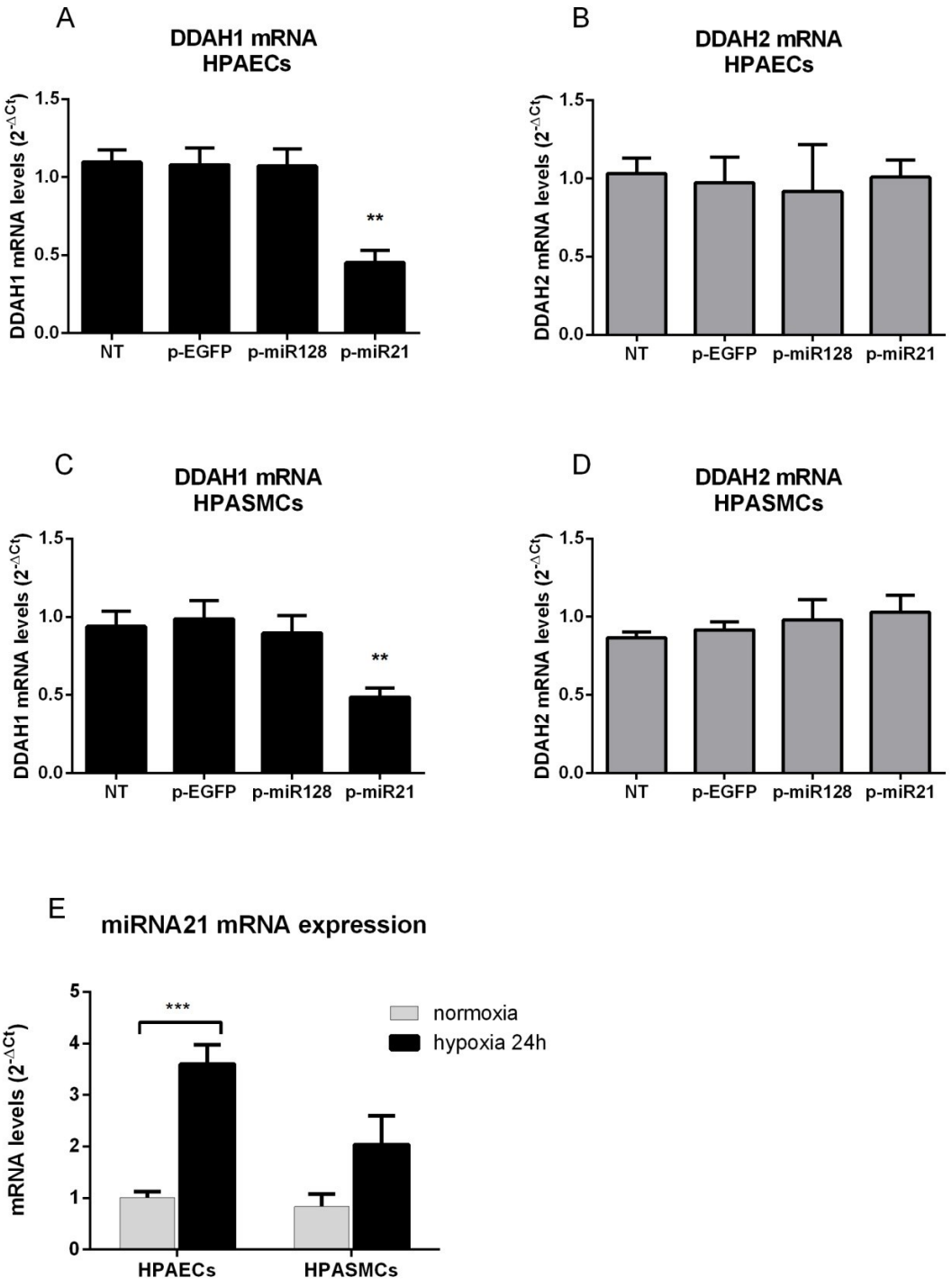

Figure 20: The effect of miR-21 and miR-128 overexpression on DDAH1 and DDAH2 mRNA levels in HPAECs and HPASMCs. Overexpression of miR-21 inhibits DDAH1 expression in HPAECs (A), and in HPASMCs (C). (B and D), miR-21 and miR128 have no effect on DDAH2 expression in HPAECs or HPASMCs, respectively; (E) miR-21 levels are increased by hypoxia in HPAECs but not in HPASMCs. NT: untreated controls; EGFP: transfection control; p-miR21: cells overexpressing miR-21; p-miR128: cells overexpressing miR-128. In (A-D) cells were kept in normoxic conditions, in (E) cells were cultured in normoxia or were exposed to hypoxia for 24 hours. N=5. ***P<0.0001; **P<0.001, comparison with no transfected cells or normoxic controls as indicated.

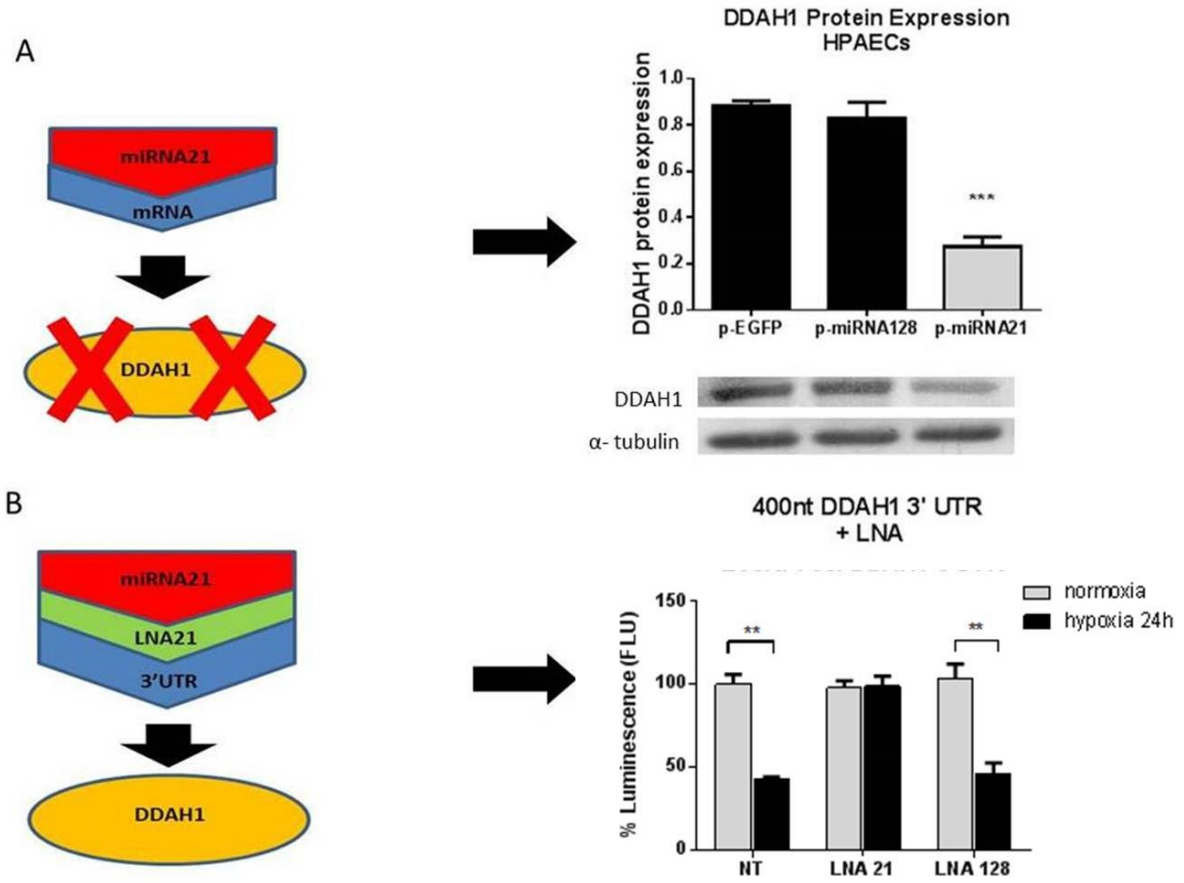

Figure 21: The role of miR21 and miR128 in the regulation of DDAH1 expression in HPAECs. (A) Overexpression of miR-21 mimics the effect of hypoxia on DDAH1 protein expression, while miR-128 has no effect. MiR-21 and miR-128 were overexpressed in normoxic HPAECs using shuttle plasmids (p-miR-21 and p-miR-128). (B) Lock nucleic acid (LNA) probes against miR-21 (LNA21) repress the effects of hypoxia on DDAH1 3'UTR, while LNA128 has no effect. The luciferase reporter vector containing 400nt fragment of DDAH1 3' UTR was co-transfected into HPAECs with 10 nmol of lock nucleic acid against miR-21 or miR-128 (LNA21 and LNA128). N=5; ***P<0.0001; **P<0.001, comparison with transfection controls or as indicated.

4.3. Discussion

The results described in this chapter can be summarised as follows:

1. Hypoxia downregulates DDAH1 expression by acting on the 3'-UTR of DDAH1 mRNA.
2. The first 400nt of DDAH1 3'-UTR confer the effects of hypoxia on DDAH1 mRNA stability.
3. MiR-21 regulates DDAH1 mRNA stability in HPAECs
4. Hypoxia triggers the endothelial cell-specific increase of miR-21 expression
5. The competitive inhibition of miR-21 protects against hypoxia-induced downregulation of DDAH1

Regulation of protein expression under hypoxic conditions may involve various transcriptional and post-transcriptional mechanisms (Levy, Levy et al. 1996; Paulding and Czyzyk-Krzeska 2000; Bail, Swerdel et al. 2010). However, the absence of AREs sequences on the 3'-UTR of DDAH1 and the large amount of data present in the literature regarding miRs, led our study to focus on miR-mediated control of DDAH mRNA stability.

The 3' untranslated region has been shown to play a major role in the regulation of mRNA turnover (Benjamin and Moroni 2007; Bail, Swerdel et al. 2010; Fabian, Sonenberg et al. 2010; Yamakuchi 2012). The mRNA turnover rate can be determined by the interaction between miRs and consensus sequences located within the 3'-UTR of the target mRNA.

MiRs regulate normal cell and tissue function but their dysregulation contributes to development of various diseases, including cancer, diabetes, hypertension, or atherosclerosis (Sassen, Miska et al. 2008; Kantharidis, Wang et al. 2011; Batkai and Thum 2012; Yamakuchi 2012). MiRs profiling and pathway analysis has led to the identification of disease-specific changes in single miRs or miR clusters linked to pathological changes observed during disease development (Ikeda, Kong et al. 2007).

The analysis of the DDAH1 3'-UTR showed that the first 400nt confer the effects of hypoxia on DDAH mRNA stability. Using three algorithms (DIANA, Target Scan and microRNA), a total of 13 microRNAs were predicted to target DDAH1 3'-UTR within this region. We focused our study on 2 microRNA candidates with previously reported links to hypoxia and pulmonary hypertension, miR-128 and miR-21 (Rhodes, Wharton et al. 2013; Yang, Banerjee et al. 2012). Limited studies regarding miR-128 have been shown. Indeed, it has been

demonstrated that miR-128 may play a critical role in human cell lung cancer tumourigenesis, angiogenesis and lymphangiogenesis by directly targeting vascular endothelial growth factor C (Hu, Cheng et al. 2014). In addition, miR-128 levels were found increased in plasma of PAH patients, but its regulatory effects have not been characterised (Rhodes, Wharton et al. 2013). By contrast, miR-21 is one of the most studied microRNAs in cardiovascular diseases (Cheng and Zhang 2010). In fact, it has been shown that expression of miR-21 in human hearts from patients with heart failure was much higher than that in normal human control hearts (Thum, Galuppo et al. 2007) and that miR-21 contributes to myocardial disease by stimulating MAP kinase signalling (Thum, Gross et al. 2008). In the arteries of rats with neointimal growth after angioplasty, miR-21 expression showed more than a fivefold increase, compared with normal control vessels (Ji, Cheng et al. 2007). Relevant to our investigation, Fleissner et al observed that patients with coronary disease show elevated levels of ADMA and miR-21. In this study miR-21 overexpression significantly repressed superoxide dismutase 2 (SOD2) in angiogenic progenitor cells (APCs), which resulted in increased intracellular reactive oxygen species concentration and impaired nitric oxide bioavailability. Therefore, the authors hypothesized that miR-21 inhibition can reduce ADMA-mediated oxidative stress and improve APCs functions (Fleissner, Jazbutyte et al. 2010).

Interestingly, a recent study has shown that the downregulation of DDAH1 expression occurs via miR-21-dependent mechanism in human umbilical venous endothelial cells (HUVECs)(Chen, Zhou et al. 2013). Chen et al in this study observed that 4-hydroxynonenal (4-HNE), a major active product formed by lipid peroxidation, can dysregulate intracellular ADMA through a miR-21 dependent pathway. However, the authors showed that miR-21 inhibition only partially inhibited ADMA metabolism by DDAH1, indicating that miR-21-independent mechanisms are also involved in the regulation of ADMA metabolism under 4-HNE exposure (Chen, Zhou et al. 2013). Curiously, in this study the overexpression of miR-21 also led to a decrease of DDAH2 expression despite the fact that DDAH2 3'-UTR has not miR-21 recognition site. The downregulation of DDAH2 by miR-21 observed in this study could be explained by the fact that DDAH2 expression is ROS sensitive (Wang, Hu et al. 2009), while miR-21 can regulate ROS formation by attenuating superoxide dismutase 2 and 3 (SOD2 and SOD3) (Fleissner, Jazbutyte et al. 2010; Zhang, Ng et al. 2012). Although the

authors suggested a possible regulation of DDAH1 and DDAH2 by miR-21 and ROS-dependent pathways, the exact mechanisms involved remain unclear.

Another study involving a network biology approach combined with experimental data in cultured cells, animal models, and diseased human tissue recognised miR-21 as an important regulatory factor that controls the downstream development of PH (Parikh, Jin et al. 2012). In this bioinformatics-based study the authors showed a molecular model of the pulmonary vasculature where hypoxia, inflammation and BMP-dependent signalling can up-regulate miR-21. In response, miR-21 can downregulate other genes involved in PH, such as RhoB and BMPR2. RhoB has been shown to suppress angiogenesis (Sabatel, Malvaux et al. 2011) and induce a vasoconstriction by activation of endothelin-1 (Hernandez-Perera, Perez-Sala et al. 2000) and inhibition of eNOS (Laufs and Liao 1998). Thus, RhoB modulation may reflect a key function of miR-21 in PH. Regarding BMPR2 it has been observed that miR-21 can be up-regulated by hypoxia and BMPR2 signaling, linking in this way miR-21 with two major pathogenic triggers of PH, hypoxia and BMPR2-dependent signaling (Parikh, Jin et al. 2012).

In summary, although the modulation of miR-21 targets may differ depending on the biological context, we showed that changes in ADMA metabolism induced by hypoxia can be mediated by the endothelium-specific downregulation of DDAH1 by miR-21.

5. The Role of DDAH-ADMA pathway in Hypoxia-Induced Vascular Dysfunction

5.1. Introduction

Endothelial dysfunction manifested by an imbalance in the levels of vasodilators and vasoconstrictors and the breakdown of endothelial barrier function is thought to play a critical role in the pathogenesis of PH (Budhiraja, Tuder et al. 2004). We hypothesized that hypoxia-induced endothelial dysfunction may result, at least in part, from inhibition of DDAH expression and activity.

We have established so far that hypoxia induces endothelium-specific downregulation of DDAH1 expression via miR-21 overexpression, resulting in an inhibition of ADMA metabolism and increase in ADMA levels. In order to investigate functional implications of hypoxia-induced decrease in ADMA metabolism, we studied cell death, proliferation, endothelial junctional organization, permeability and angiogenesis in pulmonary endothelial cells overexpressing DDAH1, DDAH2 and their inactive DDAH mutants. Pharmacological inhibition of DDAH1 activity was achieved by treating the cells with the DDAH 1 inhibitor, L-257 (Rossiter, Smith et al. 2005).

5.2. Results

5.2.1. Hypoxia Does Not Induce Cell Death in Pulmonary Vascular Cells

The Propidium Iodide (PI) assay combined with flow cytometry was used to evaluate the effect of hypoxia (1-48 hours) on cell death in HPAECs and HPASMCs. PI assay is based on the principle that apoptotic cells are characterized by DNA fragmentation and loss of nuclear DNA content. PI is capable of binding and labelling DNA, allowing a precise evaluation of cellular DNA content by flow cytometric analysis, followed by identification of apoptotic cells (Riccardi and Nicoletti 2006).

As shown in figure 22 A and B, hypoxia did not induce any significant changes in cell survival in either HPAECs or in HPASMCs. A positive control, arsenic trioxide (As2O3) induced a >4-fold increase in cell death.

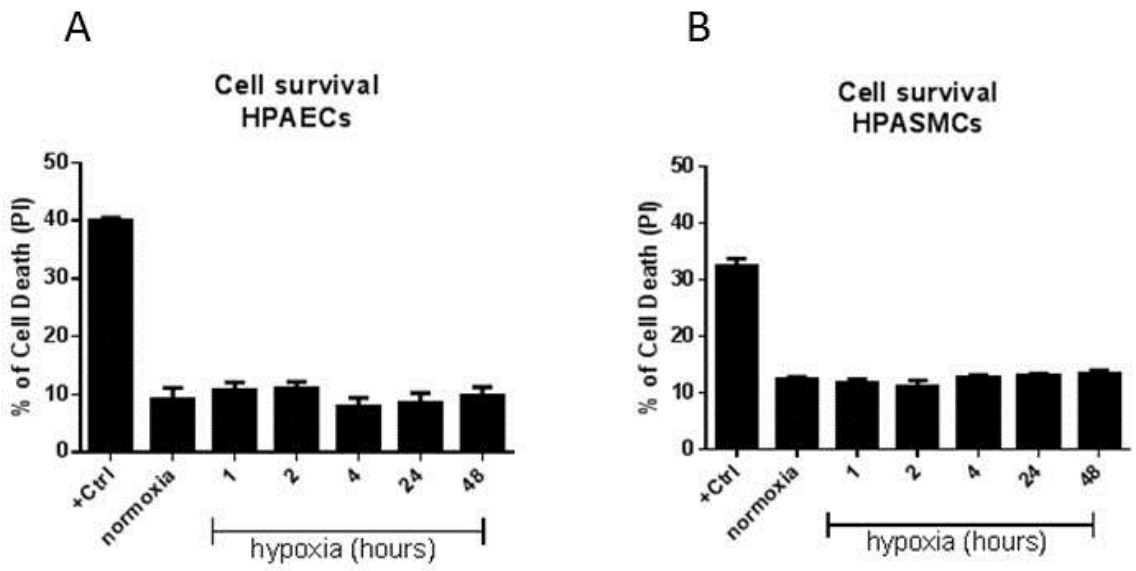

Figure 22: The effects of hypoxia (1-48 hr) on HPAECs and HPASMCs cell death. Hypoxia did not affect cell death in pulmonary vascular cells. Cell cell death was measured by PI staining and As2O3 was used as positive control. n= 6

5.2.2. HPASMCs Proliferation is controlled by endothelial DDAH1 expression

BrdU incorporation assay was used to measure changes in cell proliferation in endothelial and smooth muscle cells cultured under normoxic or hypoxic conditions.

Hypoxia (24h) increased smooth muscle cell proliferation by 2-fold, while endothelial cell proliferation was unaffected (figure 23 A, B). In order to verify the role of ADMA-DDAH1 pathway, HPAECs and HPASMCs were infected with adenoviruses to induce overexpression of DDAH1 (Ad-DDAH1) or DDAH1 inactive mutant (Ad-ΔDDAH1). Overexpression of DDAH1 or ΔDDAH1 did not affect HPAEC or HPASMC proliferation in normoxic or in hypoxic conditions (figure 23 C, D). In addition, incubation of both cell types with ADMA (100 μM) or DDAH inhibitor L-257 (100 μM) did not considerably affect cell proliferation in either normoxic or hypoxic conditions (figure 23 E-F), demonstrating that vascular cell proliferation may not be directly regulated by the ADMA-DDAH pathway.

The endothelium-derived NO inhibits smooth muscle cells proliferation *in vitro* and *in vivo* (Ambalavanan, Mariani et al. 1999). We hypothesized that this NO-dependent effect may be attenuated in hypoxia as a result of DDAH downregulation.

In order to study the effect of endothelium on smooth muscle cell proliferation *in vitro*, HPAECs and HPASMCs were co-cultured in Transwell chambers (details in material and methods 2.8.1). Briefly, HPASMCs were cultured in the bottom chambers of Transwell dishes, while HPAECs were seeded in the top chambers. The cells were separated by porous membrane (3μm pore size), allowing diffusion of soluble mediators between the two cell layers (figure 24 A). To manipulate DDAH expression, the endothelial cells were infected with AdDDAH1, AdDDAH2 or inactive DDAH mutants. On the following day, HPAECs and HPASMCs were co-cultured for 24 hours before the measurement of BrdU incorporation. As can be seen in figure 24 B, HPAECs had an inhibitory effect on HPASMC proliferation in normoxic conditions, while in hypoxic conditions this inhibitory effect was significantly attenuated. Overexpression of DDAH1, but not DDAH2 or DDAH mutants, in hypoxic HPAECs restored the endothelium-dependent inhibition of HPASMC proliferation (figure 24 B). Furthermore, the protective effects of endothelial DDAH1 overexpression were attenuated in the presence of protein kinase G (PKG) inhibitor, phosphodiesterase-resistant cGMP antagonist, Rp-8-cGMP (figure 24 C), suggesting the involvement of the NO/cGMP pathway.

Taken together, the results demonstrate that hypoxia-induced changes in DDAH1 expression have a critical impact on endothelium-dependent modulation of HPASMC proliferation.

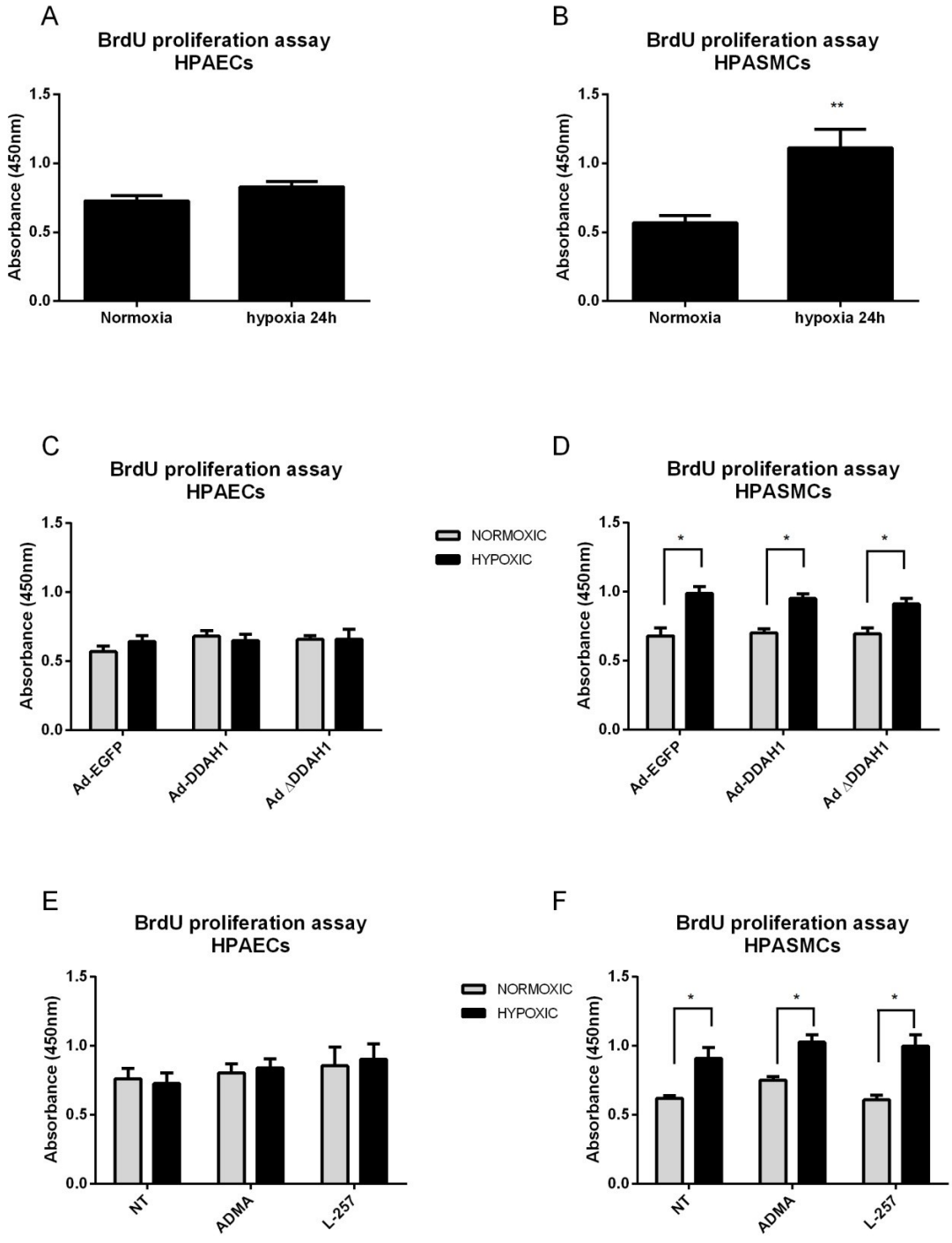

Figure 23: The effects of hypoxia and DDAH on cell proliferation. Proliferation (BrDU incorporation) in HPAECs (A) and HPASMCs (B) in normoxia or after 24 hours of hypoxia, as indicated. (C) Overexpression of DDAH1 and ΔDDAH1 had no effects on HPAECs and (D) HPASMCs proliferation. (E-F) Addition of ADMA (100μM) or inhibition of DDAH using L-257 (100μM) did not show any significant change on either HPAECs or HPASMCs proliferation. N= 6; *P<0.05, **P<0.01 comparison with the normoxic control.

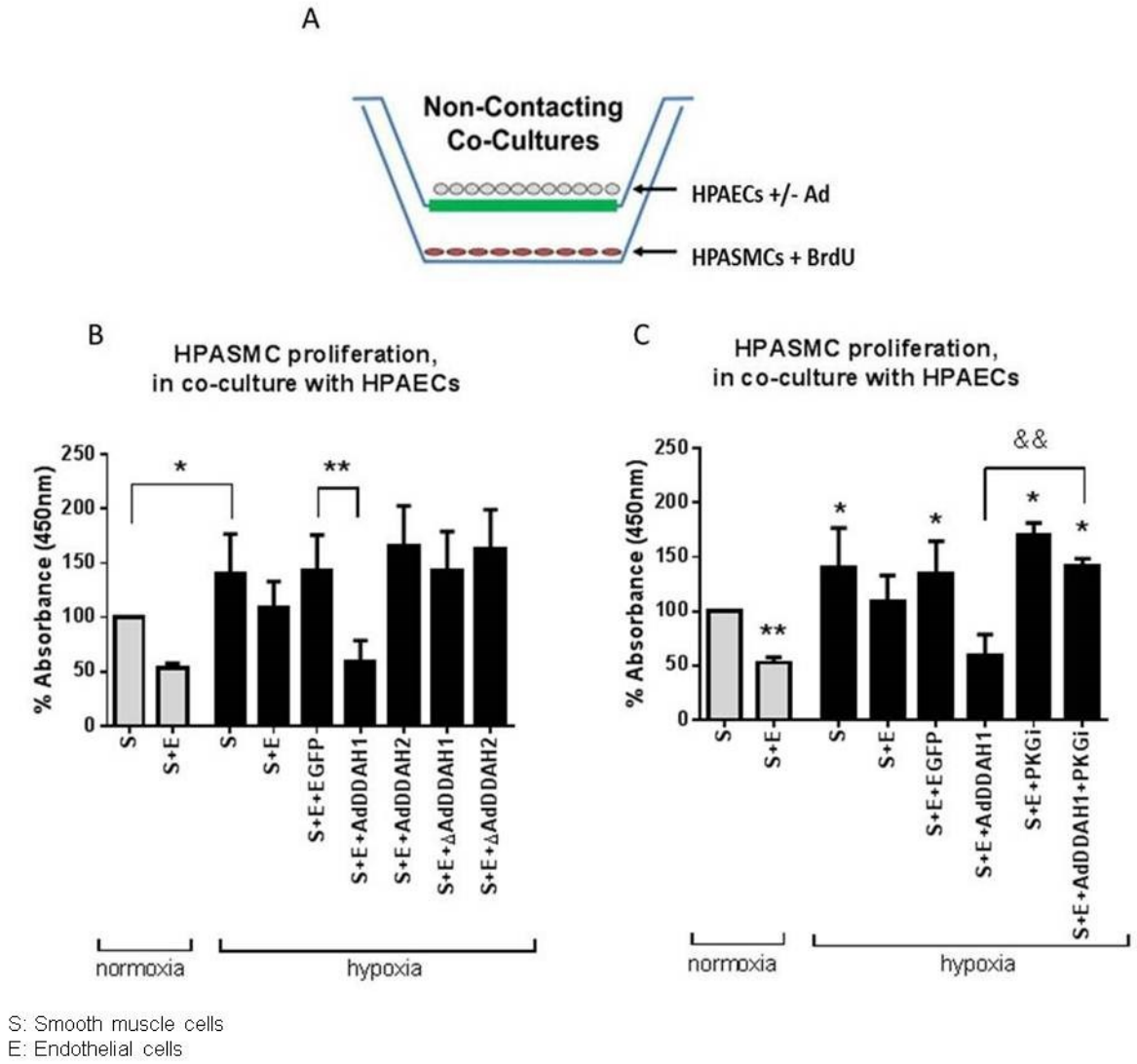

Figure 24: DDAH1 endothelium-dependent modulation of HPASMC proliferation. In (A) illustration of non-contact co-culture using Transwell system: HPAECs and HPAECs cells were co-cultured in two different compartments of Transwell dishes allowing exchange of soluble signalling mediators through the pores in the Transwell membrane (3µm) (green). (B) HPASMCs proliferation was inhibited in the presence of HPAECs in normoxia, while this inhibitory effect was attenuated in hypoxia. DDAH1 overexpression in HPAECs restored the endothelium-dependent inhibition of HPASMC proliferation in hypoxia. In (C) PKG inhibitor Rp-8-Br-PET-cGMPS (PKGi), attenuates the effects of endothelial DDAH1 overexpression on HPASMC proliferation HPAECs were left untreated or were over expressing GFP (adenoviral control) or AdDDAH1. PKGi (0.5 µM) was added to the cells 24h before the end of experiment. S: HPASMCs; E: HPAECs. AdEGFP (EGFP) was used in all the experiments as adenoviral control. *P<0.05; **P<0.01, comparisons with normoxic control; &&P<0.01 comparison, as indicated; n=6.

5.2.3. DDAH1 Regulates Endothelial Barrier Function

Endothelial barrier disruption is thought to occur early during PH development (Predescu, Predescu et al. 2005). Endothelial barrier function depends on the integrity of endothelial cell-cell contacts, in particular adherens and tight junctions (Wojciak-Stothard and Ridley 2002; Brunner, Cockcroft et al. 2005).

In order to investigate the effects of hypoxia on the organization of intercellular junctions, confluent HPAECs overexpressing DDAH1, DDAH2 (AdDDAH1-2) or their inactive mutants (ΔDDAH1-2), were exposed to normoxic or hypoxic conditions for 24h. The cells were then fixed and stained for adherens junctions protein, vascular endothelial (VE)-cadherin and filamentous (F) actin and examined under the confocal microscope.

Normoxic HPAECs showed cobblestone morphology with a predominant localization of F-actin and VE-cadherin at the cell periphery (figure 25). Hypoxia (24h) induced a loss of peripheral F-actin, dispersion of VE-cadherin from the inter-cellular junctions and the formation of central stress fibres (figure 25). Interestingly, DDAH1 prevented stress fibre formation in HPAECs, restored cortical localization of F-actin and junctional localization of VE-cadherin, while overexpression of DDAH2 had a lesser effect.

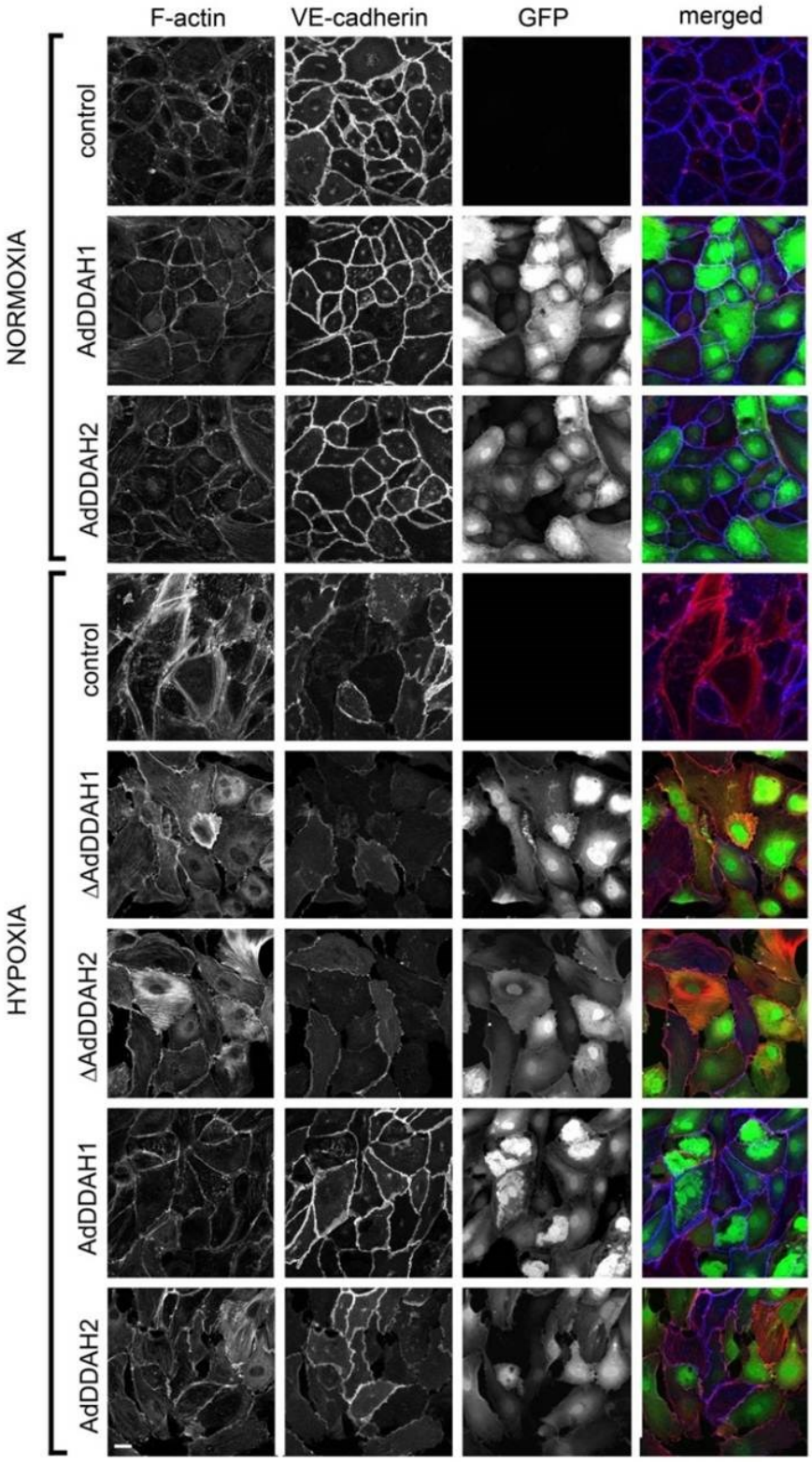

Figure 25: DDAH1 prevents hypoxia-induced dispersion of inter-cellular adherens junctions in HPAECs. HPAECs were infected with AdDDAH1, AdDDAH2, AdΔDDAH1 or AdΔDDAH2 and exposed to normoxia or to hypoxia (24h), as indicated. The columns of images from the left show localization of F-actin, VE-cadherin and GFP staining in cells, as indicated. Corresponding merged images are shown on the right (F-actin-red, VE-cadherin-blue and GFP-green). Bar=10 µm

In order to measure changes in endothelial barrier function, HPAECs were plated in the top chamber of Transwell dishes (3 µm pore size) and grown until confluence. Passage of FITC-dextran across the confluent endothelial layer was quantified after 1 hour incubation in normoxic or in hypoxic conditions (figure 26 A).

Consistent with the observed dispersion of endothelial adherens junctions, HPAECs showed a 1.5-fold increase in endothelial permeability following hypoxic exposure (figure 26 B). DDAH1 overexpression significantly attenuated the effect of hypoxia, while the overexpression of DDAH2 or inactive DDAH mutants did not have a significant effect on endothelial barrier function. The protective effects of endothelial DDAH1 overexpression were attenuated in the presence of protein kinase G inhibitor (PKGi), phosphodiesterase-resistant cGMP antagonist (figure 26 C), indicating that NO/cGMP/PKG signaling is required for the maintenance of endothelial barrier function.

In summary, these results demonstrate that the hypoxic insult compromises endothelial barrier integrity and that the effects of hypoxia are attenuated by endothelial overexpression of DDAH1 via the increase of NO/cGMP bioavailability.

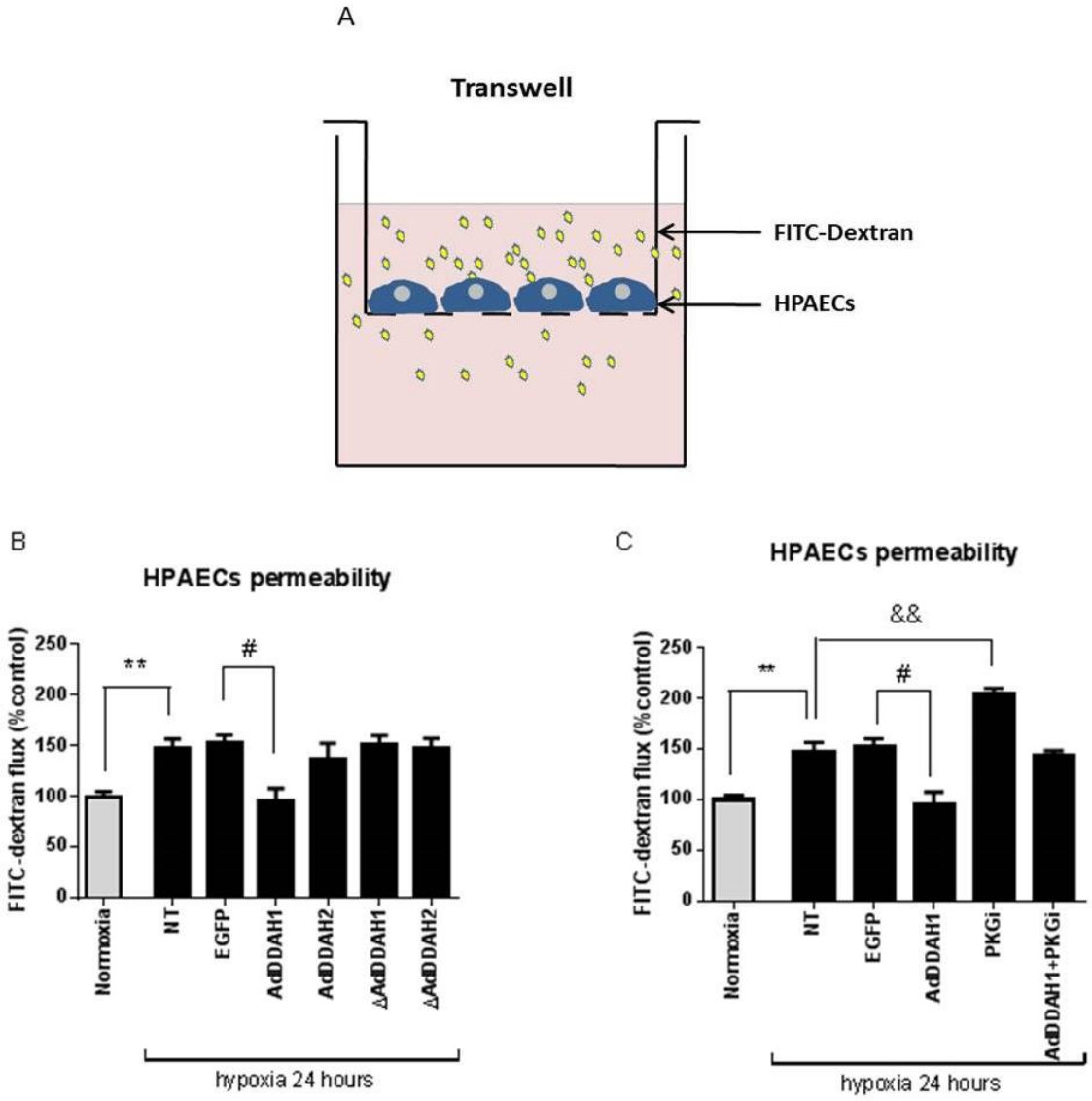

Figure 26: The effects of hypoxia and DDAH on endothelial barrier function. In (A) Transwell permeability assay in vitro measuring passage of FITC-dextran (yellow dots) across the endothelial cell layer). In (B) over expression of DDAH1 (AdDDAH1) but not DDAH2 (AdDDAH2) or inactive DDAH mutants (AdΔDDAH) attenuates hypoxia-induced increase in endothelial permeability; NT-untreated cells. (C) PKG inhibitor Rp-8-Br-PET-GMPS (PKGi) attenuates the effects of endothelial DDAH1 overexpression on endothelial permeability. HPAECs were left untreated, were over expressing EGFP (adenoviral control), AdDDAH1 or were treated with PKGi (0.5 µM). PKGi was added to the cells 24h before the end of experiment. **P<0.01, comparisons with normoxic control; #P<0.05, &&P<0.01 comparison as indicated; n=5.

5.2.4. DDAH1-ADMA Pathway Can Regulate *In Vitro* Angiogenesis

Abnormal endothelial angiogenic responses are thought to play a role in plexiform lesion formation in human PAH (Tuder, Chacon et al. 2001). Hypoxia is a contributing factor in PAH (Stenmark, Fagan et al. 2006) and therefore we studied the role of DDAH in hypoxia-induced changes in pulmonary endothelial angiogenesis *in vitro*.

The effects of hypoxia on angiogenesis were studied using the Matrigel tube formation assay, as described in materials and methods chapter 2.8.4.

24h after plating on Matrigel, endothelial cells showed formation of tubular structures (figure 27 C). There were no significant differences in tube formation between the cells cultured under normoxic or hypoxic conditions (24 hours). However, while the untreated cells showed a well-formed tubular network (figure 27 C), the addition of ADMA (100 µmol/L) or the DDAH inhibitor L-257 (100µmol/L) markedly reduced the endothelial tube formation (30% reduction for ADMA- and 50% reduction for L-257-treated cells in both, normoxic and hypoxic conditions) (figure 27 A).

To further investigate the role of DDAH enzymes in endothelial tube formation, both DDAH enzymes and their corresponding inactive mutants were overexpressed in HPAECs prior to the tube formation assay. As can be seen in figure 27 B, DDAH1 was able to increase the tubulogenesis under normoxic and hypoxic conditions, while either DDAH2 or the inactive mutants did not showed any noticeable effects, compared to the non-treated (NT) cells.

These results indicate that ADMA inhibits pulmonary endothelial angiogenesis *in vitro*, while DDAH1 has a stimulatory effect.

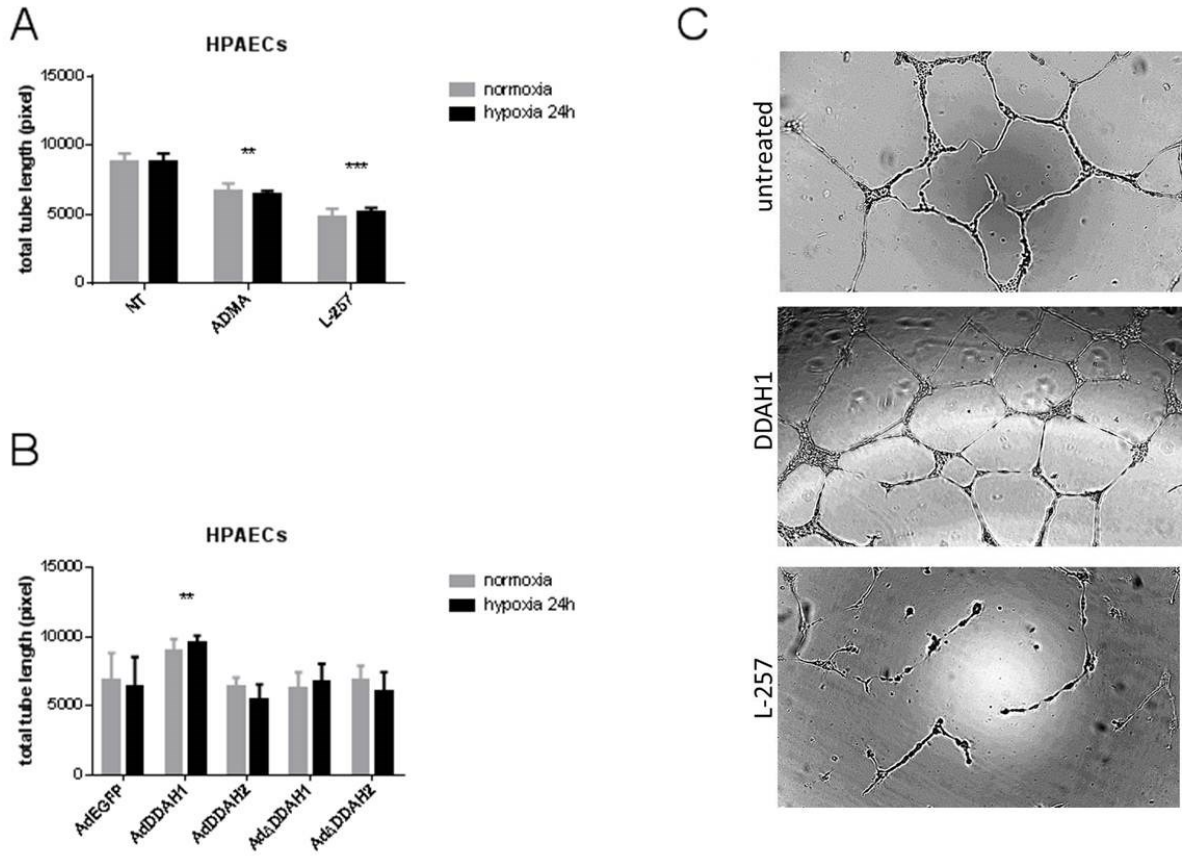

Figure 27: DDAH1 activity regulates angiogenesis in HPAECs. The cells were left untreated (NT) or were treated with ADMA (100µM, 24hr), L-257 (100µM, 24hr), or were over expressing AdEGFP, AdDDAH1, AdDDAH2 or inactive AdΔDDAH1/DDAH2 mutants (24 hr overexpression), under normoxic or hypoxic conditions, as indicated. Tube formation was measured in Matrigel assay. Representative images in (C) show tube formation in normoxic untreated, AdDDAH1 or L-257-treated cells, as indicated. N=6; **P<0.001; ***P<0.0001, comparison with normoxic control

5.2.5. NF-kB inhibition prevents hypoxia-induced changes in miR-21 and DDAH1

Inflammation plays an important role in the pathogenesis of PH (Dorfmuller, Perros et al. 2003; Selimovic, Sakiniene et al. 2008; Hall, Brogan et al. 2009; Crosswhite and Sun 2010; Price, Wort et al. 2012; Hassoun 2014). Recent studies demonstrated that inflammation is a characteristic feature of many forms of PH in both humans and animals, including chronic hypoxia-induced PH (Lu and He 2009; Price, Wort et al. 2012). Among several pro-inflammatory mediators, the transcriptional factor NF-κB (Sawada, Mitani et al. 2007) has been implicated in the progression of pulmonary hypertension.

Recently, it has been reported that NFkB is activated by hypoxia (Culver, Sundqvist et al. 2010) and that it can regulate miR-21 expression in cancer cells (Shin, Jin et al. 2011). In order to verify the potential role of NF-KB in the regulation of miR-21/DDAH pathway, hypoxic HPAECs were treated with the NF-KB inhibitor BAY-117085 (Sigma Aldrich) and DDAH1/miR-21 mRNA expression levels were quantified.

This experiment showed that BAY-117085 had an inhibitory effect on hypoxia-induced increase in mir-21 levels and downregulation of DDAH1 expression (figure 28 A, B). This preliminary observation might indicate a potential role of NFκB as an upstream activator of miR-21 signaling in hypoxic endothelial cells (figure 28 C).

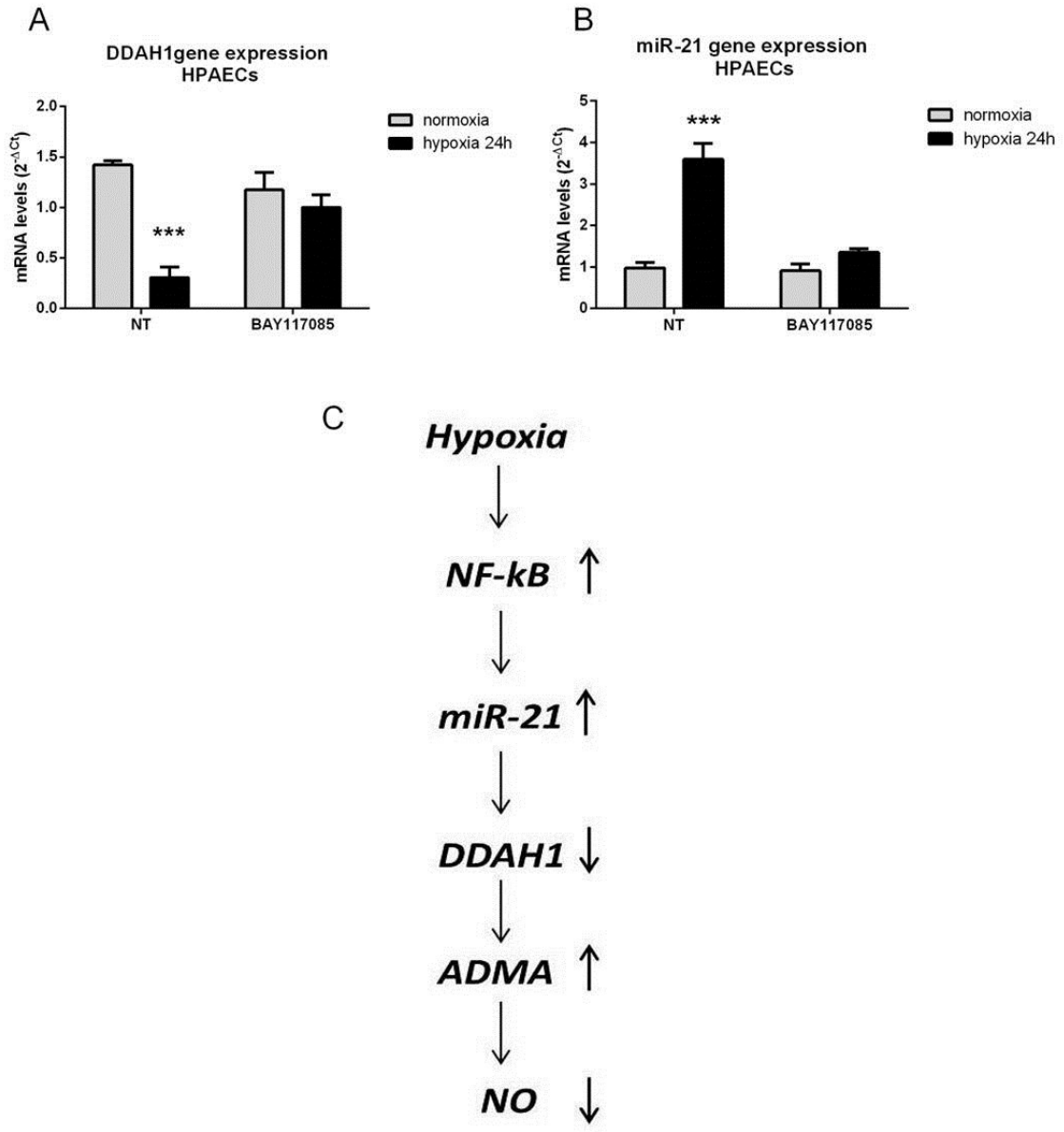

Figure 28: NF-κB inhibitor, BAY117085 prevents hypoxia-induced changes in miR-21 and DDAH1 mRNA levels in HPAECs. HPAECs were incubated with BAY 117085 (10 µmol/L) for 24h in normoxic or in hypoxic conditions, before the measurement of DDAH1 mRNA levels (A) and miR-21 (B). In (C) proposed mechanism of NF-kB in the regulation of DDAH1 signaling. ***P<0.001, comparison with normoxic controls; n=4.

5.3. Discussion

Hypoxia-induced endothelial dysfunction plays a role in pulmonary vascular remodeling. Hypoxia causes endothelial dysfunction, at least in part, via decreased bioavailability of nitric oxide.

In this chapter, we studied the role of ADMA metabolism in the regulation of hypoxia-induced changes in pulmonary vascular endothelial and smooth muscle cell death, proliferation, endothelial barrier function and angiogenesis. The results show that:

1. Hypoxia (2% O_2, 2-24h) does not induce apoptosis in pulmonary vascular cells.
2. Endothelium-dependent inhibition of HPASMC proliferation in *vitro* is lost in hypoxia and is restored by endothelial overexpression of DDAH1
3. DDAH1/NO signaling is important for the maintenance of endothelial barrier function.
4. DDAH1-ADMA pathway regulates HPAEC angiogenesis *in vitro*.
5. NFkB inhibition prevents hypoxia-induced increase in miR-21 expression in HPAECs.

Endothelial cell death is regarded as an important contributory factor in the pathogenesis of PH (Jurasz, Courtman et al. 2010). Extensive endothelial damage and a subsequent inflammatory response contribute to pulmonary vascular remodelling in the rat monocrotaline model of PH (Arcot, Lipke et al. 1993; Schultze and Roth 1998) . Combination of the VEGFR2 inhibitor Sugen and hypoxia, in rats and mice induce endothelial apoptosis followed by the formation of occlusive plexiform lesions, resulting from a local expansion of apoptosis-resistant endothelial cells (Taraseviciene-Stewart, Kasahara et al. 2001). It has been reported that hypoxia can trigger apoptosis in several ways. In fact, hypoxia can induce apoptosis by causing hyper-permeability of the inner mitochondrial membrane that leads to the release of cytochrome C, an essential component of the mitochondrial electron transport chain (Saikumar, Dong et al. 1998; de Moissac, Gurevich et al. 2000) or via activation of the caspase 9 pathway. In this mechanism, in response to hypoxia inactive caspase 9 is cleaved and activated by caspases 3 and 12, without the involvement of cytochrome C (Morishima, Nakanishi et al. 2002). Another mechanism by which hypoxia can

induce apoptosis has been reported in melanoma cells, where hypoxia can activate c-Jun NH2-terminal kinase (JNK) and induce apoptosis (Basu and Kolesnick 1998). Hypoxia-induced apoptosis has been reported in solid tumors (Graeber, Osmanian et al. 1996) and cardiovascular diseases (Mehta, Kang et al. 2002; Zhang, Li et al. 2004). Matsushita et al demonstrated that activation of NF-κβ by hypoxia induced human aortic endothelial cell (HAEC) apoptosis through the suppression of an anti-apoptotic molecule, bcl-2 (Matsushita, Morishita et al. 2000).

In this chapter, we have shown that hypoxia had no effect on pulmonary endothelial or smooth muscle cells apoptosis *in vitro*. The discrepancies in hypoxia-induced effects reported in different studies may result from differences in the experimental design: the cell type used (HPAECs versus HAECs) or the level of hypoxia (2% O_2 in our study versus near-anoxia, undetectable O_2 level in the study of Matsushita), (Matsushita, Morishita et al. 2000).

Endothelial NO can act as a vasorelaxant and an inhibitor of VSMC proliferation in *vitro* and in *vivo* (Dzau and Gibbons 1991; Peiro, Redondo et al. 1995; Ambalavanan, Mariani et al. 1999). Our results indicate that functional endothelial DDAH1 is indispensable for the endothelial control of SMC proliferation. In fact, we observed that hypoxia stimulated the growth of smooth muscle cells co-cultured with endothelial cells, but the effect was inhibited in the presence of endothelial cells overexpressing DDAH1, while DDAH2 or DDAH mutants had no effect. We used a Transwell co-culture system to study the effect of endothelium-derived diffusible factors on HPASMC proliferation. The distance separating the two cell layers was <1mm, a distance sufficiently small to ensure free diffusion of NO between the cell layers (Lancaster 1996; Lancaster 1997). However, the possibility that DDAH1 may affect the release of other factors involved in the regulation of SMC growth cannot be excluded. Indeed, hypoxic endothelial cells can exhibit chemoattractant and mitogenic activity towards smooth muscle cells and fibroblasts as a result of increased production of ET-1, VEGF and PDGF (Dawes, Peacock et al. 1994).

ADMA compromises endothelial barrier function *in vitro* and *in vivo* (Mundy and Dorrington 2000; Predescu, Predescu et al. 2005; Wojciak-Stothard, Torondel et al. 2009). Here, we showed that hypoxia-induced breakdown of pulmonary endothelial function *in vitro* was attenuated by the overexpression of DDAH1. The breakdown of endothelial barrier function

by hypoxia can lead to leukocyte adhesion, platelet activation, SMC proliferation, migration and ECM deposition (Garg and Hassid 1989; Garg and Hassid 1990; Scottburden, Schini et al. 1992; Sarkar, Webb et al. 1995; Lau and Ma 1996), contributing to pulmonary vascular remodelling.

Hypoxia induces endothelial angiogenesis by activating HIF and increasing VEGF release (Forsythe, Jiang et al. 1996), an essential mechanism in tumour growth (Forsythe, Jiang et al. 1996). HIF activation and increased endothelial production of VEGF are also features of cells in plexiform lesions in human PAH (Hirose, Hosoda et al. 2000; Humbert, Morrell et al. 2004). However, exposure to chronic hypoxia alone does not induce plexiform lesion formation, suggesting that hypoxia acts only as a contributory factor in this process. We observed that hypoxia did not significantly affect pulmonary endothelial angiogenesis in vitro. However, exogenous ADMA and DDAH1 inhibitor, L-257 inhibited pulmonary endothelial angiogenesis in vitro, while DDAH1 overexpression had a stimulatory effect, suggesting that intact ADMA metabolism is required for the maintenance of correct angiogenic responses.

NFkB is activated by hypoxia (Culver, Sundqvist et al. 2010) and has been shown to regulate miR-21 expression in cancer cells (Shin, Jin et al. 2011). Recently, Hosokawa et al demonstrated the pathophysiological role of NFkB in pulmonary hypertension (Hosokawa, Haraguchi et al. 2013). In this study, the authors observed that the use of a synthetic selective NF-kB inhibitor, N-(3,5-Bis-trifluoromethyl-phenyl)-5-chloro-2-hydroxy-benzamide (IMD-0354) can reduce RV pressure in MCT animal model and inhibit PASMCs proliferation. Accordingly, our *in vitro* experiments showed that NFkB inhibition can prevent hypoxia-induced increase in miR-21 expression in HPAECs and therefore protect DDAH1 protein from downregulation.

In summary, these results demonstrate the importance of DDAH1 in hypoxia-induced changes in ADMA levels, endothelial barrier function, angiogenesis and endothelium-dependent proliferative responses in pulmonary vascular cells. Furthermore, we also show that NFkB activity is required for the hypoxia-induced upregulation of miR-21 levels, suggesting a plausible mechanism of action of hypoxia-induced changes in DDAH1 levels.

6. Chronic Hypoxia-Induced Pulmonary Hypertension

6.1. Introduction

Hypoxia is frequently used to induce PH in a wide variety of animal species. This model is useful since it is very predictable and reproducible within the selected animal strain. Following 2-3 weeks of hypoxic exposure, mice and rats develop pulmonary hypertension characterised by remodelling, increased right ventricular systolic pressure and right ventricular hypertrophy (Stenmark, Meyrick et al. 2009).

Overexpression of the human DDAH1 (hDDAH1) gene was reported to have beneficial cardiovascular effects in mice (Schwedhelm, von Leitner et al. 2009). Indeed, the DDAH1 transgenic mice (DDAH1tg) show reduced systolic blood pressure and systemic vascular resistance (Dayoub, Achan et al. 2003). Furthermore, DDAH1tg mice exhibit a reduction of the myocardial reperfusion injury (Stuhlinger, Conci et al. 2007).

These beneficial effects may be related to an improved metabolic clearance of ADMA by kidney and liver tissues or an increased DDAH1 expression in cardiovascular tissues.

Our experiments *in vitro* suggest that DDAH1 plays an important regulatory role in pulmonary vascular responses to hypoxia. In the next series of experiments we verified the role of DDAH1 in mouse model of chronic hypoxia-induced pulmonary hypertension. We also investigated potential suitability of miR-21 targeting in treatment of PH induced by chronic hypoxia.

6.2. Results

6.2.1. Increased Expression of DDAH1 Attenuates Development of Chronic Hypoxia-Induced Pulmonary Hypertension

In order to study the effects of hDDAH1 overexpression on hypoxia-induced PH, the wildtype and the transgenic DDAH1- overexpressing mice (DDAH1tg) were exposed to hypoxia for 2 weeks. Development of pulmonary hypertension was confirmed by the measurement of right ventricular pressure and pulmonary vascular remodelling, as described in material and methods 2.9.1.

No significant differences in the right ventricular hypertrophy (RVH, 0.79±0.15 versus 0.82±0.19) or systolic pressure (RVSP, 15.7±0.12 versus 16.3±0.23) were observed between the wild type and the DDAH1tg mice kept in normal air (figure 29 A and B), consistent with previous reports (Bakr, Pak et al. 2013). Hypoxia increased RVH in the wild type mice, while in DDAH1tg mice the response was comparable to normoxic wildtype controls (1.09±0.35 versus 0.84±0.39) (figure 29 A). Consistently, RVSP was raised in both the wildtype mice and the DDAHtg mice, but DDAH1tg mice showed a significantly attenuated response (30.68 ± 0.82 mmHg in wildtype mice versus 26.93 ± 0.86 mmHg in DDAH1tg mice) (figure 29 B). In addition, an increase in pulmonary vascular muscularization was observed in both genotypes but the number of fully and partially muscularised vessels in DDAHtg mice was significantly reduced ($P < 0.05$), when compared with wildtype controls (79% SD 2.4 in wildtype mice versus 65% SD 1.04 in DDAH1tg mice)(figure 29 C, E). As expected, chronic hypoxia enhanced the expression of PCNA, a nuclear protein that participates in active cell proliferation (Takahashi, Strutton et al. 1991), in wild-type mice, whereas in DDAH1tg animals the expression levels of PCNA were reduced (figure 29 D).

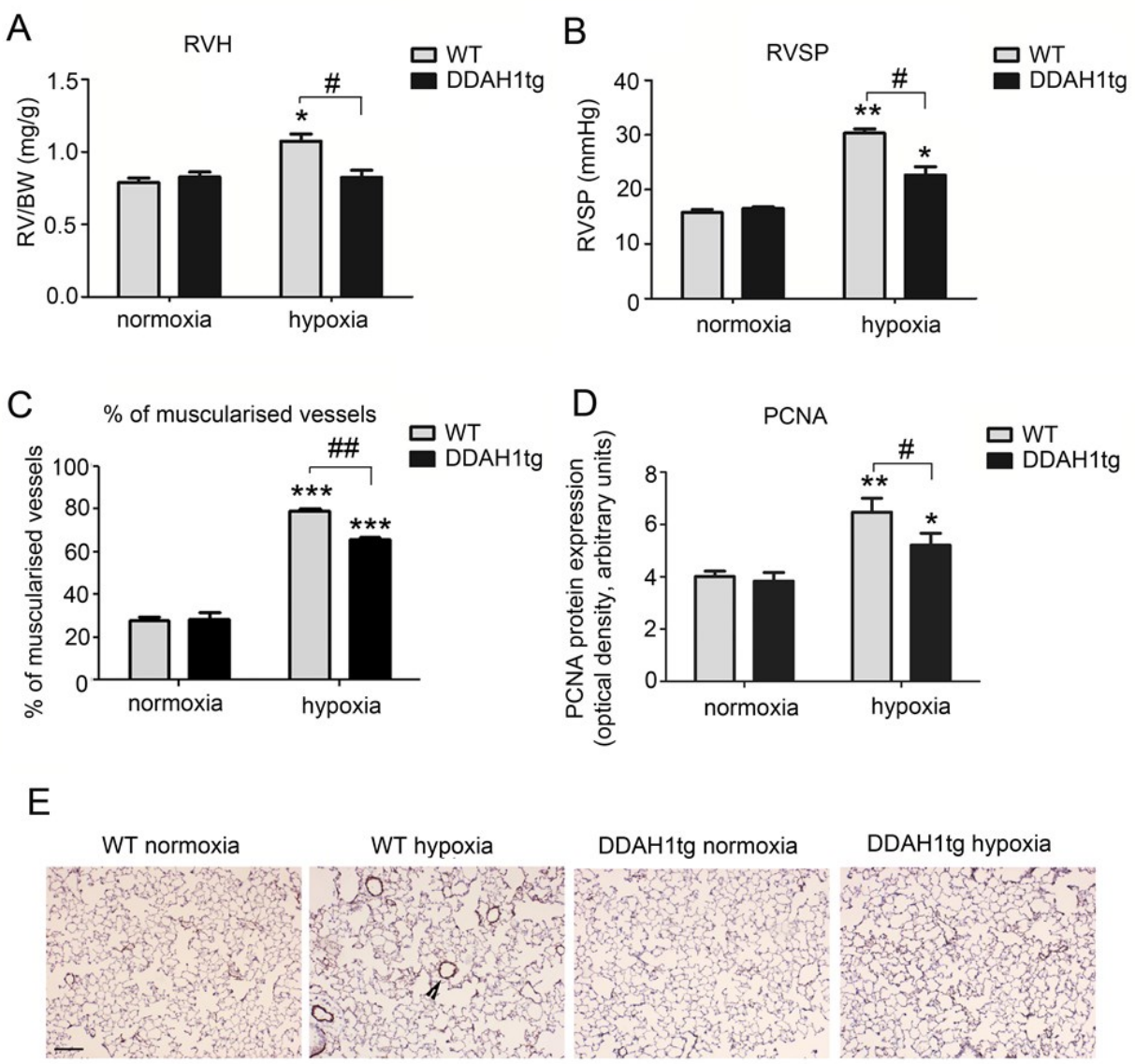

Figure 29: Increased expression of DDAH1 attenuates development of chronic hypoxia-induced pulmonary hypertension. (A) Right ventricular hypertrophy (RVH) and (B) right ventricular systolic pressure (RVSP) were measured in wild type control mice (WT; grey bars) and DDAH1 transgenic mice (DDAHtg; black bars) following 2-week exposure to normoxia or hypoxia. (C) Percentage of muscularized vessels diameter <50 µm in lung sections in the wildtype (WT) or DDAHtg mice. *P<0.05, comparison with hypoxic controls; n=8. (D) PCNA levels in normoxic and hypoxic WT (grey bars) and DDAHtg mice (black bars) in normoxia and hypoxia, as indicated. (E) Representative images of lung sections showing αSM actin staining in normoxic or the hypoxic wild type (WT) or DDAHtg mice. The arrowhead points to the fully muscularized artery in hypoxic WT mice. Bar=50 µm.

Consistent with the effects of hypoxia on DDAH expression observed *in vitro*, DDAH1 protein expression was reduced 5-fold in the lungs of pulmonary hypertensive mice (figure 30 A) and although DDAH2 protein expression was also reduced, the change was less pronounced (figure 30 B). By contrast, the lungs of DDAH1tg mice showed elevated DDAH1 expression which was reduced to normoxic control levels following exposure to chronic hypoxia (figure 30 A). Hypoxic wildtype mice showed a significant reduction in cGMP lung content (2-fold decrease), compared with wildtype normoxic controls, while DDAH1tg mice showed elevated levels of cGMP in the lung in both normoxic and in hypoxic conditions (figure 30 D). Protein expression levels of Rho GTPase RhoA, known to promote vasoconstriction and pulmonary vascular remodelling (Wojciak-Stothard, Torondel et al. 2009; Nossaman, Nossaman et al. 2010) were elevated in both the wild type and DDAH1tg mice (figure 30 C). NO/PKG-mediated phosphorylation of RhoA on Ser188 inhibits RhoA activity and reduces actomyosin contractility in pulmonary vascular smooth muscle and endothelial cells (Sauzeau, Le Jeune et al. 2000; Wojciak-Stothard, Torondel et al. 2007). Consistent with changes in lung DDAH expression and cGMP levels, pSer188RhoA levels were reduced in hypoxic wild type mice, while in hypoxic DDAHtg mice, the pSer188RhoA levels were comparable to normoxic controls (figure 30 E).

DDAHtg mice did not show any changes in DDAH2 expression under basal conditions or following hypoxic exposure (Schwedhelm, von Leitner et al. 2009).

In summary, we observed that the overexpression of DDAH1 *in vivo* attenuated development of pulmonary hypertension in chronically hypoxic mice, suggested a protective function of DDAH/NO/cGMP in PH.

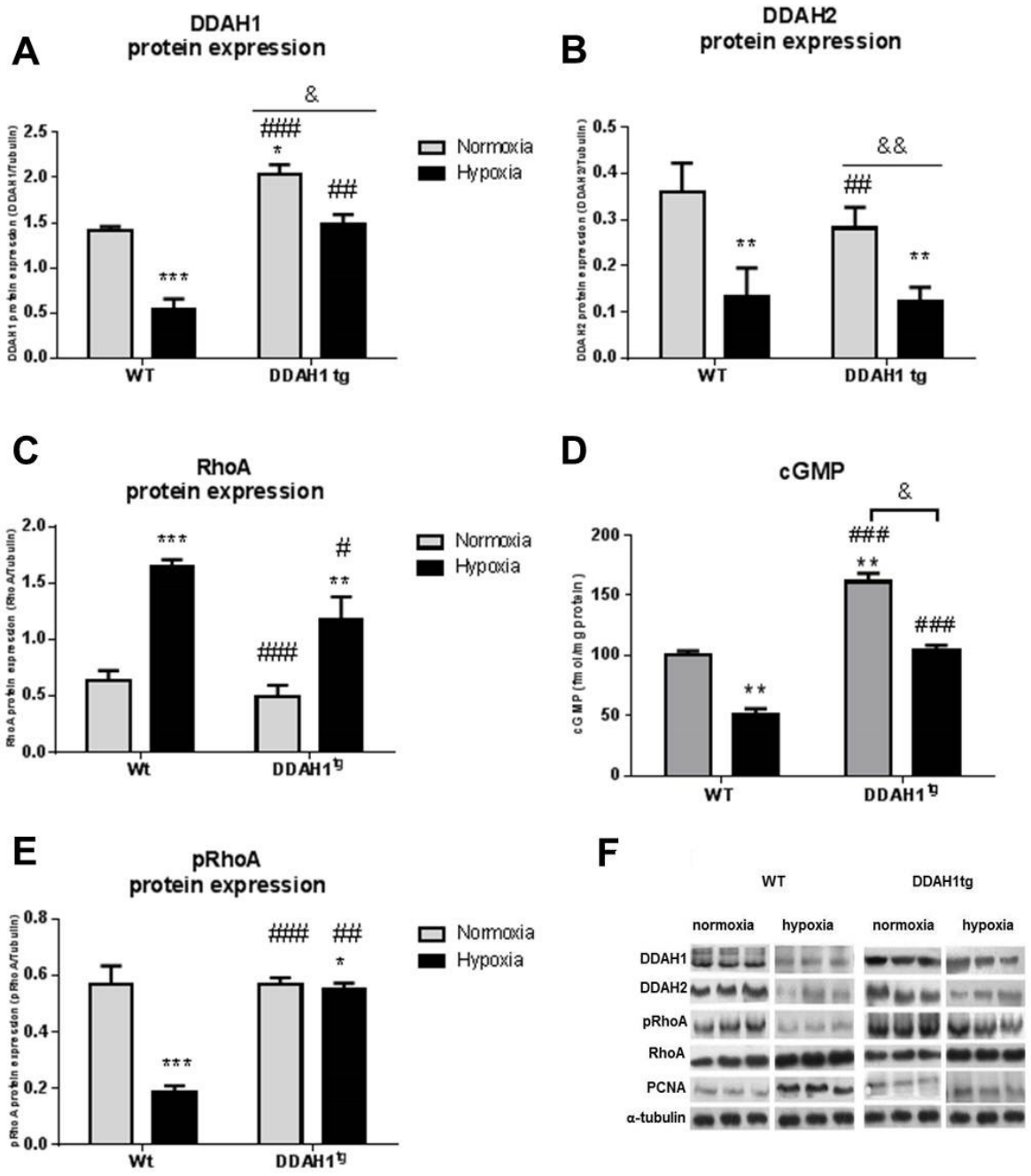

Figure 30: **Expression of DDAH1, DDAH2, RhoA, pSer188RhoA in the wildtype (WT) and DDAH1 overexpressing transgenic mice (DDAH1tg) kept for 2 weeks in normoxia (grey bars) or in hypoxia (black bars). (D) cGMP levels in lungs of normoxic and hypoxic mice. *P<0.05; **P<0.001, ***P<0.0001 comparison with normoxic controls; representative examples of western blots are shown in (F). #P<0.05, ##P<0.01, comparisons with hypoxic control, &P<0.05 comparisons as indicated. N= 8, in (A-E); N=4 in (F).**

6.2.2. MiR-21 Inhibition Moderates Development of Chronic Hypoxia-Induced Pulmonary Hypertension

MiR-21 levels are increased in hypoxic mice and in hypoxic human lung (Parikh, Jin et al. 2012; Pullamsetti, Doebele et al. 2012) and in HPAECs cultured under hypoxic conditions (as showed in results 4.2.2 figure 20E). I hypothesized that inhibition of miR-21 in hypoxic mice may result in an increase in DDAH1 expression and may have a protective effect in development PH. C57BL/6 male mice were given intraperitoneal (IP) injection of 100 µl of vehicle or 100 µl locked nucleic acid anti-miR-21 probes (LNA21) in PBS at 10 mg/kg once every other day for a total of 8 times during the 2 weeks of hypoxia exposure. Following hypoxic exposure, the mice were phenotyped as described in 2.9.1. The *in vivo* inhibition of miR-21 with specific LNA probes, reduced chronic hypoxia-induced increase in RVSP (figure 31 A), RVH (figure 31 B), and muscularization of small intrapulmonary arteries (figure 31 C and D).

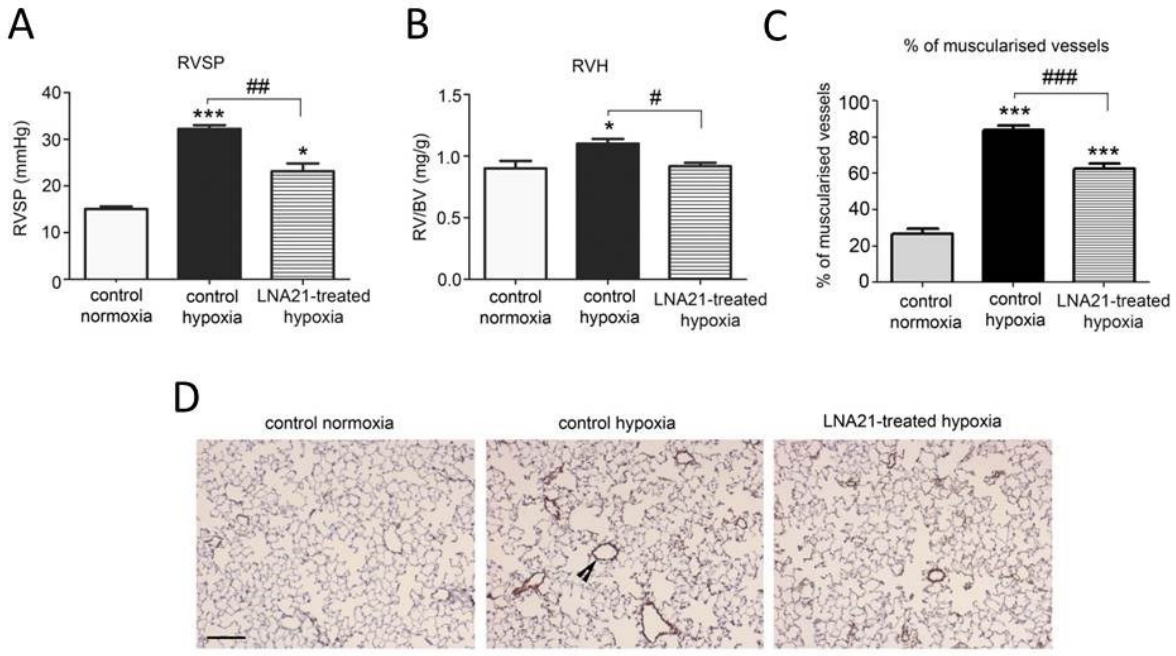

Figure 31: Treatment with LNA21 reduces (a) right ventricular systolic pressure (RVSP), (b) right ventricular hypertrophy (RVH), (c) muscularisation of intrapulmonary arteries in hypoxic pulmonary hypertensive wildtype (WT) mice. *P<0.05, ***P<0.001, comparison with untreated normoxic controls; #P<0.05 and ##P<0.01, ###P<0.001, comparisons with untreated hypoxic controls, as indicated. n=6-8. (d) Representative images of lung sections showing αSM actin staining in normoxic or the hypoxic wild type (WT) untreated or LNA21-treated mice. The arrowhead points to the fully muscularized artery in hypoxic WT mice. Bar=50 µm.

DDAH1 protein expression was reduced 5-fold in the lungs of pulmonary hypertensive mice and was restored to control levels upon treatment with LNA-21 (figure 32 A). Consistent with the observed reduction in pulmonary vascular remodelling, LNA-21 treatment also reduced protein expression levels of PCNA (figure 32 E). LNA21-treated hypoxic mice showed significantly elevated pSer188RhoA, indicative of improved nitric oxide signalling (Sauzeau, Le Jeune et al. 2000), (figure 32 B). Protein expression of RhoA was elevated in hypoxic lung and was not affected by LNA-21 treatment (Figure 32 C). RhoB, a protein homologous to RhoA, has been implicated in the pathogenesis of PH and is a predicted target of miR-21 (Sabatel, Malvaux et al. 2011; Parikh, Jin et al. 2012; Wojciak-Stothard and Wilkins 2012). However, we did not observe any significant changes in RhoB expression levels upon treatment with LNA-21 (figure 32 D), which suggests that this protein is unlikely to mediate the protective effects of LNA-21.

In order to understand the role of miR-21 in the regulation of NO/cGMP signaling, LNA21-treated mice exposed to hypoxia and subsequently cGMP was quantified.

As showed in figure 33 F, a significant increase in cGMP levels was noted in lungs of hypoxia LNA21-treated mice, compared with untreated hypoxic controls (figure 33 F). Oddly, this result is in contrast with the postulated role of ANP during hypoxia (Chen, Feng et al. 2006). In fact, ANP synthesis and secretion are enhanced during hypoxia (Raffestin, Levame et al. 1992), increasing the synthesis of cGMP and protecting against the development of pulmonary hypertension (Sciarretta, Marchitti et al. 2013).

Both the hypoxic LNA21-treated and DDAH1tg mice showed a significant reduction in ADMA levels in the lung, compared with hypoxic controls (figure 33 A). Interestingly, the effects of hypoxia on miR-21/DDAH1 expression appeared to be lung-specific, as hypoxia increased miR-21 mRNA levels and decreased DDAH1 protein levels by 3-fold in lung tissues, while miR-21/DDAH1 levels in kidney and liver remained unaffected (figure 33 B and C).

In summary, these results show that the inhibition of miR-21 can prevent hypoxia-induced reduction in DDAH1 expression in the lung and attenuate the development of chronic hypoxia-induced PH.

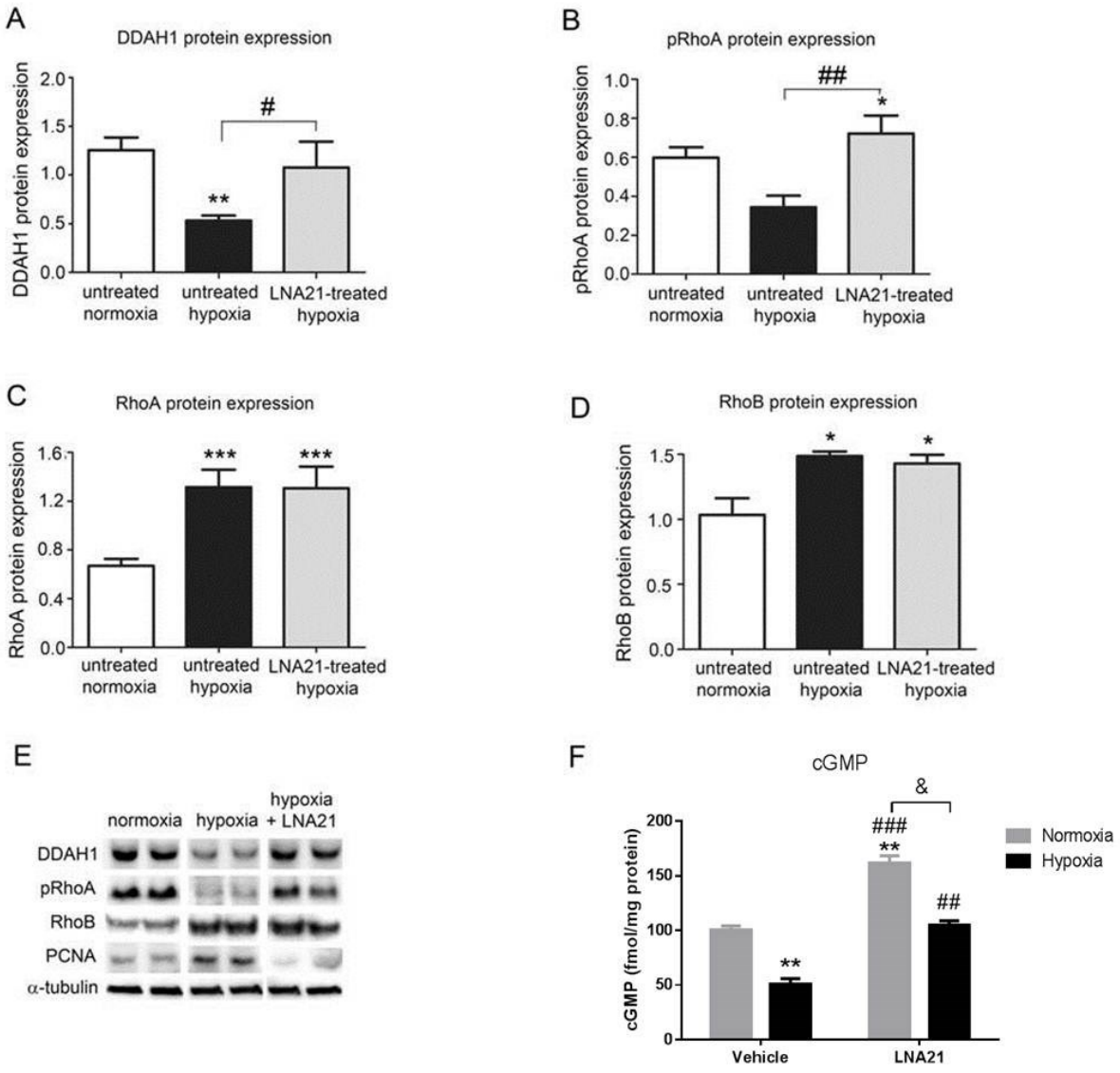

Figure 32: LNA21 treatment increased expression of DDAH1 and attenuates development of chronic hypoxia-induced PH in mice. Protein expression levels of (A) DDAH1, (B) RhoA phosphorylated on Ser 188 (pRhoA); (C) RhoA; (D) RhoB and (E) representative Western blots corresponding to (A-D). (F) cGMP levels in lungs of normoxic and hypoxic mice. *P<0.05; **P<0.001, ***P<0.0001 comparison with normoxic controls; #P<0.05, ##P<0.01, ###P<0.001comparisons with hypoxic control, &P<0.05 comparisons as indicated; n= 8, in (A-E); n=4 in (F).

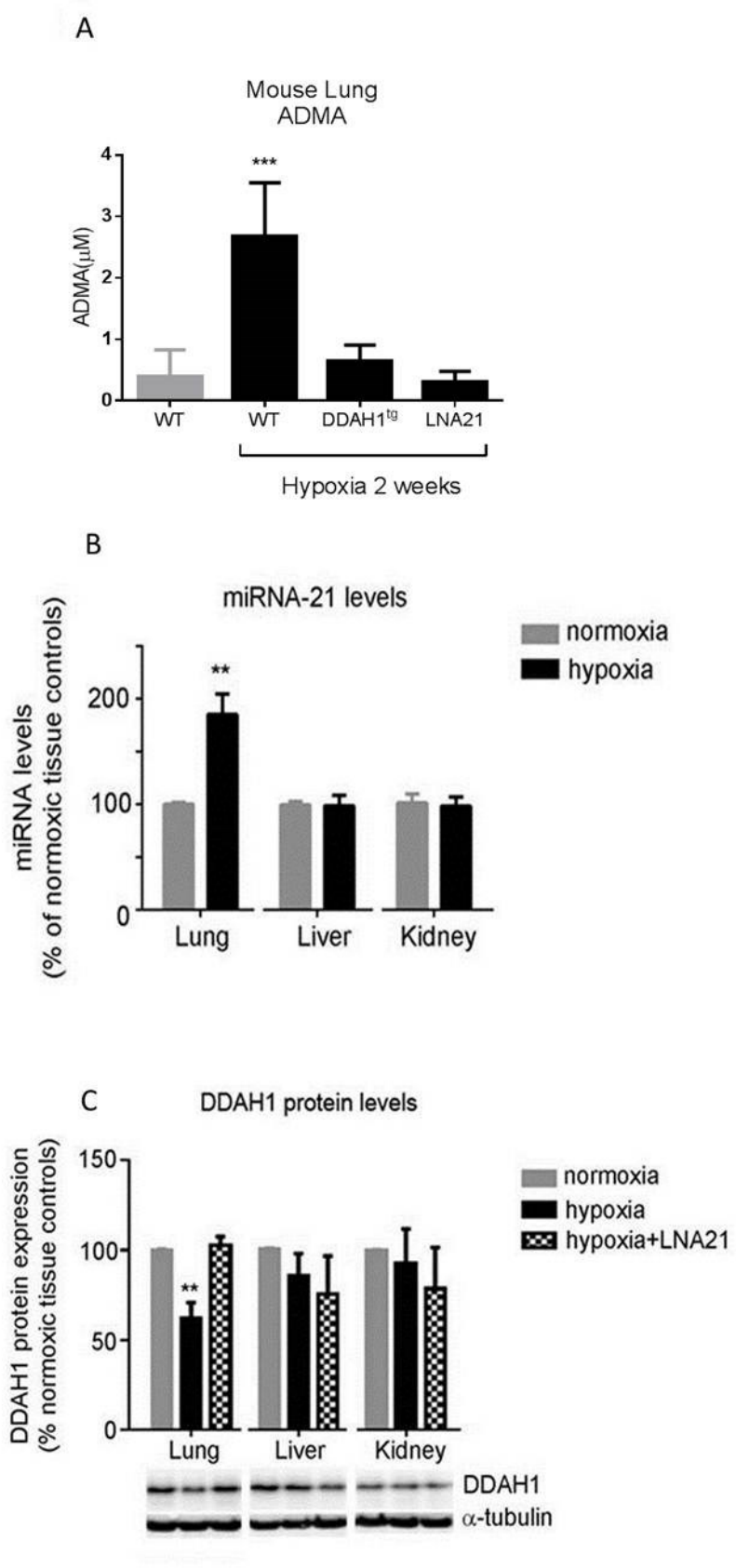

Figure 33: ADMA, miR-21 and DDAH1 levels in normoxic or hypoxic (2 weeks hypoxia) wild type (WT), LNA21-treated or DDAHtg mice, as indicated. (b) Hypoxia-induced changes in miR-21 mRNA levels and in DDAH1 protein levels in mouse lung, liver and kidney tissues. (c) ***P<0.001, **P<0.01, comparison with normoxic WT controls; n=8.

6.2.3. DDAH1 is Reduced and MiR-21 is Increased in Human Pulmonary Arterial Hypertensive (PAH) Lungs.

Data from the *in vivo* experiments suggest that microRNA21-mediated downregulation of DDAH1 might play a role in human PH. MiR-21 and DDAH1 mRNA levels were measured in lung tissues from patients with IPAH and compared with healthy controls.

Since pulmonary hypertension is a disease of the lung vasculature with secondary effects on the heart, investigations of this pathology require samples of lung tissue from patients with PH. As shown in table 3 in the material and methods chapter, the samples used were obtained from end-stage IPAH patients (n=12, 9 female patients) unresponsive to available PH therapies that underwent lung transplantation. The lung tissues from treatment-naïve patients we used were obtained in the early 1990s, before epoprostenol (Flolan) was approved for clinical use in PAH and several years before the introduction of endothelin receptor antagonists and phosphodiesterase inhibitors as therapies for PAH. As control tissues, we used samples from healthy sections of lung from patients undergoing a lobectomy (n=12, 5 female patients) usually with lung cancer.

Lung tissues from treatment-naïve patients with IPAH patients showed significantly reduced levels of DDAH1 mRNA (1.1 ± 0.4 in PAH and 2.1 ± 0.8 in healthy control group; P<0.001) and elevated levels of miR-21, (23.5 ± 5 in IPAH and 7 ± 5.3 in healthy control group; P<0.001), while DDAH2 mRNA levels remained unchanged (figure 34 A-C).

Together, these results suggest that dysregulation of the miR-21/DDAH1 pathway may be common to different forms of pulmonary hypertension.

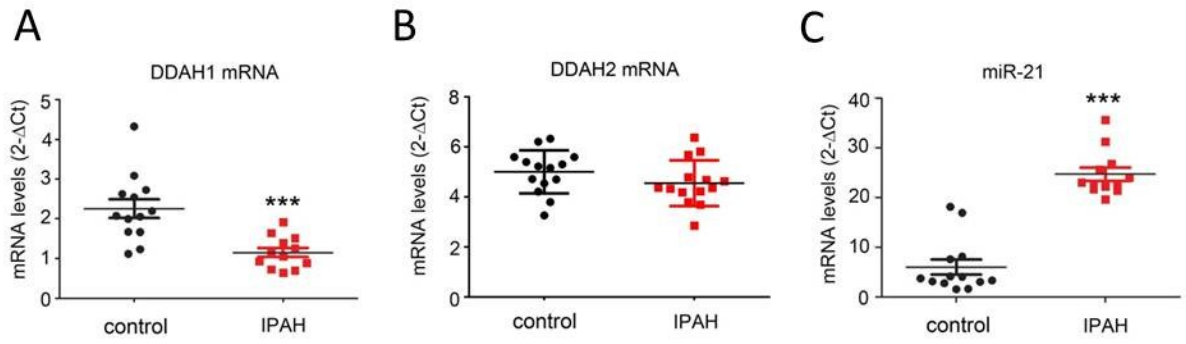

Figure 34: DDAH1, DDAH2 and miR-21 levels in IPAH patients. DDAH1 mRNA (a), DDAH2 mRNA (b) and miR-21 (c) levels were measured in lung surgical samples from IPAH patients and control subjects, as indicated. ***P<0.001, comparison with control subjects. N=12.

6.3. Discussion

The experiments in this chapter demonstrated the following findings:

1. DDAH1 overexpression and inhibition of miR-21 attenuate the development of chronic hypoxia-induced PH.
2. Hypoxia-induced, miR-21-mediated reduction in DDAH1 expression is lung-specific.
3. DDAH1 is reduced and miR-21 is increased in human PAH lungs, suggesting a possible role of miR-21/DDAH1 signaling in human pathology.

Overexpression of the human DDAH1 (hDDAH1) gene was reported to have beneficial cardiovascular effects in mice (Schwedhelm, von Leitner et al. 2009).

The authors observed that DDAH1tg mice did not show an improved DDAH-metabolic capacity of kidney and liver under normoxic conditions (Schwedhelm, von Leitner et al. 2009). However, they demonstrated that the decrease in circulating ADMA levels was due to a decreased release of ADMA from aorta and heart. The authors postulated that the protective cardiovascular effects observed in DDAH1tg mice may therefore result from improved activity of the DDAH enzyme in the cardiovascular system and not from improved renal or hepatic clearance of ADMA and L-NMMA.

Accordingly, in our study DDAH1tg mice showed attenuated pulmonary hypoxic phenotype when compared with the wild type controls, suggesting a protective role for DDAH1 in pulmonary hypertension. Furthermore, our results indicate that the protective effects of miR-21/DDAH1 may be mediated by NO/cGMP/PKG pathway, consistent with the increase in lung cGMP levels and increased phosphorylation of RhoA on Ser188.

However, further experiments will be required in order to understand the role of ANP signaling in hypoxic mice treated with LNA21. In fact, studies have shown that mice exposed to chronic hypoxia exhibit increased pulmonary arterial pressure and RV hypertrophy associated with increasing levels of plasma ANP (Preston, Hill et al. 2004). Thus, it has been postulated that increased secretion of ANP may provide a compensatory mechanism to modulate the development of hypoxia-induced pulmonary hypertension and vascular remodeling (Hirata, Suzuki et al. 1992).

Relevant to the postulated protective role of DDAH1, Bakr et al. (Bakr, Pak et al. 2013) have recently demonstrated that DDAH1tg mice show attenuated acute and sustained hypoxic pulmonary arterial vasoconstriction. Interestingly, exposure of these mice to 4 weeks (instead of 2 weeks used in our study) of hypoxia did not prevent pulmonary vascular remodelling (Bakr, Pak et al. 2013). The loss of a protective effect of DDAH1 after a more prolonged exposure to hypoxia might be associated with the gradual, hypoxia-induced decrease in β-actin promoter-driven expression (Brown and Davis 2005) of the DDAH1 transgene, noted in both studies (Bakr, Pak et al. 2013).

MiR-21 has been implicated in the pathogenesis of several diseases, including pancreatic and lung cancer (Mace, Collins et al. 2013; Haigl, Vanas et al. 2014), kidney fibrosis (Glowacki, Savary et al. 2013) and diabetic cardiomyopathy associated with cardiac fibrosis (Kumar, Raut et al. 2011). However the role of miR-21 in pulmonary hypertension remains controversial.

Liu et al found elevated levels of miR-21 in the lungs of mice with experimental pulmonary fibrosis and in the lungs of patients with idiopathic pulmonary fibrosis (Liu, Friggeri et al. 2010). Accordingly, Yang et al showed that miR-21 overexpression enhanced the expression of cell proliferation- associated proteins such as PCNA and cyclin D1, suggesting that miR-21 may play an important role in the pathogenesis of chronic hypoxia-induced pulmonary vascular remodeling (Yang, Banerjee et al. 2012). Mir-21 has been linked with several target proteins involved in pulmonary vascular remodelling and vascular smooth muscle proliferation, such as RhoB, BMPR2 (bone morphogenetic protein receptor type II), PDCD4 (programmed cell death 4) or SPRY2 (Sarkar, Gou et al. 2010)

Interestingly, Parikh et al presented a molecular model in which hypoxia, inflammation, and BMP-dependent signaling up-regulate miR-21 in the pulmonary vasculature (Parikh, Jin et al. 2012). The authors observed that miR-21 expression was increased in various *in vivo* models of pulmonary hypertension. Indeed, it has been shown that miR-21 expression was increased in lungs of IL-6-overexpressing mice and in chronic hypoxia mice treated with SU5416. Furthermore, miR-21–null mice used in the study exhibited exaggerated manifestations of PH. Parikh and colleagues suggested that the absence of miR-21 can enhance Rho-kinase activation, and probably other pathways exacerbating the PH phenotype *in vivo* (Parikh, Jin et al. 2012).

By contrast, Caruso et al observed differences in miR-21 regulation between monocrotaline and chronic hypoxia models, where miR-21 was downregulated in monocrotaline-induced PH (Caruso, MacLean et al. 2010). In addition, the authors reported a downregulation of miR-21 in lung homogenates of idiopathic PAH patients (Caruso, MacLean et al. 2010). However, in our study we observed that both, DDAH1 overexpression and the inhibition of miR-21, attenuate the development of chronic hypoxia-induced PH. These discrepancies may result from different experimental approach and *in vivo* conditions used in other studies. Moreover, the global miR-21 knockout, used by Parikh and colleagues, is expected to have a persistent effect on multiple targets in several tissues, while in our approach the intravenous injection of LNA-21 would predominantly affect the vasculature within a chosen timeframe. Although miR-21 is expressed in multiple tissues in adult mice (Syed 2014), our experiments showed a lung- specific increase of miR-21 in response to hypoxia. Our results suggest that the regulation of miR-21 expression may be tissue-dependent. The precise mechanism of differential hypoxia-induced regulation of miR-21 expression in various tissues and organs will require further investigation.

We observed increased levels of miR-21 and decreased levels of DDAH1 in lung tissues of IPAH patients, a pattern similar to the one observed in hypoxic mouse lungs. However, the roles of miR-21 and DDAH1 may be different in these two forms of pulmonary hypertension. In contrast to its protective role in chronic hypoxia-induced PH, increased NO signalling may have a detrimental effect on the development of plexiform lesions in human PAH, by promoting angiogenesis and iNOS-induced tissue damage associated with increased protein nitrosylation, metabolic stress and formation of reactive oxygen species (Hampl and Herget 2000).

In summary, we show that hypoxia induces pulmonary vascular dysfunction by selective miR-21-mediated inhibition of DDAH1 expression *in vivo* and that the overexpression of DDAH1 and/or the inhibition of miR-21 attenuate chronic hypoxia-induced PH in mice.

7. Conclusions

This study addresses for the first time the mechanism of hypoxia-induced regulation of DDAH expression and function in pulmonary vasculature *in vitro* and *in vivo*. Hypoxia inhibited the expression of DDAH1, at both the mRNA and protein level, and increased ADMA levels in pulmonary endothelial but not vascular smooth muscle cells, while DDAH2 expression was inhibited in both cell types. The selective change in endothelial DDAH1 abundance accounted for the increase in ADMA levels, led to HPAEC dysfunction and modulated HPASMC proliferation *in vitro*, whereas DDAH2 had little effect. The selective reduction of DDAH1 expression in hypoxic endothelial cells resulted from miR-21-induced degradation of DDAH1 mRNA and was prevented by inhibition of miR-21. We further show that reduced DDAH expression in the chronic hypoxic mouse lung is associated with elevated miR-21 levels and decreased NO signaling. DDAH1 overexpression or treatment of mice with the miR-21 inhibitor, LNA-21 attenuated the development of chronic hypoxia-induced pulmonary hypertension. Evidence that these observations might have translational significance is provided by the demonstration that miR-21 levels are also increased and DDAH1 levels decreased in IPAH lung tissues. Hypoxia-induced increases in ADMA levels have been attributed to increased protein arginine methylation and decreased DDAH activity but the mechanisms are not well understood (Yildirim, Bulau et al. 2006). Our data show that both DDAH1 and DDAH2 are reduced in hypoxic lung, but only pulmonary endothelial DDAH1 contributes to hypoxia-induced changes in ADMA metabolism.

The development of pulmonary arterial vasculopathy in animals and humans with pulmonary hypertension has been linked to dysregulation of microRNA (Grant, White et al. 2013). We identified 13 candidate miRs that can bind to the 3'UTR region of DDAH1, and two of these, miR-21 (Parikh, Jin et al. 2012; Chen, Zhou et al. 2013) and miR-128 (Rhodes, Wharton et al. 2013), have been implicated in the pathogenesis of pulmonary hypertension. Our data show that miR-21 but not miR-128 contributes to the hypoxia-induced degradation of DDAH1 mRNA in pulmonary endothelial cells. MiR-21 expression was elevated while DDAH1 expression was reduced in the cultured HPAECs and in the lungs of chronically hypoxic pulmonary hypertensive mice. Importantly, similar differences were observed in lung tissues of treatment-naïve IPAH patients, suggesting that dysregulation of the miR-21/DDAH1 pathway may be common to different forms of pulmonary hypertension. In

support of the postulated role of miR-21, other studies have documented a progressive increase in miR-21 in chronic hypoxia-induced pulmonary hypertensive mice and localization of miR-21 to distal small arteries in the animal and human hypoxic lung (Parikh, Jin et al. 2012; Pullamsetti, Doebele et al. 2012; Wojciak-Stothard and Wilkins 2012). Of interest, published data from our laboratory have shown that blood-derived endothelial-like cells from idiopathic PAH patients display a distinct disease-related phenotype characterised by reduced DDAH1 expression, increased ADMA production and abnormal angiogenesis and that this phenotype is ameliorated by over expression of DDAH1 or stimulation of NO/cGMP pathway (Tsang, Leiper et al. 2013).

While overexpression of miR-21 has been implicated in the hypoxia-induced proliferation and migration of cultured PASMCs (Sarkar, Gou et al. 2010), we found that the manipulation of miR-21/DDAH1 expression had no direct effect on the hypoxia-induced proliferative responses of HPASMCs. Exposure to hypoxia induced a relatively small increase in miR-21 levels in HPASMCs compared to HPAECs (1.5 and 4-fold increase, respectively) and, consistent with this, no significant changes in DDAH1 expression were observed in HPASMCs. Furthermore, neither DDAH enzymes nor their inactive mutants affected PASMC growth *in vitro*. In agreement with the postulated role of endothelial DDAH1 in pulmonary vascular homeostasis, hypoxia-induced stimulation of HPASMC proliferation was also significantly reduced in cells co-cultured with DDAH1-overexpressing HPAECs. *In vivo* studies in DDAHtg mice demonstrated that overexpression of DDAH1 was accompanied by reduced levels of the cell proliferation marker, PCNA, in the lung and reduced pulmonary artery muscularization, without compensatory changes in DDAH2 expression. Reduced PASMC proliferation in DDAHtg mice might result from increased bioavailability of the endothelium-derived NO, which is known to have an inhibitory effect on vascular smooth muscle growth *in vitro* (Peiro, Redondo et al. 1995). In addition, we also observed an inhibitory effect of ADMA on endothelial angiogenesis in normoxia and in hypoxia, rescued by over expression of DDAH1. However, the rationale of performing this assay in future studies needs to be considered. There is no evidence of increased angiogenesis in chronic hypoxia-induced PH, although hypoxia may be a contributory factor in plexiform lesion formation in human PAH.

DDAH1tg mice exhibit greater tissue DDAH activity, reduced plasma ADMA levels, increased NOS activity and reduced systemic vascular resistance (Schwedhelm, von Leitner et al.

2009). These mice are also more resistant to the inhibitory effect of ADMA on angioadaptation after hindlimb ischemia, show increased blood vessel formation in the fibrovascular disc system and improved endothelial regeneration after femoral artery injury (Jacobi, Sydow et al. 2005). Bakr et al (Bakr, Pak et al. 2013) have recently shown that sustained hypoxic pulmonary vasoconstriction was attenuated in DDAH1tg mice, but pulmonary vascular remodelling was unaffected following 4 weeks of hypoxia. At 4 weeks, there were no significant differences in DDAH1 activity, ADMA levels, NO or cGMP between the two groups of mice. In contrast, we observed a protective effect from DDAH1 overexpression at an earlier time point, before the disease was well established (2 week hypoxia). While DDAH1 expression decreased overtime in both WT and DDAH1tg mice, the levels in hypoxic DDAH1tg mice were still significantly higher compared to hypoxic WT controls, likely to warrant sufficient level of DDAH1 activity and NO signalling. The loss of a protective effect observed in mice exposed to hypoxia for 4 weeks, it is probably associated with the hypoxia-induced decrease in β-actin promoter-driven expression (Brown and Davis 2005) of the DDAH1 transgene, noted in both studies (Bakr, Pak et al. 2013).

Consistent with this, at 2 weeks of hypoxia ADMA levels were reduced and the levels of cGMP target, pSer188RhoA, were increased in hypoxic DDAHtg mice compared to hypoxic WT controls. Furthermore, treatment of mice with LNA21 prior to and during exposure to 2 weeks hypoxia increased DDAH1 expression, reduced ADMA levels and attenuated development of pulmonary hypertension, while previously reported treatment of mice with established disease (2 -4 weeks after hypoxic exposure) had little effect on hemodynamic performance or pulmonary vascular remodelling (Pullamsetti, Doebele et al. 2012).

Interestingly, both the hypoxic LNA21-treated and DDAH1tg mice showed a significant reduction in ADMA levels in the lung, compared with hypoxic controls.

Altogether, these observations show that DDAH1 overexpression attenuates early development of chronic hypoxia-induced PH but continuous supplementation of miR-21 inhibitors may be required to retain DDAH/NO signalling.

The finding that DDAH2 expression was unaffected by miR-21 in hypoxic HPAECs, is consistent with the fact that the human DDAH2 mRNA does not have a miR-21 recognition sequence in the 3'UTR region. Although the DDAH2 mRNA and protein levels were reduced in hypoxia, these changes had no apparent effect on ADMA levels, cell proliferation or

endothelial permeability. On the other hand, it is possible that DDAH2 has a role in cardiac response to chronic hypoxia, as DDAH2 transgenic mice show increased cardiac NO production, attenuated inflammatory responses and reduced Ang II –induced perivascular fibrosis in coronary micro-vasculature (Hasegawa, Wakino et al. 2007). In support of our observations of a predominant role for DDAH1 in pulmonary hypertension, genetic deletion of DDAH2 had no impact on basal or hypoxic pulmonary haemodynamics (Wang, Caplin et al. 2013).

The identification of additional factors that functionally integrate the miR-21/DDAH pathway in the development of pulmonary hypertension constitutes a longer-term challenge. Nonetheless, inflammation is a common feature in experimental and human pulmonary hypertension (Hassoun, Mouthon et al. 2009) and our observations suggest that pro-inflammatory transcription factor, NFκB may have a role in mediating hypoxia-induced changes in miR-21 and DDAH1 expression in HPAECs. This observation is also consistent with the reported role of NFkB as an upstream activator of miR-21 in cancer (Shin, Jin et al. 2011).

In addition to DDAH1, miR-21 may also affect other target proteins implicated in the pathogenesis of pulmonary hypertension, such as BMPR2 or RhoB (Parikh, Jin et al. 2012; Mehta, Parthasarathy et al. 2013). Our data indicate that RhoB is an unlikely target of miR-21 in hypoxia, as the total RhoB expression levels in the hypoxic lung remained elevated in spite of high miR-21 expression and were unaffected by LNA21. Instead, we show that the miR-21/DDAH pathway may affect RhoA signaling. Activation of RhoA contributes to hypoxic pulmonary vasoconstriction and pulmonary vascular remodelling (Connolly and Aaronson 2011). In contrast to untreated hypoxic controls, DDAHtg and LNA-treated hypoxic mice showed elevated Ser188 phosphorylation of RhoA, indicative of increased NO signalling. NO/PKG-mediated Ser188 phosphorylation inhibits RhoA activity by enhancing its interaction with Rho guanine-dissociation inhibitor, mechanism implicated in the protective effects of sildenafil (Guilluy, Sauzeau et al. 2005). ADMA has also been shown to induce endothelial dysfunction by reducing RhoASer188 levels and activating RhoA in cultured HPAECs (Wojciak-Stothard and Wilkins 2012).

In summary, we show that selective NFκB-dependent miR-21-mediated inhibition of DDAH1 expression in pulmonary endothelial cells plays an important role in hypoxia-induced

pulmonary hypertension. Preventing the miR-21-mediated degradation of DDAH1 mRNA reduces ADMA levels and attenuates development of chronic hypoxia-induced pulmonary hypertension. This protective effect is likely to result from increased NO bioavailability. The endothelial regulation of DDAH1 expression by miR-21 may have broader significance in the development of pulmonary hypertension.

7.1. Study Limitations

We have shown that hypoxia regulates DDAH1 expression, at least in part, via the miR-21-mediated degradation of DDAH1mRNA in cultured endothelial cells. The significance of this pathway in chronic hypoxia-induced effects *in vivo* will require further analysis. Apart from the DDAH1-NO pathway, miR-21 has been linked to BMP and inflammatory signaling (Ahmed, Mardaryev et al. 2011) and therefore it is likely that miR-21 effects are complex and will depend on the cell/tissue type and the stage of the disease. It has been shown that activation of miR-21 leads to the activation of mir-181b-1 in epithelial cells (Iliopoulos, Jaeger et al. 2010), suggesting that the analysis of miR clusters rather than single miRs would be more appropriate in future studies.

We have identified 13 miRs potentially targeting 3'UTR of DDAH1 and selected two miRs implicated in the pathogenesis of PAH, miR-21 and miR-128, for further analysis. While manipulation of miR-21 levels was sufficient to convey the effects of hypoxia on DDAH1 expression, other miRs may also play a role and their potential involvement will need to be verified experimentally.

Our *in vitro* model involved exposure of cells to 1h-48h hypoxia (PO2 14-30 mmHg, 2% O2). Longer exposure times were not feasible in our conditions because of the re-oxygenation accompanying changes of culture medium. A plausible solution to this problem would be the use of chambers allowing manipulation of cells in a continuous hypoxic environment. The effects induced by 48h hypoxia (2% O2) may differ from the effects of chronic hypoxia (2 weeks at 10% O2).

In order to better understand the role of miR-21/DDAH1 in human PAH, other animal models, such as MCT and Sugen/hypoxia should be used. Hypoxic model does not show a strong inflammatory response or plexiform lesion formation, a key characteristic of severe human PAH.

7.2. Future Work

The mechanism behind hypoxia-induced DDAH2 downregulation in pulmonary vascular cells will require further studies. Apart from competing with L-arginine, the hypoxia-induced increase in ADMA, it has been suggested that ADMA can also "uncouple" NOS, leading to Reactive Oxygen Species (ROS) generation, implicated in the pathogenesis of PH (Wilcox 2012). ROS can, in turn, decrease the expression and activity of DDAH2, as shown in cancer cells (Wang, Hu et al. 2009). Additionally, it is possible that DDAH2 might play a role in the cardiac response to hypoxia, since DDAH2 transgenic mice show increased cardiac NO production (Hasegawa, Wakino et al. 2007). Therefore, the potential role of DDAH2 in the pulmonary and cardiac responses to chronic hypoxia will require further investigation.

It has been reported that pathophysiological concentrations of ADMA can regulate gene expression in human endothelial cells by what appears to be an NO-independent mechanism (Smith, Anthony et al. 2005). Further studies will be needed to clarify the NO-independent effects of ADMA in pulmonary vascular cells.

Furthermore, miR cluster analysis and network-based bioinformatic approach combined with experimental studies in *vitro* and *in vivo* will be needed to delineate the role for miR-21 in the pathogenesis of PAH. Potential role of NFkB in the regulation of miR-21 expression and DDAH pathway will also require further studies. Our preliminary data shows that the NFkB inhibitor, BAY-117085 attenuates hypoxia-induced increase in miR-21 expression in HPAECs. It will need to be established whether NFkB affects miR-21 expression directly, by binding to consensus sequences in the miR-21 promoter, or indirectly, via the changes in the levels of pro-inflammatory cytokines or growth factors. MiR-21 expression changes in the lung/lung vasculature will also need to be investigated in inflammatory models of PH, such as MCT.

Finally, regarding the human data presented in this study, we observed a similar pattern of changes in miR-21/DDAH1 levels in lung tissues from chronically hypoxic PH mice and lung tissues from treatment-naïve IPAH patients. A more detailed miR-21/DDAH1 pathway analysis using a larger number of human samples will be needed to draw conclusions regarding the importance of miR-21/DDAH1/NO signalling in human PH.

8. Therapeutic perspectives

8.1. Activation of DDAH as a Therapeutic Target

Our study, along with others, demonstrated that a reduction of DDAH1 expression or activity and an increase in ADMA levels occur in animal model of PH. In fact, the deficiency of DDAH1 or its pharmacological inhibition can raise the right ventricular pressure in animal models, demonstrating a strong relationship between DDAH1, ADMA and pulmonary vascular dysfunction (Leiper, Nandi et al. 2007). These data collectively demonstrate that the DDAH-ADMA pathway can have a central role in the development of PH (Millatt, Whitley et al. 2003; Sasaki, Doi et al. 2007).

The modulation of DDAH expression or activity may be beneficial in diseases where there is an altered ADMA metabolism that consequently deregulates NO signalling.

Potentially, there are several way of increasing DDAH activity such as, gene delivery, increasing transcription, attenuation of redox inactivation and allosteric activation (Leiper and Nandi 2011). *In vitro* studies, including the results presented in this thesis, demonstrated that the overexpression of DDAH1 is sufficient to reduce ADMA levels and increase NO production (Torondel, Nandi et al. 2010; Iannone, Zhao et al. 2014). Furthermore, studies using adenoviral-mediated delivery of DDAH1 restore vascular function *in vivo* (Shibata, Ueda et al. 2009; Ueda, Yamagishi et al. 2009). In fact, the overexpression of DDAH1 resulted in restoration of physiological ADMA concentrations and significantly attenuated systemic hypertension. Although these studies indicate that DDAH gene delivery may have therapeutic utility, this approach could have complications due to the fact that DDAH may have additional ADMA-independent related activities that could be mediated via protein–protein interactions (Tokuo, Yunoue et al. 2001; Hasegawa, Wakino et al. 2006).

The Increased transcription of DDAH can result in reduced ADMA concentrations and increased NO production from cells in culture. For example, functional farnesoid X receptor (FXR) agonists have been shown to increase DDAH1 expression and reduce ADMA concentrations by acting on the FXR response element within the intron 1 of the DDAH1 gene (Hu, Chouinard et al. 2006). Similarly, IL-1β induces expression of DDAH1 with an associated reduction in ADMA levels in vascular smooth muscle cells (Ueda, Kato et al.

2003). However, selective activators of DDAH1 transcription have not been identified and therefore this approach has not been tested experimentally.

Both DDAH enzymes activities can be regulated by oxidative and nitrosative modification on the cysteine residue (Leiper, Murray-Rust et al. 2002; Knipp, Braun et al. 2003). *In vitro* studies showed that antioxidant treatment of cells can attenuate DDAH inhibition that is induced by oxidized low-density lipoprotein (Lin, Ito et al. 2002), and similar observations have been made in an *in vivo* model of hypercholesterolemia, suggesting that the redox inactivation of DDAH may be an important mechanism of vascular dysfunction (Tan, Jiang et al. 2007). These results indicate that antioxidant therapies might have beneficial effects on vasculature preserving DDAH activity.

Another potential way to protect DDAH activity could be the inhibition of specific miRNA acting on the 3'-UTR of DDAH.

Once the role of a specific miRNA in pathology is established, selecting specific anti-miRNA inhibitors combined with delivery strategies could be straightforward. Nevertheless, effective and safe delivery of anti-miRNA drugs can be difficult for many cell types.

Although individual miRNAs are often produced only in specific cell types or developmental stages, the human genome contains more than 500 miRNAs, and each miRNA can potentially repress hundreds of genes (Bartel 2009; Chiang, Schoenfeld et al. 2010).

Thus, treating diseases with anti-miRNA oligonucleotides will require the development of novel modification or formulation strategies able to increase tissue-specific modulation of the targeted miRNA. However, since miRNAs constitute a class of drug targets unrelated to any others, new additional technologies and methods are also required.

Ideally, small molecules able to activate DDAH1 will be able to lower ADMA levels and restore NO signalling in disease states. Although several compounds have been clinically tested (as described in the next paragraph) in order to increase DDAH activity, a more comprehensive understanding of the role and regulation of DDAH enzymes is necessary for determining whether or not DDAH modulation has therapeutic potential.

8.2. Clinically utilized compounds and DDAH

Several compounds have been clinically used in order to alter the activity of DDAH and thus the effects on ADMA and NO in various disease models (Wadham and Mangoni 2009; Leiper and Nandi 2011). For example, vitamin A (retinol) is known to regulate cell proliferation, differentiation and apoptosis (Pan and Baker 2007). Although all trans-retinoic acid can prevent cardiovascular remodelling in animal models of hypertension by increasing DDAH2 expression (Achan, Tran et al. 2000), recently Bjelakovic et al failed to demonstrate any significant benefit of vitamin A on mortality prevention in healthy individuals (Bjelakovic, Nikolova et al. 2012).

Vitamin E has also been implicated in the progression of atherosclerosis by modulation of angiogenesis and inhibition of vascular smooth muscle cell proliferation (Munteanu and Zingg 2007). Although a pilot study suggested that an antioxidant therapy with vitamin E could potentially lower ADMA levels, another study filed to show a significant reduction in plasma ADMA concentration *in vivo* (Tan, Jiang et al. 2007; Onozato, Tojo et al. 2008).

In addition, it has been demonstrated that the amino acid taurine can lower blood pressure through effects mediated by the central nervous system (Militante and Lombardini 2002). Tan et al showed protective effects of taurine on the endothelium related to a decrease in ADMA level by an increased DDAH activity (Tan, Jiang et al. 2007). Although the effects of taurine on DDAH activity might provide further beneficial effects in the peripheral circulation, nowadays there are no clinical data on the effects of taurine supplementation on cardiovascular outcomes.

The antiplatelet drug aspirin, extensively used in secondary cardiovascular prevention, has been reported to upregulate DDAH activity, providing new insights on its antioxidant and vasculoprotective effects (Deng, Deng et al. 2004). Interestingly, the effects of aspirin on DDAH activity were only observed at low doses (Deng, Deng et al. 2004). Furthermore, statins such as atorvastatin, probucol and pravastatin, which exert beneficial effects on endothelial function and vascular remodelling, have been reported to enhance DDAH and eNOS activity (Yang, Chen et al. 2005; Yin and Xiong 2005; Jiang, Zhang et al. 2006). Despite the fact that no significant reduction of ADMA plasma level were observed in patients with chronic heart failure and diabetes, it has been shown that atorvastatin can reduce plasma ADMA concentration in animal models (Valkonen, Laakso et al. 2003; Tanaka, Katayama et

al. 2007; Young, Strey et al. 2008). Interestingly, a statin with powerful antioxidant effects such as fluvastatin (Yamaguchi, Matsuno et al. 2002) showed a significant reduction of ADMA plasma levels in patients with metabolic syndrome (Oguz and Uzunlulu 2008). Although the antioxidant effects of fluvastatin need to be confirmed in human (Nagase, Hirayama et al. 2005), this effect could explain its efficacy in lowering plasma ADMA concentration compared with other statins.

The angiotensin receptor blockers exert several protective effects on vascular homeostasis independently of angiotensin receptor 1 blockade (Ando, Ushijima et al. 2013). In fact, it has been demonstrated that angiotensin blocker such as losartan and telemistran can upregulate DDAH activity and expression (Wakino, Hayashi et al. 2005). Clinical trials have demonstrated the effectiveness of angiotensin receptor blockers in reducing cardiovascular morbidity and mortality in several patient groups (Kjeldsen, Dahlof et al. 2002; Yusuf, Teo et al. 2008)

In summary, several compounds used to treat cardiovascular risk factors showed to have little effect on circulating ADMA levels, suggesting that the relationship between plasma ADMA levels and common cardiovascular risk factors can be complex. While drugs such as aspirin telemistran and losartan showed a good correlation between basic studies on DDAH and cardiovascular outcomes, other compounds such as vitamin A, vitamin E and probucol have not been translated into cardiovascular protection. Plausible reasons for the lack of clinical beneficial effects could be due to variability of the targeted population or incorrect dosing. At the present, there are no current drugs that have specific effects on the DDAH/ADMA pathway and therefore it is not currently possible to understand the likely effect of DDAH activation in humans.

9. References

Aaronson, P. I., T. P. Robertson, et al. (2006). "Hypoxic pulmonary vasoconstriction: mechanisms and controversies." J Physiol **570**(Pt 1): 53-58.

Abe, K., M. Toba, et al. (2010). "Formation of Plexiform Lesions In Experimental Severe Pulmonary Arterial Hypertension." Am J Respir Crit Care Med **181**.

Abud, E. M., J. Maylor, et al. (2012). "Digoxin inhibits development of hypoxic pulmonary hypertension in mice." Proc Natl Acad Sci U S A **109**(4): 1239-1244.

Achan, V., M. Broadhead, et al. (2003). "Asymmetric dimethylarginine causes hypertension and cardiac dysfunction in humans and is actively metabolized by dimethylarginine dimethylaminohydrolase." Arterioscler Thromb Vasc Biol **23**(8): 1455-1459.

Achan, V., M. Broadhead, et al. (2003). "Asymmetric dimethylarginine causes hypertension and cardiac dysfunction in humans and is actively metabolized by dimethylarginine dimethylaminohydrolase." Arteriosclerosis Thrombosis and Vascular Biology **23**(8): 1455-1459.

Achan, V., C. Tran, et al. (2000). "All-trans-retinoic acid increases nitric oxide synthesis by endothelial cells: A role for the induction of dimethylarginine dimethylaminohydrolase." Circulation **102**(18): 17-17.

Agarwal, R. and M. Gomberg-Maitland (2011). "Current therapeutics and practical management strategies for pulmonary arterial hypertension." Am Heart J **162**(2): 201-213.

Ahmed, M. I., A. N. Mardaryev, et al. (2011). "MicroRNA-21 is an important downstream component of BMP signalling in epidermal keratinocytes." J Cell Sci **124**(20): 3399-3404.

Aiello, L. P., R. L. Avery, et al. (1994). "Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders." N Engl J Med **331**(22): 1480-1487.

Alavi, A., J. D. Hood, et al. (2003). "Role of Raf in vascular protection from distinct apoptotic stimuli." Science **301**(5629): 94-96.

Albsmeier, J., D. Tsikas, et al. (2002). "Asymmetric dimethylarginine (ADMA), an explanation for the L-arginine-paradox." Naunyn-Schmiedebergs Archives of Pharmacology **365**: R14-R14.

Ali, N., N. Mah, et al. (2012). "Identification of a Hypoxia-Responsive MicroRNA Signature in Lung Endothelial Cells." Irish Journal of Medical Science **181**: S414-S414.

Ambalavanan, N., G. Mariani, et al. (1999). "Role of nitric oxide in regulating neonatal porcine pulmonary artery smooth muscle cell proliferation." Biol Neonate **76**(5): 291-300.

Ambartsumian, N., J. Klingelhofer, et al. (1998). "Tissue-specific posttranscriptional downregulation of expression of the S100A4(mts1) gene in transgenic animals." Invasion Metastasis **18**(2): 96-104.

Ando, H., K. Ushijima, et al. (2013). "Relationship between the receptor occupancy profile and pleiotropic effects of angiotensin II receptor blockers." Br J Clin Pharmacol **75**(2): 415-422.

Arcot, S. S., D. W. Lipke, et al. (1993). "Alterations of Growth-Factor Transcripts in Rat Lungs during Development of Monocrotaline-Induced Pulmonary-Hypertension." Biochemical Pharmacology **46**(6): 1086-1091.

Arnaoutova, I. and H. K. Kleinman (2010). "In vitro angiogenesis: endothelial cell tube formation on gelled basement membrane extract." Nature Protocols **5**(4): 628-635.

Bail, S., M. Swerdel, et al. (2010). "Differential regulation of microRNA stability." Rna-a Publication of the Rna Society **16**(5): 1032-1039.

Bakr, A., O. Pak, et al. (2013). "Effect of DDAH1 Overexpression on the Response of the Pulmonary Vasculature to Hypoxia." Am J Respir Cell Mol Biol.

Bakr, A., O. Pak, et al. (2013). "Effects of dimethylarginine dimethylaminohydrolase-1 overexpression on the response of the pulmonary vasculature to hypoxia." Am J Respir Cell Mol Biol **49**(3): 491-500.

Baliga, R. S., R. J. MacAllister, et al. (2011). "New perspectives for the treatment of pulmonary hypertension." Br J Pharmacol **163**(1): 125-140.

Baliga, R. S., R. J. MacAllister, et al. (2011). "New perspectives for the treatment of pulmonary hypertension." Br J Pharmacol **163**(1): 125-140.

Bartel, D. P. (2004). "MicroRNAs: genomics, biogenesis, mechanism, and function." Cell **116**(2): 281-297.

Bartel, D. P. (2009). "MicroRNAs: Target Recognition and Regulatory Functions." Cell **136**(2): 215-233.

Basu, S. and R. Kolesnick (1998). "Stress signals for apoptosis: ceramide and c-Jun kinase." Oncogene **17**(25): 3277-3285.

Batkai, S. and T. Thum (2012). "MicroRNAs in Hypertension: Mechanisms and Therapeutic Targets." Current Hypertension Reports **14**(1): 79-87.

Bauer, M., H. C. Wilkens, et al. (2002). "Selective upregulation of endothelin B receptor gene expression in severe pulmonary hypertension." Circulation **105**(9): 1034-1036.

Bedford, M. T. and S. Richard (2005). "Arginine methylation: An emerging regulator of protein function." Molecular Cell **18**(3): 263-272.

Belik, J. (2009). "Riociguat, an oral soluble guanylate cyclase stimulator for the treatment of pulmonary hypertension." Curr Opin Investig Drugs **10**(9): 971-979.

Bender, A. T. and J. A. Beavo (2006). "Cyclic nucleotide phosphodiesterases: Molecular regulation to clinical use." Pharmacol Rev **58**(3): 488-520.

Benjamin, D. and C. Moroni (2007). "mRNA stability and cancer: an emerging link?" Expert Opin Biol Ther **7**(10): 1515-1529.

Beppu, H., F. Ichinose, et al. (2004). "BMPR-II heterozygous mice have mild pulmonary hypertension and an impaired pulmonary vascular remodeling response to prolonged hypoxia." Am J Physiol Lung Cell Mol Physiol **287**(6): L1241-1247.

Bernatchez, P. N., S. Soker, et al. (1999). "Vascular endothelial growth factor effect on endothelial cell proliferation, migration, and platelet-activating factor synthesis is Flk-1-dependent." Journal of Biological Chemistry **274**(43): 31047-31054.

Bjelakovic, G., D. Nikolova, et al. (2012). "Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases." Cochrane Database of Systematic Reviews(3).

Black, S. M., L. S. Sanchez, et al. (2001). "sGC and PDE5 are elevated in lambs with increased pulmonary blood flow and pulmonary hypertension." American Journal of Physiology-Lung Cellular and Molecular Physiology **281**(5): L1051-L1057.

Boger, R. H., S. M. Bode-Boger, et al. (2000). "An endogenous inhibitor of nitric oxide synthase regulates endothelial adhesiveness for monocytes." J Am Coll Cardiol **36**(7): 2287-2295.

Boudreau, N., E. Turley, et al. (1991). "Fibronectin, hyaluronan, and a hyaluronan binding protein contribute to increased ductus arteriosus smooth muscle cell migration." Dev Biol **143**(2): 235-247.

Brock, M., V. J. Samillan, et al. (2012). "AntagomiR directed against miR-20a restores functional BMPR2 signalling and prevents vascular remodelling in hypoxia-induced pulmonary hypertension." Eur Heart J.

Brock, M., M. Trenkmann, et al. (2009). "Interleukin-6 modulates the expression of the bone morphogenic protein receptor type II through a novel STAT3-microRNA cluster 17/92 pathway." Circ Res **104**(10): 1184-1191.

Brown, R. C. and T. P. Davis (2005). "Hypoxia/aglycemia alters expression of occludin and actin in brain endothelial cells." Biochem Biophys Res Commun **327**(4): 1114-1123.

Brunner, H., J. R. Cockcroft, et al. (2005). "Endothelial function and dysfunction. Part II: Association with cardiovascular risk factors and diseases. A statement by the Working Group on Endothelins and Endothelial Factors of the European Society of Hypertension." J Hypertens **23**(2): 233-246.

Brusselmans, K., V. Compernolle, et al. (2003). "Heterozygous deficiency of hypoxia-inducible factor-2alpha protects mice against pulmonary hypertension and right ventricular dysfunction during prolonged hypoxia." J Clin Invest **111**(10): 1519-1527.

Budhiraja, R., R. M. Tuder, et al. (2004). "Endothelial dysfunction in pulmonary hypertension." Circulation **109**(2): 159-165.

Bull, T. M., C. D. Coldren, et al. (2007). "Gene expression profiling in pulmonary hypertension." Proc Am Thorac Soc **4**(1): 117-120.

Burke, D. L., M. G. Frid, et al. (2009). "Sustained hypoxia promotes the development of a pulmonary artery-specific chronic inflammatory microenvironment." American Journal of Physiology-Lung Cellular and Molecular Physiology **297**(2): L238-L250.

Burke, D. L., M. G. Frid, et al. (2009). "Sustained hypoxia promotes the development of a pulmonary artery-specific chronic inflammatory microenvironment." Am J Physiol Lung Cell Mol Physiol **297**(2): L238-250.

Calver, A., J. Collier, et al. (1993). "Effect of local intra-arterial asymmetric dimethylarginine (ADMA) on the forearm arteriolar bed of healthy volunteers." J Hum Hypertens **7**(2): 193-194.

Campbell, A. I. M., Y. D. Zhao, et al. (2001). "Cell-based gene transfer of vascular endothelial growth factor attenuates monocrotaline-induced pulmonary hypertension." Circulation **104**(18): 2242-2248.

Caruso, P., M. R. MacLean, et al. (2010). "Dynamic changes in lung microRNA profiles during the development of pulmonary hypertension due to chronic hypoxia and monocrotaline." Arterioscler Thromb Vasc Biol **30**(4): 716-723.

Chan, K. L., P. J. Currie, et al. (1987). "Comparison of three Doppler ultrasound methods in the prediction of pulmonary artery pressure." J Am Coll Cardiol **9**(3): 549-554.

Chen, L., J. P. Zhou, et al. (2013). "4-HNE Increases Intracellular ADMA Levels in Cultured HUVECs: Evidence for miR-21-Dependent Mechanisms." PLoS One **8**(5): e64148.

Chen, Y. F., J. A. Feng, et al. (2006). "Atrial natriuretic peptide-dependent modulation of hypoxia-induced pulmonary vascular remodeling." Life Sci **79**(14): 1357-1365.

Cheng, Y. H. and C. X. Zhang (2010). "MicroRNA-21 in Cardiovascular Disease." J Cardiovasc Transl Res **3**(3): 251-255.

Chester, M., P. Tourneux, et al. (2009). "Cinaciguat, a soluble guanylate cyclase activator, causes potent and sustained pulmonary vasodilation in the ovine fetus." Am J Physiol Lung Cell Mol Physiol **297**(2): L318-325.

Chiang, H. R., L. W. Schoenfeld, et al. (2010). "Mammalian microRNAs: experimental evaluation of novel and previously annotated genes." Genes Dev **24**(10): 992-1009.

Christman, B. W., C. D. Mcpherson, et al. (1992). "An Imbalance between the Excretion of Thromboxane and Prostacyclin Metabolites in Pulmonary-Hypertension." New England Journal of Medicine **327**(2): 70-75.

Collier, J. and P. Vallance (1989). "2nd Messenger Role for No Widens to Nervous and Immune-Systems." Trends in Pharmacological Sciences **10**(11): 427-431.

Connolly, M. J. and P. I. Aaronson (2011). "Key role of the RhoA/Rho kinase system in pulmonary hypertension." Pulm Pharmacol Ther **24**(1): 1-14.

Cooke, J. P. (2004). "Asymmetrical dimethylarginine: the Uber marker?" Circulation **109**(15): 1813-1818.

Cooke, J. P. and Y. T. Ghebremariam (2011). "DDAH says NO to ADMA." Arterioscler Thromb Vasc Biol **31**(7): 1462-1464.

Cool, C. D., D. Kennedy, et al. (1997). "Pathogenesis and evolution of plexiform lesions in pulmonary hypertension associated with scleroderma and human immunodeficiency virus infection." Hum Pathol **28**(4): 434-442.

Courboulin, A., R. Paulin, et al. (2011). "Role for miR-204 in human pulmonary arterial hypertension." J Exp Med **208**(3): 535-548.

Crosswhite, P. and Z. J. Sun (2010). "Nitric oxide, oxidative stress and inflammation in pulmonary arterial hypertension." J Hypertens **28**(2): 201-212.

Culver, C., A. Sundqvist, et al. (2010). "Mechanism of hypoxia-induced NF-kappaB." Mol Cell Biol **30**(20): 4901-4921.

Czyzykrzeska, M. F., Z. Dominski, et al. (1994). "Hypoxia Stimulates Binding of a Cytoplasmic Protein to a Pyrimidine-Rich Sequence in the 3'-Untranslated Region of Rat Tyrosine-Hydroxylase Messenger-Rna." Journal of Biological Chemistry **269**(13): 9940-9945.

Davies, R. J. and N. W. Morrell (2008). "Molecular mechanisms of pulmonary arterial hypertension: role of mutations in the bone morphogenetic protein type II receptor." Chest **134**(6): 1271-1277.

Davis, B. N., A. C. Hilyard, et al. (2008). "SMAD proteins control DROSHA-mediated microRNA maturation." Nature **454**(7200): 56-61.

Dawes, K. E., A. J. Peacock, et al. (1994). "Characterization of Fibroblast Mitogens and Chemoattractants Produced by Endothelial-Cells Exposed to Hypoxia." Am J Respir Cell Mol Biol **10**(5): 552-559.

Dayoub, H., V. Achan, et al. (2003). "Dimethylarginine dimethylaminohydrolase regulates nitric oxide synthesis: genetic and physiological evidence." Circulation **108**(24): 3042-3047.

de Jesus Perez, V. A., E. Rosenzweig, et al. (2012). "Safety and efficacy of transition from systemic prostanoids to inhaled treprostinil in pulmonary arterial hypertension." Am J Cardiol **110**(10): 1546-1550.

de Moissac, D., R. M. Gurevich, et al. (2000). "Caspase activation and mitochondrial cytochrome c release during hypoxia-mediated apoptosis of adult ventricular myocytes." Journal of Molecular and Cellular Cardiology **32**(1): 53-63.

Dejana, E., E. Tournier-Lasserve, et al. (2009). "The Control of Vascular Integrity by Endothelial Cell Junctions: Molecular Basis and Pathological Implications." Dev Cell **16**(2): 209-221.

Deng, S., P. Y. Deng, et al. (2004). "Aspirin protected against endothelial damage induced by LDL: role of endogenous NO synthase inhibitors in rats." Acta Pharmacol Sin **25**(12): 1633-1639.

Denko, N. C., L. A. Fontana, et al. (2003). "Investigating hypoxic tumor physiology through gene expression patterns." Oncogene **22**(37): 5907-5914.

Di Luozzo, G., J. Bhargava, et al. (2000). "Vascular smooth muscle cell effect on endothelial cell endothelin-1 production." Journal of Vascular Surgery **31**(4): 781-789.

Ding, H., B. Wu, et al. (2010). "A Novel Loss-of-Function DDAH1 Promoter Polymorphism Is Associated With Increased Susceptibility to Thrombosis Stroke and Coronary Heart Disease." Circ Res **106**(6): 1145-U1312.

Dorfmuller, P., F. Perros, et al. (2003). "Inflammation in pulmonary arterial hypertension." European Respiratory Journal **22**(2): 358-363.

Dorfmuller, P., V. Zarka, et al. (2002). "Chemokine RANTES in severe pulmonary arterial hypertension." Am J Respir Crit Care Med **165**(4): 534-539.

Dumitrascu, R., N. Weissmann, et al. (2006). "Activation of soluble guanylate cyclase reverses experimental pulmonary hypertension and vascular remodeling." Circulation **113**(2): 286-295.

Dzau, V. J. and G. H. Gibbons (1991). "Endothelium and Growth-Factors in Vascular Remodeling of Hypertension." Hypertension **18**(5): S115-S121.

Ebralidze, A., E. Tulchinsky, et al. (1989). "Isolation and characterization of a gene specifically expressed in different metastatic cells and whose deduced gene product has a high degree of homology to a Ca2+-binding protein family." Genes Dev **3**(7): 1086-1093.

Egermayer, P., G. I. Town, et al. (1999). "Role of serotonin in the pathogenesis of acute and chronic pulmonary hypertension." Thorax **54**(2): 161-168.

Elahi, M. M., Y. X. Kong, et al. (2009). "Oxidative stress as a mediator of cardiovascular disease." Oxidative Medicine and Cellular Longevity **2**(5): 259-269.

Ellerbroek, S. M., K. Wennerberg, et al. (2003). "Serine phosphorylation negatively regulates RhoA in vivo." J Biol Chem **278**(21): 19023-19031.

Elmen, J., M. Lindow, et al. (2008). "LNA-mediated microRNA silencing in non-human primates." Nature **452**(7189): 896-899.

Etienne-Manneville, S. and A. Hall (2002). "Rho GTPases in cell biology." Nature **420**(6916): 629-635.

Evgenov, O. V., P. Pacher, et al. (2006). "NO-independent stimulators and activators of soluble guanylate cyclase: discovery and therapeutic potential." Nature Reviews Drug Discovery **5**(9): 755-768.

Fabian, M. R., N. Sonenberg, et al. (2010). "Regulation of mRNA Translation and Stability by microRNAs." Annual Review of Biochemistry, Vol 79 **79**: 351-379.

Ferrara, N., H. P. Gerber, et al. (2003). "The biology of VEGF and its receptors." Nat Med **9**(6): 669-676.

Flam, B. R., D. C. Eichler, et al. (2007). "Endothelial nitric oxide production is tightly coupled to the citrulline-NO cycle." Nitric Oxide-Biology and Chemistry **17**(3-4): 115-121.

Fleissner, F., V. Jazbutyte, et al. (2010). "Short communication: asymmetric dimethylarginine impairs angiogenic progenitor cell function in patients with coronary artery disease through a microRNA-21-dependent mechanism." Circ Res **107**(1): 138-143.

Forsythe, J. A., B. H. Jiang, et al. (1996). "Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1." Mol Cell Biol **16**(9): 4604-4613.

Frasch, H. F., C. Marshall, et al. (1999). "Endothelin-1 is elevated in monocrotaline pulmonary hypertension." American Journal of Physiology-Lung Cellular and Molecular Physiology **276**(2): L304-L310.

Gabbiani, G. (2003). "The myofibroblast in wound healing and fibrocontractive diseases." Journal of Pathology **200**(4): 500-503.

Galie, N., B. H. Brundage, et al. (2009). "Tadalafil therapy for pulmonary arterial hypertension." Circulation **119**(22): 2894-2903.

Galie, N., H. A. Ghofrani, et al. (2005). "Sildenafil citrate therapy for pulmonary arterial hypertension." New England Journal of Medicine **353**(20): 2148-2157.

Galie, N., M. M. Hoeper, et al. (2009). "Guidelines for the diagnosis and treatment of pulmonary hypertension." Eur Respir J **34**(6): 1219-1263.

Galie, N., M. Humbert, et al. (2002). "Effects of beraprost sodium, an oral prostacyclin analogue, in patients with pulmonary arterial hypertension: a randomized, double-blind, placebo-controlled trial." J Am Coll Cardiol **39**(9): 1496-1502.

Gangopahyay, A., M. Oran, et al. (2011). "Bone morphogenetic protein receptor II is a novel mediator of endothelial nitric-oxide synthase activation." J Biol Chem **286**(38): 33134-33140.

Garg, U. C. and A. Hassid (1989). "Nitric Oxide-Generating Vasodilators and 8-Bromo-Cyclic Guanosine-Monophosphate Inhibit Mitogenesis and Proliferation of Cultured Rat Vascular Smooth-Muscle Cells." Journal of Clinical Investigation **83**(5): 1774-1777.

Garg, U. C. and A. Hassid (1990). "Nitric Oxide-Generating Vasodilators Inhibit Mitogenesis and Proliferation of Balb/C3t3 Fibroblasts by a Cyclic Gmp-Independent Mechanism." Biochem Biophys Res Commun **171**(1): 474-479.

Gebb, S. A. and P. L. Jones (2003). "Hypoxia and lung branching morphogenesis." Hypoxia: Through the Lifecycle **543**: 117-125.

Gerasimovskaya, E. V., S. Ahmad, et al. (2002). "Extracellular ATP is an autocrine/paracrine regulator of hypoxia-induced adventitial fibroblast growth - Signaling through extracellular signal-regulated kinase-1/2 and the Egr-1 transcription factor." Journal of Biological Chemistry **277**(47): 44638-44650.

Ghofrani, H. A., M. M. Hoeper, et al. (2010). "Riociguat for chronic thromboembolic pulmonary hypertension and pulmonary arterial hypertension: a phase II study." Eur Respir J **36**(4): 792-799.

Ghofrani, H. A., W. Seeger, et al. (2005). "Imatinib for the treatment of pulmonary arterial hypertension." N Engl J Med **353**(13): 1412-1413.
Ghofrani, H. A., R. Voswinckel, et al. (2006). "Hypoxia- and non-hypoxia-related pulmonary hypertension - established and new therapies." Cardiovasc Res **72**(1): 30-40.
Giaid, A. and D. Saleh (1995). "Reduced Expression of Endothelial Nitric-Oxide Synthase in the Lungs of Patients with Pulmonary-Hypertension." New England Journal of Medicine **333**(4): 214-221.
Giaid, A. and D. Saleh (1995). "Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension." N Engl J Med **333**(4): 214-221.
Giaid, A., M. Yanagisawa, et al. (1993). "Expression of Endothelin-1 in the Lungs of Patients with Pulmonary-Hypertension." New England Journal of Medicine **328**(24): 1732-1739.
Gilboa, L., A. Nohe, et al. (2000). "Bone morphogenetic protein receptor complexes on the surface of live cells: a new oligomerization mode for serine/threonine kinase receptors." Mol Biol Cell **11**(3): 1023-1035.
Giordano, F. J. (2005). "Oxygen, oxidative stress, hypoxia, and heart failure." J Clin Invest **115**(3): 500-508.
Glisovic, T., J. L. Bachorik, et al. (2008). "RNA-binding proteins and post-transcriptional gene regulation." FEBS Lett **582**(14): 1977-1986.
Glowacki, F., G. Savary, et al. (2013). "Increased Circulating miR-21 Levels Are Associated with Kidney Fibrosis." PLoS One **8**(2).
Goldberg, M. A., C. C. Gaut, et al. (1991). "Erythropoietin Messenger-Rna Levels Are Governed by Both the Rate of Gene-Transcription and Posttranscriptional Events." Blood **77**(2): 271-277.
Goldberg, M. A. and T. J. Schneider (1994). "Similarities between the Oxygen-Sensing Mechanisms Regulating the Expression of Vascular Endothelial Growth-Factor and Erythropoietin." Journal of Biological Chemistry **269**(6): 4355-4359.
Goldstrohm, A. C. and M. Wickens (2008). "Multifunctional deadenylase complexes diversify mRNA control." Nat Rev Mol Cell Biol **9**(4): 337-344.
Gould, S. J. and S. Subramani (1988). "Firefly Luciferase as a Tool in Molecular and Cell Biology." Analytical Biochemistry **175**(1): 5-13.
Graeber, T. G., C. Osmanian, et al. (1996). "Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours." Nature **379**(6560): 88-91.
Grant, J. S., K. White, et al. (2013). "MicroRNAs in pulmonary arterial remodeling." Cell Mol Life Sci.
Granville, D. J., C. M. Carthy, et al. (1998). "Apoptosis: molecular aspects of cell death and disease." Lab Invest **78**(8): 893-913.
Guilluy, C., V. Sauzeau, et al. (2005). "Inhibition of RhoA/Rho kinase pathway is involved in the beneficial effect of sildenafil on pulmonary hypertension." Br J Pharmacol **146**(7): 1010-1018.
Haigl, B., V. Vanas, et al. (2014). "Expression of microRNA-21 in non- small cell lung cancer tissue increases with disease progression and is likely caused by growth conditional changes during malignant transformation." Int J Oncol **44**(4): 1325-1334.
Hall, A. (1998). "Rho GTPases and the actin cytoskeleton." Science **279**(5350): 509-514.
Hall, S., P. Brogan, et al. (2009). "Contribution of inflammation to the pathology of idiopathic pulmonary arterial hypertension in children." Thorax **64**(9): 778-783.
Hampl, V. and J. Herget (2000). "Role of nitric oxide in the pathogenesis of chronic pulmonary hypertension." Physiol Rev **80**(4): 1337-1372.
Han, Z. S., A. J. Pope, et al. (2009). "Increased ADMA/protein methylation modulates nitric oxide generation in hypoxic human lung microvascular endothelial cells." Faseb Journal **23**.
Hasegawa, K., S. Wakino, et al. (2006). "Dimethylarginine dimethylaminohydrolase 2 increases vascular endothelial growth factor expression through Sp1 transcription factor in endothelial cells." Arteriosclerosis Thrombosis and Vascular Biology **26**(7): 1488-1494.

Hasegawa, K., S. Wakino, et al. (2007). "Role of asymmetric dimethylarginine in vascular injury in transgenic mice overexpressing dimethylarginie dimethylaminohydrolase 2." Circulation Research **101**(2): e2-10.

Hassoun, P. M. (2014). "Inflammation in pulmonary arterial hypertension: is it time to quell the fire?" European Respiratory Journal **43**(3): 685-688.

Hassoun, P. M., L. Mouthon, et al. (2009). "Inflammation, growth factors, and pulmonary vascular remodeling." J Am Coll Cardiol **54**(1 Suppl): S10-19.

Hatabu, H., W. B. Gefter, et al. (1991). "Magnetic resonance approaches to the evaluation of pulmonary vascular anatomy and physiology." Magn Reson Q **7**(3): 208-225.

Hatano, S. and T. Strasser (1975). Primary pulmonary hypertension : report on a WHO meeting, Geneva, 15-17 October 1973. Geneva

Albany, N.Y., World Health Organization ;

distributed by Q Corporation.

Hernandez-Perera, O., D. Perez-Sala, et al. (2000). "Involvement of Rho GTPases in the transcriptional inhibition of preproendothelin-1 gene expression by simvastatin in vascular endothelial cells." Circ Res **87**(7): 616-622.

Herrmann, J., S. Samee, et al. (2005). "Differential effect of experimental hypertension and hypercholesterolemia on adventitial remodeling." Arterioscler Thromb Vasc Biol **25**(2): 447-453.

Hilgers, R. H. and R. C. Webb (2005). "Molecular aspects of arterial smooth muscle contraction: focus on Rho." Exp Biol Med (Maywood) **230**(11): 829-835.

Hirata, Y., E. Suzuki, et al. (1992). "Role of endogenous ANP in sodium excretion in rats with experimental pulmonary hypertension." Am J Physiol **262**(6 Pt 2): H1684-1689.

Hirose, S., Y. Hosoda, et al. (2000). "Expression of vascular endothelial growth factor and its receptors correlates closely with formation of the plexiform lesion in human pulmonary hypertension." Pathol Int **50**(6): 472-479.

Hislop, A. and L. Reid (1976). "New Findings in Pulmonary-Arteries of Rats with Hypoxia-Induced Pulmonary-Hypertension." Br J Exp Pathol **57**(5): 542-554.

Hislop, A. A. and C. M. Pierce (2000). "Growth of the vascular tree." Paediatr Respir Rev **1**(4): 321-327.

Hobbs, A. J. (1997). "Soluble guanylate cyclase: the forgotten sibling." Trends in Pharmacological Sciences **18**(12): 484-491.

Hong, I. S., H. V. Coe, et al. (2014). "Macitentan for the Treatment of Pulmonary Arterial Hypertension." Ann Pharmacother.

Hong, K. H., Y. J. Lee, et al. (2008). "Genetic ablation of the BMPR2 gene in pulmonary endothelium is sufficient to predispose to pulmonary arterial hypertension." Circulation **118**(7): 722-730.

Hosokawa, S., G. Haraguchi, et al. (2013). "Pathophysiological roles of nuclear factor kappaB (NF-kB) in pulmonary arterial hypertension: effects of synthetic selective NF-kB inhibitor IMD-0354." Cardiovasc Res **99**(1): 35-43.

Hu, J., Y. Cheng, et al. (2014). "microRNA-128 plays a critical role in human non-small cell lung cancer tumourigenesis, angiogenesis and lymphangiogenesis by directly targeting vascular endothelial growth factor-C." Eur J Cancer.

Hu, T. H., M. Chouinard, et al. (2006). "Farnesoid X receptor agonist reduces serum asymmetric dimethylarginine levels through hepatic dimethylarginine dimethylaminohydrolase-1 gene regulation." Journal of Biological Chemistry **281**(52): 39831-39838.

Hu, X., X. Xu, et al. (2009). "Vascular endothelial-specific dimethylarginine dimethylaminohydrolase-1-deficient mice reveal that vascular endothelium plays an important role in removing asymmetric dimethylarginine." Circulation **120**(22): 2222-2229.

Hu, X. L., D. Atzler, et al. (2011). "Dimethylarginine Dimethylaminohydrolase-1 Is the Critical Enzyme for Degrading the Cardiovascular Risk Factor Asymmetrical Dimethylarginine." Arteriosclerosis Thrombosis and Vascular Biology **31**(7): 1540-U1145.

Huang, X., L. H. Ding, et al. (2009). "Hypoxia-Inducible mir-210 Regulates Normoxic Gene Expression Involved in Tumor Initiation." Molecular Cell **35**(6): 856-867.

Humbert, M., G. Monti, et al. (1995). "Increased Interleukin-1 and Interleukin-6 Serum Concentrations in Severe Primary Pulmonary-Hypertension." Am J Respir Crit Care Med **151**(5): 1628-1631.

Humbert, M., N. W. Morrell, et al. (2004). "Cellular and molecular pathobiology of pulmonary arterial hypertension." J Am Coll Cardiol **43**(12): 13s-24s.

Humbert, M., N. W. Morrell, et al. (2004). "Cellular and molecular pathobiology of pulmonary arterial hypertension." J Am Coll Cardiol **43**(12 Suppl S): 13S-24S.

Humbert, M., O. Sitbon, et al. (2010). "Survival in patients with idiopathic, familial, and anorexigen-associated pulmonary arterial hypertension in the modern management era." Circulation **122**(2): 156-163.

Huntzinger, E. and E. Izaurralde (2011). "Gene silencing by microRNAs: contributions of translational repression and mRNA decay." Nat Rev Genet **12**(2): 99-110.

Iannone, L., L. Zhao, et al. (2014). "MiRNA-21/DDAH1 pathway regulates pulmonary vascular responses to hypoxia." Biochemical Journal.

Ikeda, S., S. W. Kong, et al. (2007). "Altered microRNA expression in human heart disease." Physiol Genomics **31**(3): 367-373.

Iliopoulos, D., S. A. Jaeger, et al. (2010). "STAT3 Activation of miR-21 and miR-181b-1 via PTEN and CYLD Are Part of the Epigenetic Switch Linking Inflammation to Cancer." Molecular Cell **39**(4): 493-506.

Iyer, N. V., L. E. Kotch, et al. (1998). "Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha." Genes Dev **12**(2): 149-162.

Jackson, R. J. (1993). "Cytoplasmic regulation of mRNA function: the importance of the 3' untranslated region." Cell **74**(1): 9-14.

Jacobi, J., K. Sydow, et al. (2005). "Overexpression of dimethylarginine dimethylaminohydrolase reduces tissue asymmetric dimethylarginine levels and enhances angiogenesis." Circulation **111**(11): 1431-1438.

Jaffe, A. B. and A. Hall (2005). "Rho GTPases: biochemistry and biology." Annu Rev Cell Dev Biol **21**: 247-269.

Jasmin, J. F., M. Lucas, et al. (2001). "Effectiveness of a nonselective ET(A/B) and a selective ET(A) antagonist in rats with monocrotaline-induced pulmonary hypertension." Circulation **103**(2): 314-318.

Jeffery, T. K. and N. W. Morrell (2002). "Molecular and cellular basis of pulmonary vascular remodeling in pulmonary hypertension." Prog Cardiovasc Dis **45**(3): 173-202.

Jensen, S. G., P. Lamy, et al. (2011). "Evaluation of two commercial global miRNA expression profiling platforms for detection of less abundant miRNAs." Bmc Genomics **12**.

Ji, R. R., Y. H. Cheng, et al. (2007). "MicroRNA expression signature and antisense-mediated depletion reveal an essential role of microRNA in vascular neointimal lesion formation." Circ Res **100**(11): 1579-1588.

Jiang, J. L., X. H. Zhang, et al. (2006). "Probucol decreases asymmetrical dimethylarginine level by alternation of protein arginine methyltransferase I and dimethylarginine dimethylaminohydrolase activity." Cardiovascular Drugs and Therapy **20**(4): 281-294.

Jing, Z. C., K. Parikh, et al. (2013). "Efficacy and safety of oral treprostinil monotherapy for the treatment of pulmonary arterial hypertension: a randomized, controlled trial." Circulation **127**(5): 624-633.

Jonigk, D., H. Golpon, et al. (2011). "Plexiform Lesions in Pulmonary Arterial Hypertension Composition, Architecture, and Microenvironment." American Journal of Pathology **179**(1): 167-179.

Jonigk, D., H. Golpon, et al. (2011). "Plexiform lesions in pulmonary arterial hypertension composition, architecture, and microenvironment." Am J Pathol **179**(1): 167-179.

Jurasz, P., D. Courtman, et al. (2010). "Role of apoptosis in pulmonary hypertension: From experimental models to clinical trials." Pharmacol Ther **126**(1): 1-8.
Kantharidis, P., B. Wang, et al. (2011). "Diabetes Complications: The MicroRNA Perspective." Diabetes **60**(7): 1832-1837.
Kato, M. and N. C. Staub (1966). "Response of small pulmonary arteries to unilobar hypoxia and hypercapnia." Circ Res **19**(2): 426-440.
Kekewska, A., T. Gornemann, et al. (2012). "Antiserotonergic properties of terguride in blood vessels, platelets, and valvular interstitial cells." J Pharmacol Exp Ther **340**(2): 369-376.
Keogh, A. M., E. Mayer, et al. (2009). "Interventional and surgical modalities of treatment in pulmonary hypertension." J Am Coll Cardiol **54**(1 Suppl): S67-77.
Kerr, J. F. R., A. H. Wyllie, et al. (1972). "Apoptosis - Basic Biological Phenomenon with Wide-Ranging Implications in Tissue Kinetics." British Journal of Cancer **26**(4): 239-&.
Kjeldsen, S. E., B. Dahlof, et al. (2002). "Effects of losartan on cardiovascular morbidity and mortality in patients with isolated systolic hypertension and left ventricular hypertrophy - A Losartan Intervention For Endpoint Reduction (LIFE) substudy." Jama-Journal of the American Medical Association **288**(12): 1491-1498.
Knipp, M., O. Braun, et al. (2003). "Zn(II)-free dimethylargininase-1 (DDAH-1) is inhibited upon specific Cys-S-nitrosylation." Journal of Biological Chemistry **278**(5): 3410-3416.
Konishi, H., K. Sydow, et al. (2007). "Dimethylarginine dimethylaminohydrolase promotes endothelial repair after vascular injury." J Am Coll Cardiol **49**(10): 1099-1105.
Krick, S., J. Hanze, et al. (2005). "Hypoxia-driven proliferation of human pulmonary artery fibroblasts: cross-talk between HIF-1 alpha and an autocrine angiotensin system." Faseb Journal **19**(2): 857-+.
Kuhn, M. (2012). "Endothelial actions of atrial and B-type natriuretic peptides." Br J Pharmacol **166**(2): 522-531.
Kumar, A., S. K. Raut, et al. (2011). "Microrna-21 Contributes to Diabetic Cardiomyopathy Associated Cardiac Fibrosis." Circulation **124**(21).
Kwapiszewska, G., J. Wilhelm, et al. (2005). "Expression profiling of laser-microdissected intrapulmonary arteries in hypoxia-induced pulmonary hypertension." Respir Res **6**: 109.
Lajer, M., L. Tarnow, et al. (2008). "Plasma concentration of asymmetric dimethylarginine (ADMA) predicts cardiovascular morbidity and mortality in type 1 diabetic patients with diabetic nephropathy." Diabetes Care **31**(4): 747-752.
Lancaster, J. R., Jr. (1996). "Diffusion of free nitric oxide." Methods Enzymol **268**: 31-50.
Lancaster, J. R., Jr. (1997). "A tutorial on the diffusibility and reactivity of free nitric oxide." Nitric Oxide **1**(1): 18-30.
Lane, K. B., R. D. Machado, et al. (2000). "Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension." Nat Genet **26**(1): 81-84.
Langleben, D., R. J. Barst, et al. (1999). "Continuous infusion of epoprostenol improves the net balance between pulmonary endothelin-1 clearance and release in primary pulmonary hypertension." Circulation **99**(25): 3266-3271.
Langleben, D., B. W. Christman, et al. (2002). "Effects of the thromboxane synthetase inhibitor and receptor antagonist terbogrel in patients with primary pulmonary hypertension." Am Heart J **143**(5): E4.
Lau, Y. T. and W. C. Ma (1996). "Nitric oxide inhibits migration of cultured endothelial cells." Biochem Biophys Res Commun **221**(3): 670-674.
Laufs, U. and J. K. Liao (1998). "Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by rho GTPase." Journal of Biological Chemistry **273**(37): 24266-24271.
Le Cras, T. D., N. E. Markham, et al. (2002). "Treatment of newborn rats with a VEGF receptor inhibitor causes pulmonary hypertension and abnormal lung structure." Am J Physiol Lung Cell Mol Physiol **283**(3): L555-562.

Lee, S. D., K. R. Shroyer, et al. (1998). "Monoclonal endothelial cell proliferation is present in primary but not secondary pulmonary hypertension." Journal of Clinical Investigation **101**(5): 927-934.

Leiper, J., J. Murray-Rust, et al. (2002). "S-nitrosylation of dimethylarginine dimethylaminohydrolase regulates enzyme activity: Further interactions between nitric oxide synthase and dimethylarginine dimethylaminohydrolase." Proc Natl Acad Sci U S A **99**(21): 13527-13532.

Leiper, J., J. Murray-Rust, et al. (2002). "S-nitrosylation of dimethylarginine dimethylaminohydrolase regulates enzyme activity: further interactions between nitric oxide synthase and dimethylarginine dimethylaminohydrolase." Proc Natl Acad Sci U S A **99**(21): 13527-13532.

Leiper, J. and M. Nandi (2011). "The therapeutic potential of targeting endogenous inhibitors of nitric oxide synthesis." Nat Rev Drug Discov **10**(4): 277-291.

Leiper, J., M. Nandi, et al. (2007). "Disruption of methylarginine metabolism impairs vascular homeostasis." Nat Med **13**(2): 198-203.

Leiper, J. M., J. S. Maria, et al. (1999). "Identification of two human dimethylarginine dimethylaminohydrolases with distinct tissue distributions and homology with microbial arginine deiminases." Biochemical Journal **343**: 209-214.

Lepine, J. P., R. Mordasini, et al. (1982). "Aminorex and Pulmonary-Hypertension - a Long-Term Follow-up-Study." Bulletin Europeen De Physiopathologie Respiratoire-Clinical Respiratory Physiology **18**(4): P88-P89.

Levy, A. P., N. S. Levy, et al. (1996). "Post-transcriptional regulation of vascular endothelial growth factor by hypoxia." J Biol Chem **271**(5): 2746-2753.

Levy, A. P., N. S. Levy, et al. (1995). "Regulation of Vascular Endothelial Growth-Factor in Cardiac Myocytes." Circ Res **76**(5): 758-766.

Levy, A. P., N. S. Levy, et al. (1995). "Regulation of vascular endothelial growth factor in cardiac myocytes." Circ Res **76**(5): 758-766.

Levy, M., C. Maurey, et al. (2007). "Impaired apoptosis of pulmonary endothelial cells is associated with intimal proliferation and irreversibility of pulmonary hypertension in congenital heart disease." J Am Coll Cardiol **49**(7): 803-810.

Levy, N. S., S. M. Chung, et al. (1998). "Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR." Journal of Biological Chemistry **273**(11): 6417-6423.

Li, D. C., V. E. Laubach, et al. (2001). "Upregulation of lung soluble guanylate cyclase during chronic hypoxia is prevented by deletion of eNOS." American Journal of Physiology-Lung Cellular and Molecular Physiology **281**(2): L369-L376.

Li, D. C., N. Zhou, et al. (1999). "Soluble guanylate cyclase gene expression and localization in rat lung after exposure to hypoxia." American Journal of Physiology-Lung Cellular and Molecular Physiology **277**(4): L841-L847.

Li, H. B., S. J. Chen, et al. (1994). "Enhanced Endothelin-1 and Endothelin Receptor Gene-Expression in Chronic Hypoxia." J Appl Physiol **77**(3): 1451-1459.

Li, M., S. Vattulainen, et al. (2014). "Loss-of BMPR2 is Associated With Abnormal DNA Repair in Pulmonary Arterial Hypertension." Am J Respir Cell Mol Biol.

Lin, K. Y., A. Ito, et al. (2002). "Impaired nitric oxide synthase pathway in diabetes mellitus - Role of asymmetric dimethylarginine and dimethylarginine dimethylaminohydrolase." Circulation **106**(8): 987-992.

Lincoln, T. M., N. Dey, et al. (2001). "Invited review: cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression." J Appl Physiol (1985) **91**(3): 1421-1430.

Liu, C. Y., C. H. Wang, et al. (1998). "Increased level of exhaled nitric oxide and up-regulation of inducible nitric oxide synthase in patients with primary lung cancer." British Journal of Cancer **78**(4): 534-541.

Liu, D., J. Wang, et al. (2007). "Dosage-dependent requirement of BMP type II receptor for maintenance of vascular integrity." Blood **110**(5): 1502-1510.

Liu, G., A. Friggeri, et al. (2010). "miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis." Journal of Experimental Medicine **207**(8): 1589-1597.
Liu, L. H., Z. Guo, et al. (2012). "Protection of DDAH2 Overexpression Against Homocysteine-Induced Impairments of DDAH/ADMA/NOS/NO Pathway in Endothelial Cells." Cellular Physiology and Biochemistry **30**(6): 1413-1422.
Liu, Y., S. R. Cox, et al. (1995). "Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer." Circ Res **77**(3): 638-643.
Loirand, G., C. Guilluy, et al. (2006). "Regulation of Rho proteins by phosphorylation in the cardiovascular system." Trends Cardiovasc Med **16**(6): 199-204.
Loirand, G. and P. Pacaud (2010). "The role of rho protein signaling in hypertension." Nature Reviews Cardiology **7**(11): 637-647.
Long, L., M. R. MacLean, et al. (2006). "Serotonin increases susceptibility to pulmonary hypertension in BMPR2-deficient mice." Circ Res **98**(6): 818-827.
Louis, S. F. and P. Zahradka (2010). "Vascular smooth muscle cell motility: From migration to invasion." Experimental & Clinical Cardiology **15**(4): E75-E85.
Lowery, J. W. and M. P. de Caestecker (2010). "BMP signaling in vascular development and disease." Cytokine Growth Factor Rev **21**(4): 287-298.
Lu, A., J. F. Clark, et al. (2009). "Down-regulation of interleukin 7 mRNA by hypoxia is calcium dependent." Neurological Research **31**(5): 545-549.
Lucas, K. A., G. M. Pitari, et al. (2000). "Guanylyl cyclases and signaling by cyclic GMP." Pharmacol Rev **52**(3): 375-413.
MacAllister, R. J., H. Parry, et al. (1996). "Regulation of nitric oxide synthesis by dimethylarginine dimethylaminohydrolase." Br J Pharmacol **119**(8): 1533-1540.
Mace, T. A., A. L. Collins, et al. (2013). "Hypoxia induces the overexpression of microRNA-21 in pancreatic cancer cells." Journal of Surgical Research **184**(2): 855-860.
Mack, C. P., A. V. Somlyo, et al. (2001). "Smooth muscle differentiation marker gene expression is regulated by RhoA-mediated actin polymerization." Journal of Biological Chemistry **276**(1): 341-347.
Maclean, M. R. and Y. Dempsie (2010). "The serotonin hypothesis of pulmonary hypertension revisited." Adv Exp Med Biol **661**: 309-322.
Madhani, M., M. Okorie, et al. (2006). "Reciprocal regulation of human soluble and particulate guanylate cyclases in vivo." Br J Pharmacol **149**(6): 797-801.
Manalo, D. J., A. Rowan, et al. (2005). "Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1." Blood **105**(2): 659-669.
Matsushita, H., R. Morishita, et al. (2000). "Hypoxia-induced endothelial apoptosis through nuclear factor-kappa B (NF-kappa B)-mediated bcl-2 suppression - In vivo evidence of the importance of NF-kappa B in endothelial cell regulation." Circ Res **86**(9): 974-981.
McQuaid, K. E. and A. K. Keenan (1997). "Endothelial barrier dysfunction and oxidative stress: roles for nitric oxide?" Exp Physiol **82**(2): 369-376.
Mehta, D. and A. B. Malik (2006). "Signaling mechanisms regulating endothelial permeability." Physiol Rev **86**(1): 279-367.
Mehta, J., P. T. Parthasarathy, et al. (2013). "New hope for a microRNA therapy for pulmonary arterial hypertension." Front Genet **4**: 137.
Mehta, U., B. P. S. Kang, et al. (2002). "Studies of apoptosis and bcl-2 in experimental atherosclerosis in rabbit and influence of selenium supplementation." General Physiology and Biophysics **21**(1): 15-29.
Merklinger, S. L., R. A. Wagner, et al. (2005). "Increased fibulin-5 and elastin in S100A4/Mts1 mice with pulmonary hypertension." Circ Res **97**(6): 596-604.
Meyrick, B. and L. Reid (1980). "Hypoxia-Induced Structural-Changes in the Media and Adventitia of the Rat Hilar Pulmonary-Artery and Their Regression." American Journal of Pathology **100**(1): 151-178.

Meyrick, B. and L. Reid (1980). "Hypoxia-induced structural changes in the media and adventitia of the rat hilar pulmonary artery and their regression." Am J Pathol **100**(1): 151-178.
Meyrick, B. and L. Reid (1983). "Pulmonary hypertension. Anatomic and physiologic correlates." Clin Chest Med **4**(2): 199-217.
Meyrick, B. O. and E. A. Perkett (1989). "The Sequence of Cellular and Hemodynamic-Changes of Chronic Pulmonary-Hypertension Induced by Hypoxia and Other Stimuli." American Review of Respiratory Disease **140**(5): 1486-1489.
Militante, J. D. and J. B. Lombardini (2002). "Treatment of hypertension with oral taurine: experimental and clinical studies." Amino Acids **23**(4): 381-393.
Millatt, L. J., G. S. Whitley, et al. (2003). "Evidence for dysregulation of dimethylarginine dimethylaminohydrolase I in chronic hypoxia-induced pulmonary hypertension." Circulation **108**(12): 1493-1498.
Millatt, L. J., G. S. Whitley, et al. (2003). "Evidence for Dysregulation of dimethylarginine dimethylaminohydrolase I in chronic hypoxia-induced pulmonary hypertension." Circulation **108**(12): 1493-1498.
Miyata, M., F. Sakuma, et al. (1995). "Pulmonary-Hypertension in Rats .2. Role of Interleukin-6." Int Arch Allergy Immunol **108**(3): 287-291.
Mlczoch, J. (1984). Drug and dietary induced pulmonary hypertension. Pulmonary Hypertension. W. EK. New York**:** 341-359.
Mohamed, T. M. A., D. Oceandy, et al. (2009). "Specific Role of Neuronal Nitric-oxide Synthase when Tethered to the Plasma Membrane Calcium Pump in Regulating the beta-Adrenergic Signal in the Myocardium." Journal of Biological Chemistry **284**(18): 12091-12098.
Moncada, S., E. A. Higgs, et al. (1977). "Human Arterial and Venous Tissues Generate Prostacyclin (Prostaglandin-X), a Potent Inhibitor of Platelet-Aggregation." Lancet **1**(8001): 18-21.
Moreno-Vinasco, L., M. Gomberg-Maitland, et al. (2008). "Genomic assessment of a multikinase inhibitor, sorafenib, in a rodent model of pulmonary hypertension." Physiol Genomics **33**(2): 278-291.
Morishima, N., K. Nakanishi, et al. (2002). "An endoplasmic reticulum stress-specific caspase cascade in apoptosis - Cytochrome c-independent activation of caspase-9 by caspase-12." Journal of Biological Chemistry **277**(37): 34287-34294.
Morrell, N. W. (2006). "Pulmonary hypertension due to BMPR2 mutation: a new paradigm for tissue remodeling?" Proc Am Thorac Soc **3**(8): 680-686.
Morrell, N. W., X. D. Yang, et al. (2001). "Altered growth responses of muscle cells from patients pulmonary artery smooth with primary pulmonary hypertension to transforming growth factor-beta(1) and bone morphogenetic proteins." Circulation **104**(7): 790-795.
Motley, H. L., A. Cournand, et al. (1947). "The influence of short periods of induced acute anoxia upon pulmonary artery pressures in man." Am J Physiol **150**(2): 315-320.
Mundy, A. L. and K. L. Dorrington (2000). "Inhibition of nitric oxide synthesis augments pulmonary oedema in isolated perfused rabbit lung." Br J Anaesth **85**(4): 570-576.
Munteanu, A. and J. M. Zingg (2007). "Cellular, molecular and clinical aspects of vitamin E on atherosclerosis prevention." Molecular Aspects of Medicine **28**(5-6): 538-590.
Nagase, S., A. Hirayama, et al. (2005). "Does fluvastatin really have an antioxidant effect in humans?" Kidney International **68**(3): 1373-1374.
Napoli, C., G. Paolisso, et al. (2013). "Effects of Nitric Oxide on Cell Proliferation Novel Insights." J Am Coll Cardiol **62**(2): 89-95.
Naruse, K., K. Shimizu, et al. (1994). "Long-term inhibition of NO synthesis promotes atherosclerosis in the hypercholesterolemic rabbit thoracic aorta. PGH2 does not contribute to impaired endothelium-dependent relaxation." Arterioscler Thromb **14**(5): 746-752.
Newby, A. C., K. M. Southgate, et al. (1992). "Inhibition of Vascular Smooth-Muscle Cell-Proliferation by Endothelium-Dependent Vasodilators." Herz **17**(5): 291-299.

Nijveldt, R. J., T. Teerlink, et al. (2003). "The liver is an important organ in the metabolism of asymmetrical dimethylarginine (ADMA)." Clin Nutr **22**(1): 17-22.
Nong, Z. X., M. Hoylaerts, et al. (1997). "Nitric oxide inhalation inhibits platelet aggregation and platelet-mediated pulmonary thrombosis in rats." Circ Res **81**(5): 865-869.
Nor, J. E., J. Christensen, et al. (1999). "Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression." Am J Pathol **154**(2): 375-384.
North, A. J., F. R. Moya, et al. (1995). "Pulmonary Endothelial Nitric-Oxide Synthase Gene-Expression Is Decreased in a Rat Model of Congenital Diaphragmatic-Hernia." Am J Respir Cell Mol Biol **13**(6): 676-682.
Nossaman, B. D., V. E. Nossaman, et al. (2010). "Role of the RhoA/Rho-kinase pathway in the regulation of pulmonary vasoconstrictor function." Can J Physiol Pharmacol **88**(1): 1-8.
Ogawa, T., M. Kimoto, et al. (1989). "Purification and Properties of a New Enzyme, Ng,Ng-Dimethylarginine Dimethylaminohydrolase, from Rat-Kidney." Journal of Biological Chemistry **264**(17): 10205-10209.
Oguz, A. and M. Uzunlulu (2008). "Short Term Fluvastatin Treatment Lowers Serum Asymmetric Dimethylarginine Levels in Patients with Metabolic Syndrome." Atherosclerosis Supplements **9**(1): 212-212.
Onozato, M. L., A. Tojo, et al. (2008). "Expression of N(G),N(G)-dimethylarginine dimethylaminohydrolase and protein arginine N-methyltransferase isoforms in diabetic rat kidney - Effects of angiotensin II receptor blockers." Diabetes **57**(1): 172-180.
Pak, O., A. Aldashev, et al. (2007). "The effects of hypoxia on the cells of the pulmonary vasculature." Eur Respir J **30**(2): 364-372.
Palm, F., M. L. Onozato, et al. (2007). "Dimethylarginine dimethylaminohydrolase (DDAH): expression, regulation, and function in the cardiovascular and renal systems." Am J Physiol Heart Circ Physiol **293**(6): H3227-3245.
Palmer, R. M., A. G. Ferrige, et al. (1987). "Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor." Nature **327**(6122): 524-526.
Pan, J. and K. M. Baker (2007). "Retinoic acid and the heart." Vitamin A **75**: 257-+.
Parikh, V. N., R. C. Jin, et al. (2012). "MicroRNA-21 integrates pathogenic signaling to control pulmonary hypertension: results of a network bioinformatics approach." Circulation **125**(12): 1520-1532.
Patel, C. A. and S. Rattan (2007). "Cellular regulation of basal tone in internal anal sphincter smooth muscle by RhoA/ROCK." American Journal of Physiology-Gastrointestinal and Liver Physiology **292**(6): G1747-G1756.
Patrick, D. M., R. L. Montgomery, et al. (2010). "Stress-dependent cardiac remodeling occurs in the absence of microRNA-21 in mice." J Clin Invest **120**(11): 3912-3916.
Paulding, W. R. and M. F. Czyzyk-Krzeska (2000). "Hypoxia-induced regulation of mRNA stability." Oxygen Sensing: Molecule to Man **475**: 111-121.
Peiro, C., J. Redondo, et al. (1995). "Influence of endothelium on cultured vascular smooth muscle cell proliferation." Hypertension **25**(4 Pt 2): 748-751.
Peiro, C., J. Redondo, et al. (1995). "Influence of Endothelium on Cultured Vascular Smooth-Muscle Cell-Proliferation." Hypertension **25**(4): 748-751.
Perrella, M. A., E. S. Edell, et al. (1992). "Endothelium-Derived Relaxing Factor in Pulmonary and Renal Circulations during Hypoxia." American Journal of Physiology **263**(1): R45-R50.
Petkov, V., W. Mosgoeller, et al. (2003). "Vasoactive intestinal peptide as a new drug for treatment of primary pulmonary hypertension." J Clin Invest **111**(9): 1339-1346.
Pinto, R. F. A., M. D. Higuchi, et al. (2004). "Decreased numbers of T-lymphocytes and predominance of recently recruited macrophages in the walls of peripheral pulmonary arteries from 26 patients with pulmonary hypertension secondary to congenital cardiac shunts." Cardiovascular Pathology **13**(5): 268-275.

Pope, A. J., K. Karrupiah, et al. (2009). "Role of Dimethylarginine Dimethylaminohydrolases in the Regulation of Endothelial Nitric Oxide Production." Journal of Biological Chemistry **284**(51): 35338-35347.

Pope, A. J., K. Karuppiah, et al. (2009). "Role of the PRMT-DDAH-ADMA axis in the regulation of endothelial nitric oxide production." Pharmacol Res **60**(6): 461-465.

Prabhakar, N. R. and G. L. Semenza (2012). "Adaptive and maladaptive cardiorespiratory responses to continuous and intermittent hypoxia mediated by hypoxia-inducible factors 1 and 2." Physiol Rev **92**(3): 967-1003.

Predescu, D., S. Predescu, et al. (2005). "Constitutive eNOS-derived nitric oxide is a determinant of endothelial junctional integrity." American Journal of Physiology-Lung Cellular and Molecular Physiology **289**(3): L371-L381.

Preston, I. R., N. S. Hill, et al. (2004). "Synergistic effects of ANP and sildenafil on cGMP levels and amelioration of acute hypoxic pulmonary hypertension." Exp Biol Med (Maywood) **229**(9): 920-925.

Price, L. C., S. J. Wort, et al. (2012). "Inflammation in Pulmonary Arterial Hypertension." Chest **141**(1): 210-221.

Pulido, T., I. Adzerikho, et al. (2013). "Macitentan and morbidity and mortality in pulmonary arterial hypertension." N Engl J Med **369**(9): 809-818.

Pullamsetti, S. S., C. Doebele, et al. (2012). "Inhibition of microRNA-17 improves lung and heart function in experimental pulmonary hypertension." Am J Respir Crit Care Med **185**(4): 409-419.

Pullamsetti, S. S., R. Savai, et al. (2011). "The role of dimethylarginine dimethylaminohydrolase in idiopathic pulmonary fibrosis." Sci Transl Med **3**(87): 87ra53.

Qiao, Y. N., W. Q. He, et al. (2014). "Myosin phosphatase target subunit 1 (MYPT1) regulates the contraction and relaxation of vascular smooth muscle and maintains blood pressure." J Biol Chem **289**(32): 22512-22523.

Rabinovitch, M. (2008). "Molecular pathogenesis of pulmonary arterial hypertension." Journal of Clinical Investigation **118**(7): 2372-2379.

Rabinovitch, M., C. Guignabert, et al. (2014). "Inflammation and Immunity in the Pathogenesis of Pulmonary Arterial Hypertension." Circ Res **115**(1): 165-175.

Raffestin, B., M. Levame, et al. (1992). "Pulmonary vasodilatory action of endogenous atrial natriuretic factor in rats with hypoxic pulmonary hypertension. Effects of monoclonal atrial natriuretic factor antibody." Circ Res **70**(1): 184-192.

Reddi, A. H. (1994). "Bone and cartilage differentiation." Curr Opin Genet Dev **4**(5): 737-744.

Rhodes, C. J., J. Wharton, et al. (2013). "Reduced microRNA-150 is associated with poor survival in pulmonary arterial hypertension." Am J Respir Crit Care Med **187**(3): 294-302.

Riccardi, C. and I. Nicoletti (2006). "Analysis of apoptosis by propidium iodide staining and flow cytometry." Nat Protoc **1**(3): 1458-1461.

Rich, S., D. R. Dantzker, et al. (1987). "Primary pulmonary hypertension. A national prospective study." Ann Intern Med **107**(2): 216-223.

Ridley, A. J. (2001). "Rho GTPases and cell migration." J Cell Sci **114**(Pt 15): 2713-2722.

Roberts, J. D., C. T. Roberts, et al. (1995). "Continuous Nitric-Oxide Inhalation Reduces Pulmonary Arterial Structural-Changes, Right-Ventricular Hypertrophy, and Growth-Retardation in the Hypoxic Newborn Rat." Circ Res **76**(2): 215-222.

Robertson, T. P., J. P. Ward, et al. (2001). "Hypoxia induces the release of a pulmonary-selective, Ca(2+)-sensitising, vasoconstrictor from the perfused rat lung." Cardiovasc Res **50**(1): 145-150.

Rojas, A., H. Figueroa, et al. (2006). "Oxidative stress at the vascular wall. Mechanistic and pharmacological aspects." Archives of Medical Research **37**(4): 436-448.

Rondon, I. J., L. A. MacMillan, et al. (1991). "Hypoxia up-regulates the activity of a novel erythropoietin mRNA binding protein." J Biol Chem **266**(25): 16594-16598.

Ross, J. (1995). "mRNA stability in mammalian cells." Microbiol Rev **59**(3): 423-450.
Rossiter, S., C. L. Smith, et al. (2005). "Selective substrate-based inhibitors of mammalian dimethylarginine dimethylaminohydrolase." J Med Chem **48**(14): 4670-4678.
Rubin, L. J., J. Mendoza, et al. (1990). "Treatment of primary pulmonary hypertension with continuous intravenous prostacyclin (epoprostenol). Results of a randomized trial." Ann Intern Med **112**(7): 485-491.
Russwurm, M. and D. Koesling (2004). "Guanylyl cyclase: NO hits its target." Free Radicals: Enzymology, Signalling and Disease(71): 51-63.
Russwurm, M., N. Wittau, et al. (2001). "Guanylyl cyclase/PSD-95 interaction - Targeting of the nitric oxide-sensitive alpha(2)beta(1) guanylyl cyclase to synaptic membranes." Journal of Biological Chemistry **276**(48): 44647-44652.
Sabatel, C., L. Malvaux, et al. (2011). "MicroRNA-21 exhibits antiangiogenic function by targeting RhoB expression in endothelial cells." PLoS One **6**(2): e16979.
Saikumar, P., Z. Dong, et al. (1998). "Role of hypoxia-induced Bax translocation and cytochrome c release in reoxygenation injury." Oncogene **17**(26): 3401-3415.
Sakao, S., L. Taraseviciene-Stewart, et al. (2005). "Initial apoptosis is followed by increased proliferation of apoptosis-resistant endothelial cells." Faseb Journal **19**(7): 1178-+.
Sakao, S., K. Tatsumi, et al. (2009). "Endothelial cells and pulmonary arterial hypertension: apoptosis, proliferation, interaction and transdifferentiation." Respir Res **10**.
Sarkar, J., D. Gou, et al. (2010). "MicroRNA-21 plays a role in hypoxia-mediated pulmonary artery smooth muscle cell proliferation and migration." Am J Physiol Lung Cell Mol Physiol **299**(6): L861-871.
Sarkar, R., R. C. Webb, et al. (1995). "Nitric-Oxide Inhibition of Endothelial-Cell Mitogenesis and Proliferation." Surgery **118**(2): 274-279.
Sartore, S., A. Chiavegato, et al. (2001). "Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: from innocent bystander to active participant." Circ Res **89**(12): 1111-1121.
Sasaki, A., S. Doi, et al. (2007). "Roles of accumulated endogenous nitric oxide synthase inhibitors, enhanced arginase activity, and attenuated nitric oxide synthase activity in endothelial cells for pulmonary hypertension in rats." American Journal of Physiology-Lung Cellular and Molecular Physiology **292**(6): L1480-L1487.
Sassen, S., E. A. Miska, et al. (2008). "MicroRNA - implications for cancer." Virchows Archiv **452**(1): 1-10.
Sausbier, M., R. Schubert, et al. (2000). "Mechanisms of NO/cGMP-dependent vasorelaxation." Circ Res **87**(9): 825-830.
Sauzeau, V., H. Le Jeune, et al. (2000). "Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA-induced Ca2+ sensitization of contraction in vascular smooth muscle." J Biol Chem **275**(28): 21722-21729.
Savale, L., L. Tu, et al. (2009). "Impact of interleukin-6 on hypoxia-induced pulmonary hypertension and lung inflammation in mice." Respir Res **10**: 6.
Schultze, A. E. and R. A. Roth (1998). "Chronic pulmonary hypertension - The monocrotaline model and involvement of the hemostatic system." Journal of Toxicology and Environmental Health-Part B-Critical Reviews **1**(4): 271-346.
Schwedhelm, E., E. C. von Leitner, et al. (2009). "Extensive characterization of the human DDAH1 transgenic mice." Pharmacol Res **60**(6): 494-502.
Sciarretta, S., S. Marchitti, et al. (2013). "C2238 atrial natriuretic peptide molecular variant is associated with endothelial damage and dysfunction through natriuretic peptide receptor C signaling." Circ Res **112**(10): 1355-1364.
Scotland, R., P. Foster, et al. (2004). "C-type natriuretic peptide inhibits leukocyte recruitment in vivo via natriuretic peptide receptor-C activation." Circulation **110**(17): 240-240.

Scotland, R. S., A. Ahluwalia, et al. (2005). "C-type natniuretic peptide in vascular physiology and disease." Pharmacol Ther **105**(2): 85-93.
Scottburden, T., V. B. Schini, et al. (1992). "Platelet-Derived Growth-Factor Suppresses and Fibroblast Growth-Factor Enhances Cytokine-Induced Production of Nitric-Oxide by Cultured Smooth-Muscle Cells - Effects on Cell-Proliferation." Circ Res **71**(5): 1088-1100.
Selimovic, N., E. Sakiniene, et al. (2008). "Inflammation and growth factors in patients with pulmonary arterial hypertension." Eur Heart J **29**: 559-559.
Semenza, G. L. (2005). "Pulmonary vascular responses to chronic hypoxia mediated by hypoxia-inducible factor 1." Proc Am Thorac Soc **2**(1): 68-70.
Shao, D., J. E. Park, et al. (2011). "The role of endothelin-1 in the pathogenesis of pulmonary arterial hypertension." Pharmacol Res **63**(6): 504-511.
Sharma, H. S., M. Wunsch, et al. (1992). "Molecular biology of the coronary vascular and myocardial responses to ischemia." J Cardiovasc Pharmacol **20 Suppl 1**: S23-31.
Shaul, P. W. (2002). "Regulation of endothelial nitric oxide synthase: location, location, location." Annu Rev Physiol **64**: 749-774.
Shaw, G. and R. Kamen (1986). "A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation." Cell **46**(5): 659-667.
Shibata, R., S. Ueda, et al. (2009). "Involvement of asymmetric dimethylarginine (ADMA) in tubulointerstitial ischaemia in the early phase of diabetic nephropathy." Nephrol Dial Transplant **24**(4): 1162-1169.
Shimoda, L. A. and G. L. Semenza (2011). "HIF and the Lung Role of Hypoxia-inducible Factors in Pulmonary Development and Disease." Am J Respir Crit Care Med **183**(2): 152-156.
Shimoda, L. A., J. S. Sham, et al. (2002). "Acute and chronic hypoxic pulmonary vasoconstriction: a central role for endothelin-1?" Respir Physiol Neurobiol **132**(1): 93-106.
Shin, V. Y., H. Jin, et al. (2011). "NF-kappaB targets miR-16 and miR-21 in gastric cancer: involvement of prostaglandin E receptors." Carcinogenesis **32**(2): 240-245.
Shweiki, D., A. Itin, et al. (1992). "Vascular Endothelial Growth-Factor Induced by Hypoxia May Mediate Hypoxia-Initiated Angiogenesis." Nature **359**(6398): 843-845.
Sibal, L., S. C. Agarwal, et al. (2010). "The Role of Asymmetric Dimethylarginine (ADMA) in Endothelial Dysfunction and Cardiovascular Disease." Curr Cardiol Rev **6**(2): 82-90.
Sieber, C., J. Kopf, et al. (2009). "Recent advances in BMP receptor signaling." Cytokine Growth Factor Rev **20**(5-6): 343-355.
Simonneau, G., I. M. Robbins, et al. (2009). "Updated clinical classification of pulmonary hypertension." J Am Coll Cardiol **54**(1 Suppl): S43-54.
Simonneau, G., I. M. Robbins, et al. (2009). "Updated Clinical Classification of Pulmonary Hypertension." J Am Coll Cardiol **54**(1): S43-S54.
Simonneau, G., A. Torbicki, et al. (2012). "Selexipag: an oral, selective prostacyclin receptor agonist for the treatment of pulmonary arterial hypertension." Eur Respir J **40**(4): 874-880.
Siow, R. C., C. M. Mallawaarachchi, et al. (2003). "Migration of adventitial myofibroblasts following vascular balloon injury: insights from in vivo gene transfer to rat carotid arteries." Cardiovasc Res **59**(1): 212-221.
Small, E. M. and E. N. Olson (2011). "Pervasive roles of microRNAs in cardiovascular biology." Nature **469**(7330): 336-342.
Smith, C. L., S. Anthony, et al. (2005). "Effects of ADMA upon gene expression: an insight into the pathophysiological significance of raised plasma ADMA." PLoS Med **2**(10): e264.
Smith, C. L., G. M. Birdsey, et al. (2003). "Dimethylarginine dimethylaminohydrolase activity modulates ADMA levels, VEGF expression, and cell phenotype." Biochem Biophys Res Commun **308**(4): 984-989.
Soon, E., A. M. Holmes, et al. (2010). "Elevated Levels of Inflammatory Cytokines Predict Survival in Idiopathic and Familial Pulmonary Arterial Hypertension." Circulation **122**(9): 921-U990.

Stacher, E., B. B. Graham, et al. (2012). "Modern Age Pathology of Pulmonary Arterial Hypertension." Am J Respir Crit Care Med **186**(3): 261-272.
Stan, R. V. (2009). Anatomy of the pulmonary endothelium. the pulmonary endothelium: function in health and disease. N. F. VOELKEL. Oxford, John Wiley & Sons, Ltd. **2:** 25-29.
Stasch, J. P., K. Dembowsky, et al. (2002). "Cardiovascular actions of a novel NO-independent guanylyl cyclase stimulator, BAY 41-8543: in vivo studies." Br J Pharmacol **135**(2): 344-355.
Stasch, J. P. and O. V. Evgenov (2013). "Soluble guanylate cyclase stimulators in pulmonary hypertension." Handb Exp Pharmacol **218**: 279-313.
Stasch, J. P., P. Pacher, et al. (2011). "Soluble Guanylate Cyclase as an Emerging Therapeutic Target in Cardiopulmonary Disease." Circulation **123**(20): 2263-2273.
Steiner, M. K., O. L. Syrkina, et al. (2009). "Interleukin-6 overexpression induces pulmonary hypertension." Circ Res **104**(2): 236-244, 228p following 244.
Stenmark, K. R., N. Davie, et al. (2006). "Role of the adventitia in pulmonary vascular remodeling." Physiology (Bethesda) **21**: 134-145.
Stenmark, K. R., K. A. Fagan, et al. (2006). "Hypoxia-induced pulmonary vascular remodeling - Cellular and molecular mechanisms." Circ Res **99**(7): 675-691.
Stenmark, K. R., K. A. Fagan, et al. (2006). "Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms." Circ Res **99**(7): 675-691.
Stenmark, K. R. and R. P. Mecham (1997). "Cellular and molecular mechanisms of pulmonary vascular remodeling." Annu Rev Physiol **59**: 89-144.
Stenmark, K. R., B. Meyrick, et al. (2009). "Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure." American Journal of Physiology-Lung Cellular and Molecular Physiology **297**(6): L1013-L1032.
Steudel, W., F. Ichinose, et al. (1997). "Pulmonary vasoconstriction and hypertension in mice with targeted disruption of the endothelial nitric oxide synthase (NOS 3) gene." Circ Res **81**(1): 34-41.
Storz, G., J. A. Opdyke, et al. (2004). "Controlling mRNA stability and translation with small, noncoding RNAs." Current Opinion in Microbiology **7**(2): 140-144.
Stuhlinger, M. C., E. Conci, et al. (2007). "Asymmetric dimethyl L-arginine (ADMA) is a critical regulator of myocardial reperfusion injury." Cardiovasc Res **75**(2): 417-425.
Stuhlinger, M. C., E. Conci, et al. (2007). "Asymmetric Dimethyl L-Arginine (ADMA) is a critical regulator of myocardial reperfusion injury." Cardiovasc Res **75**(2): 417-425.
Syed, B., Hidalgo,Romero (2014). "microRNA-21 expression analysis in mice " The FASEB Journal **vol. 28**(no. 1): Supplement 711.716.
Sylvester, J. T., L. A. Shimoda, et al. (2012). "Hypoxic pulmonary vasoconstriction." Physiol Rev **92**(1): 367-520.
Takahashi, H., G. M. Strutton, et al. (1991). "Determination of Proliferating Fractions in Malignant Melanomas by Anti-Pcna Cyclin Monoclonal-Antibody." Histopathology **18**(3): 221-227.
Tamargo, J., J. Duarte, et al. (2010). "Cinaciguat, a soluble guanylate cyclase activator for the potential treatment of acute heart failure." Current Opinion in Investigational Drugs **11**(9): 1039-1047.
Tamosiuniene, R., W. Tian, et al. (2011). "Regulatory T Cells Limit Lung Vascular Endothelial Injury And Prevent The Development Of Pulmonary Hypertension." Am J Respir Crit Care Med **183**.
Tan, B., D. J. Jiang, et al. (2007). "Taurine protects against low-density lipoprotein-induced endothelial dysfunction by the DDAH/ADMA pathway." Vascul Pharmacol **46**(5): 338-345.
Tanaka, M., K. Sydow, et al. (2005). "Dimethylarginine dimethylaminohydrolase overexpression suppresses graft coronary artery disease." Circulation **112**(11): 1549-1556.
Tanaka, N., Y. Katayama, et al. (2007). "Effects of long-term administration of HMG-CoA reductase inhibitor, atorvastatin, on stroke events and local cerebral blood flow in stroke-prone spontaneously hypertensive rats." Brain Research **1169**: 125-132.

Taraseviciene-Stewart, L., Y. Kasahara, et al. (2001). "Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension." FASEB J **15**(2): 427-438.

Taraseviciene-Stewart, L., Y. Kasahara, et al. (2001). "Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension." Faseb Journal **15**(2): 427-438.

Teerlink, T. (2005). "ADMA metabolism and clearance." Vascular Medicine **10**: S73-S81.

Teerlink, T., R. J. Nijveldt, et al. (2002). "Determination of arginine, asymmetric dimethylarginine, and symmetric dimethylarginine in human plasma and other biological samples by high-performance liquid chromatography." Analytical Biochemistry **303**(2): 131-137.

Teichert-Kuliszewska, K., M. J. Kutryk, et al. (2006). "Bone morphogenetic protein receptor-2 signaling promotes pulmonary arterial endothelial cell survival: implications for loss-of-function mutations in the pathogenesis of pulmonary hypertension." Circ Res **98**(2): 209-217.

Thum, T., P. Galuppo, et al. (2007). "MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure." Circulation **116**(3): 258-267.

Thum, T., C. Gross, et al. (2008). "MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts." Nature **456**(7224): 980-984.

Thyberg, J., K. Blomgren, et al. (1995). "Phenotypic Modulation of Smooth-Muscle Cells during the Formation of Neointimal Thickenings in the Rat Carotid-Artery after Balloon Injury - an Electron-Microscopic and Stereological Study." Cell Tissue Res **281**(3): 421-433.

Tokuo, H., S. Yunoue, et al. (2001). "Phosphorylation of neurofibromin by cAMP-dependent protein kinase is regulated via a cellular association of N-G,N-G-dimethylarginine dimethylaminohydrolase." FEBS Lett **494**(1-2): 48-53.

Torondel, B., M. Nandi, et al. (2010). "Adenoviral-mediated overexpression of DDAH improves vascular tone regulation." Vasc Med **15**(3): 205-213.

Tran, C. T., M. F. Fox, et al. (2000). "Chromosomal localization, gene structure, and expression pattern of DDAH1: comparison with DDAH2 and implications for evolutionary origins." Genomics **68**(1): 101-105.

Tsai, E. J. and D. A. Kass (2009). "Cyclic GMP signaling in cardiovascular pathophysiology and therapeutics." Pharmacol Ther **122**(3): 216-238.

Tsang, H., J. Leiper, et al. (2013). "Role of asymmetric methylarginine and connexin 43 in the regulation of pulmonary endothelial function." Pulm Circ **3**(3): 675-691.

Tsihlis, N. D., C. S. Oustwani, et al. (2011). "Nitric Oxide Inhibits Vascular Smooth Muscle Cell Proliferation and Neointimal Hyperplasia by Increasing the Ubiquitination and Degradation of UbcH10." Cell Biochemistry and Biophysics **60**(1-2): 89-97.

Tuder, R. M., S. H. Abman, et al. (2009). "Development and pathology of pulmonary hypertension." J Am Coll Cardiol **54**(1 Suppl): S3-9.

Tuder, R. M., M. Chacon, et al. (2001). "Expression of angiogenesis-related molecules in plexiform lesions in severe pulmonary hypertension: evidence for a process of disordered angiogenesis." J Pathol **195**(3): 367-374.

Tuder, R. M., M. Chacon, et al. (2001). "Expression of angiogenesis-related molecules in plexiform lesions in severe pulmonary hypertension: evidence for a process of disordered angiogenesis." Journal of Pathology **195**(3): 367-374.

Tuder, R. M., B. Groves, et al. (1994). "Exuberant Endothelial-Cell Growth and Elements of Inflammation Are Present in Plexiform Lesions of Pulmonary-Hypertension." American Journal of Pathology **144**(2): 275-285.

Tuder, R. M. and N. F. Voelkel (1998). "Pulmonary hypertension and inflammation." Journal of Laboratory and Clinical Medicine **132**(1): 16-24.

Ueda, S., S. Kato, et al. (2003). "Regulation of cytokine-induced nitric oxide synthesis by asymmetric dimethylarginine - Role of dimethylarginine dimethylaminohydrolase." Circ Res **92**(2): 226-233.

Ueda, S., S. Yamagishi, et al. (2009). "Involvement of asymmetric dimethylarginine (ADMA) in glomerular capillary loss and sclerosis in a rat model of chronic kidney disease (CKD)." Life Sci **84**(23-24): 853-856.

Unemori, E. N., N. Ferrara, et al. (1992). "Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells." J Cell Physiol **153**(3): 557-562.

Valkonen, V. P., J. Laakso, et al. (2003). "Asymmetrical dimethylarginine (ADMA) and risk of acute coronary events - Does statin treatment influence plasma ADMA levels?" Atherosclerosis Supplements **4**(4): 19-22.

Valkonen, V. P., H. Paiva, et al. (2001). "Risk of acute coronary events and serum concentration of asymmetrical dimethylarginine." Lancet **358**(9299): 2127-2128.

van Wetering, S., J. D. van Buul, et al. (2002). "Reactive oxygen species mediate Rac-induced loss of cell-cell adhesion in primary human endothelial cells." J Cell Sci **115**(9): 1837-1846.

Vergadi, E., M. S. Chang, et al. (2011). "Early Macrophage Recruitment and Alternative Activation Are Critical for the Later Development of Hypoxia-Induced Pulmonary Hypertension." Circulation **123**(18): 1986-U1299.

Vermeersch, P., E. Buys, et al. (2007). "Soluble guanylate cyclase-alpha1 deficiency selectively inhibits the pulmonary vasodilator response to nitric oxide and increases the pulmonary vascular remodeling response to chronic hypoxia." Circulation **116**(8): 936-943.

Vermeersch, P., E. Buys, et al. (2007). "Soluble guanylate cyclase-alpha 1 deficiency selectively inhibits the pulmonary vasodilator response to nitric oxide and increases the pulmonary vascular remodeling response to chronic hypoxia." Circulation **116**(8): 936-943.

Villamor, E., T. D. LeCras, et al. (1997). "Chronic intrauterine pulmonary hypertension impairs endothelial nitric oxide synthase in the ovine fetus." American Journal of Physiology-Lung Cellular and Molecular Physiology **272**(5): L1013-L1020.

Wada, K., K. Inoue, et al. (2002). "Identification of methylated proteins by protein arginine N-methyltransferase 1, PRMT1, with a new expression cloning strategy." Biochim Biophys Acta **1591**(1-3): 1-10.

Wadham, C. and A. A. Mangoni (2009). "Dimethylarginine dimethylaminohydrolase regulation: a novel therapeutic target in cardiovascular disease." Expert Opinion on Drug Metabolism & Toxicology **5**(3): 303-319.

Wakino, S., K. Hayashi, et al. (2005). "Pioglitazone lowers systemic asymmetric dimethylarginine by inducing dimethylarginine dimethylaminohydrolase in rats." Hypertension Research **28**(3): 255-262.

Wang, G. R., Y. Zhu, et al. (1998). "Mechanism of platelet inhibition by nitric oxide: In vivo phosphorylation of thromboxane receptor by cyclic GMP-dependent protein kinase." Proc Natl Acad Sci U S A **95**(9): 4888-4893.

Wang, S., C. P. Hu, et al. (2009). "All-Trans Retinoic Acid Inhibits Cobalt Chloride-Induced Apoptosis in PC12 Cells: Role of the Dimethylarginine Dimethylaminohydrolase/Asymmetric Dimethylarginine Pathway." Journal of Neuroscience Research **87**(8): 1938-1946.

Wang, Z., B. Caplin, et al. (2013). "The Hemodynamic Effects of Deletion of DDAH2 in Conscious Mice." Circulation **128**(22).

Waxman, A. B., L. Lawler, et al. (2008). "Cicletanine for the treatment of pulmonary arterial hypertension." Arch Intern Med **168**(19): 2164-2166.

Weissmann, N., S. Hackemack, et al. (2009). "The soluble guanylate cyclase activator HMR1766 reverses hypoxia-induced experimental pulmonary hypertension in mice." Am J Physiol Lung Cell Mol Physiol **297**(4): L658-665.

Welsh, D. J., M. Harnett, et al. (2004). "Proliferation and signaling in fibroblasts: role of 5-hydroxytryptamine2A receptor and transporter." Am J Respir Crit Care Med **170**(3): 252-259.

West, J., J. Harral, et al. (2008). "Mice expressing BMPR2R899X transgene in smooth muscle develop pulmonary vascular lesions." Am J Physiol Lung Cell Mol Physiol **295**(5): L744-755.
Wharton, J., J. W. Strange, et al. (2005). "Antiproliferative effects of phosphodiesterase type 5 inhibition in human pulmonary artery cells." Am J Respir Crit Care Med **172**(1): 105-113.
Wilcox, C. S. (2012). "Asymmetric dimethylarginine and reactive oxygen species: unwelcome twin visitors to the cardiovascular and kidney disease tables." Hypertension **59**(2): 375-381.
Wilson, D. W., H. J. Segall, et al. (1989). "Progressive inflammatory and structural changes in the pulmonary vasculature of monocrotaline-treated rats." Microvasc Res **38**(1): 57-80.
Wilson, G. M. and G. Brewer (1999). "The search for trans-acting factors controlling messenger RNA decay." Progress in Nucleic Acid Research and Molecular Biology, Vol 62 **62**: 257-291.
Wilusz, C. J., M. Wormington, et al. (2001). "The cap-to-tail guide to mRNA turnover." Nat Rev Mol Cell Biol **2**(4): 237-246.
Winter, J., S. Jung, et al. (2009). "Many roads to maturity: microRNA biogenesis pathways and their regulation." Nat Cell Biol **11**(3): 228-234.
Wojciak-Stothard, B. (2008). "New drug targets for pulmonary hypertension: Rho GTPases in pulmonary vascular remodelling." Postgrad Med J **84**(993): 348-353.
Wojciak-Stothard, B. (2012). "Role of Rho GTPases in the regulation of pulmonary endothelial barrier function and angiogenesis." Vascul Pharmacol **56**(5-6): 326-327.
Wojciak-Stothard, B., V. B. Abdul-Salam, et al. (2014). "Aberrant Chloride Intracellular Channel 4 Expression Contributes to Endothelial Dysfunction in Pulmonary Arterial Hypertension." Circulation **129**(17): 1770-1780.
Wojciak-Stothard, B. and A. J. Ridley (2002). "Rho GTPases and the regulation of endothelial permeability." Vascul Pharmacol **39**(4-5): 187-199.
Wojciak-Stothard, B., B. Torondel, et al. (2007). "The ADMA/DDAH pathway is a critical regulator of endothelial cell motility." J Cell Sci **120**(Pt 6): 929-942.
Wojciak-Stothard, B., B. Torondel, et al. (2009). "Modulation of Rac1 activity by ADMA/DDAH regulates pulmonary endothelial barrier function." Mol Biol Cell **20**(1): 33-42.
Wojciak-Stothard, B., L. Y. Tsang, et al. (2005). "Rac and Rho play opposing roles in the regulation of hypoxia/reoxygenation-induced permeability changes in pulmonary artery endothelial cells." Am J Physiol Lung Cell Mol Physiol **288**(4): L749-760.
Wojciak-Stothard, B. and M. R. Wilkins (2012). "Role of RhoB in the Regulation of Pulmonary Endothelial and Smooth Muscle Cell Responses to Hypoxia." Circ Res **110**(11): 1423-1434.
Xu, L., D. Kong, et al. (2007). "Suppression of IP3-mediated calcium release and apoptosis by Bcl-2 involves the participation of protein phosphatase 1." Mol Cell Biochem **295**(1-2): 153-165.
Yamaguchi, Y., S. Matsuno, et al. (2002). "Fluvastatin reduces modification of low-density lipoprotein in hyperlipidemic rabbit loaded with oxidative stress." Eur J Pharmacol **436**(1-2): 97-105.
Yamakuchi, M. (2012). "MicroRNAs in Vascular Biology." Int J Vasc Med **2012**: 794898.
Yang, S., S. Banerjee, et al. (2012). "miR-21 regulates chronic hypoxia-induced pulmonary vascular remodeling." Am J Physiol Lung Cell Mol Physiol **302**(6): L521-529.
Yang, S. Z., S. Banerjee, et al. (2012). "miR-21 regulates chronic hypoxia-induced pulmonary vascular remodeling." American Journal of Physiology-Lung Cellular and Molecular Physiology **302**(6): L521-L529.
Yang, T. L., M. F. Chen, et al. (2005). "Fenofibrate decreases asymmetric dimethylarginine level in cultured endothelial cells by inhibiting NF-kappa B activity." Naunyn-Schmiedebergs Archives of Pharmacology **371**(5): 401-407.
Yeager, M. E., G. R. Halley, et al. (2001). "Microsatellite instability of endothelial cell growth and apoptosis genes within plexiform lesions in primary pulmonary hypertension." Circ Res **88**(1): E2-E11.
Yi, E. S., H. Kim, et al. (2000). "Distribution of obstructive intimal lesions and their cellular phenotypes in chronic pulmonary hypertension - A morphometric and immunohistochemical study." Am J Respir Crit Care Med **162**(4): 1577-1586.

Yi, E. S., H. Kim, et al. (2000). "Distribution of obstructive intimal lesions and their cellular phenotypes in chronic pulmonary hypertension. A morphometric and immunohistochemical study." Am J Respir Crit Care Med **162**(4 Pt 1): 1577-1586.

Yildirim, A. O., P. Bulau, et al. (2006). "Increased protein arginine methylation in chronic hypoxia: role of protein arginine methyltransferases." Am J Respir Cell Mol Biol **35**(4): 436-443.

Yin, Q. F. and Y. Xiong (2005). "Pravastatin restores DDAH activity and endothelium-dependent relaxation of rat aorta after exposure to glycated protein." J Cardiovasc Pharmacol **45**(6): 525-532.

Young, J. M., C. H. Strey, et al. (2008). "Effect of atorvastatin on plasma levels of asymmetric dimethylarginine in patients with non-ischaemic heart failure." Eur J Heart Fail **10**(5): 463-466.

Young, R. A. (1940). "The Pulmonary Circulation-Before and After Harvey: Part II." Br Med J **1**(4123): 41-44.

Yu, A. Y., L. A. Shimoda, et al. (1999). "Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1alpha." J Clin Invest **103**(5): 691-696.

Yusuf, S., K. K. Teo, et al. (2008). "Telmisartan, ramipril, or both in patients at high risk for vascular events." New England Journal of Medicine **358**(15): 1547-1559.

Zalewski, A., Y. Shi, et al. (2002). "Diverse origin of intimal cells - Smooth muscle cells, myofibroblasts, fibroblasts, and beyond?" Circ Res **91**(8): 652-655.

Zhang, P., X. Hu, et al. (2011). "Dimethylarginine dimethylaminohydrolase 1 modulates endothelial cell growth through nitric oxide and Akt." Arterioscler Thromb Vasc Biol **31**(4): 890-897.

Zhang, W. G., D. Y. Li, et al. (2004). "Role of AIF in human coronary artery endothelial cell apoptosis." American Journal of Physiology-Heart and Circulatory Physiology **286**(1): H354-H358.

Zhang, X. M., W. L. Ng, et al. (2012). "MicroRNA-21 Modulates the Levels of Reactive Oxygen Species by Targeting SOD3 and TNF alpha." Cancer Res **72**(18): 4707-4713.

Zhao, L., C. N. Chen, et al. (2012). "Histone deacetylation inhibition in pulmonary hypertension: therapeutic potential of valproic acid and suberoylanilide hydroxamic acid." Circulation **126**(4): 455-467.

Zhao, L., N. A. Mason, et al. (2001). "Sildenafil inhibits hypoxia-induced pulmonary hypertension." Circulation **104**(4): 424-428.

Zhao, Y. D., A. I. M. Campbell, et al. (2003). "Protective role of angiopoietin-1 in experimental pulmonary hypertension." Circ Res **92**(9): 984-991.

Zhao, Y. D., D. W. Courtman, et al. (2005). "Rescue of monocrotaline-induced pulmonary arterial hypertension using bone marrow-derived endothelial-like progenitor cells: efficacy of combined cell and eNOS gene therapy in established disease." Circ Res **96**(4): 442-450.

Ziche, M., L. Morbidelli, et al. (1997). "Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis." Journal of Clinical Investigation **99**(11): 2625-2634.