CHARACTERIZATION AND ROLE OF KRÜPPEL-LIKE FACTOR 2 IN MODELS OF PULMONARY HYPERTENSION

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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Pulmonary arterial hypertension (PAH) results from endothelial cell (EC) damage leading to pulmonary vasoconstriction and arteriolar remodeling. Patients with PAH exhibit high pulmonary arterial pressures due to increased pulmonary vascular resistance and die of progressive right-sided heart failure. The pathogenesis of PAH is not completely understood, but involves processes which reflect abnormalities in EC function: an imbalance of vasodilators and constrictors, thrombosis, vascular smooth muscle cell (SMC) hypertrophy and proliferation, and susceptibility to EC apoptosis. Therefore, it is important to investigate possible alterations in the underlying mechanisms that regulate EC structure and function. Krüppel-like factor 2 (KLF2) is a shear-responsive transcription factor, highly expressed in the pulmonary ECs under physiological conditions, and known to maintain EC homeostasis by acting as a master switch for a quiescent profile of EC gene transcription. We hypothesized that Klf2 expression is reduced in models of pulmonary
hypertension (PH) and its down-regulation contributes to PH development; conversely, \textit{Klf2} overexpression is beneficial, and may represent a novel therapeutic target. The role of KLF2 in PH was characterized in two experimental rat models: the monocrotaline model of severe and lethal PAH, and the chronic hypoxia model of reversible hypoxic PH. In vivo \textit{Klf2} expression was manipulated using jetPEI® to enhance or reduce the activity of the KLF2 pathway. Plasmids containing \textit{short hairpin Klf2 (shKLF2)} or \textit{Klf2}, or empty plasmids were selectively delivered to the pulmonary microvasculature, and the effect on pulmonary hemodynamics, microvascular structure and function, along with various \textit{in vitro} functional and molecular assays of EC activity, were assessed. Results suggest that reduced \textit{Klf2} expression may be a critical early event in EC activation and initiation of PAH; and, its persistent downregulation may play a role in the transition to a progressive and irreversible process. Data also suggests that an early therapeutic intervention to overexpress \textit{Klf2}, can prevent the development of PH in both models tested when applied before the “irreversible” microvascular remodeling is present. However, once the full PAH phenotype is established, in particular in the presence advanced arteriolar remodeling, \textit{Klf2} gene transfer was unsuccessful in reversing the disease in the MCT model.
ACKNOWLEDGEMENTS

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<tbody>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>Ad</td>
<td>adenovirus</td>
</tr>
<tr>
<td>Alk1</td>
<td>activin receptor-like kinase 1</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>Angpt</td>
<td>angiopoietin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APAH</td>
<td>Associated Pulmonary Arterial Hypertension</td>
</tr>
<tr>
<td>ASS</td>
<td>argininosuccinate synthase</td>
</tr>
<tr>
<td>BMPR2</td>
<td>bone morphogenetic protein receptor type II</td>
</tr>
<tr>
<td>Br</td>
<td>bronchiale</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CCBs</td>
<td>calcium channel blockers</td>
</tr>
<tr>
<td>CH</td>
<td>chronic hypoxia</td>
</tr>
<tr>
<td>CycA</td>
<td>cyclophilin A</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>EBM</td>
<td>endothelial basal media</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGM</td>
<td>endothelial growth media</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin -1</td>
</tr>
<tr>
<td>ETA</td>
<td>endothelin receptor A</td>
</tr>
<tr>
<td>ETB</td>
<td>endothelin receptor B</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>F344</td>
<td>Fischer 344</td>
</tr>
<tr>
<td>FHR</td>
<td>Fawn-Hooded rats</td>
</tr>
<tr>
<td>FMA</td>
<td>fluorescent micro-angiography</td>
</tr>
<tr>
<td>FPAH</td>
<td>Familial Pulmonary Arterial Hypertension</td>
</tr>
</tbody>
</table>
GAPDH  glyceraldehyde 3-phosphate dehydrogenase
GFP    green fluorescent protein
GMP    guanosine monophosphate
GTP-ase guanosine triphosphate-ase
H₂O₂   hydrogen peroxide
HIF    hypoxia inducible factor
HIV    Human Immunodeficiency Virus
HO-1   heme oxygenase -1
HPAH   Hereditary Pulmonary Arterial Hypertension
HUVECs human umbilical vein endothelial cells
IPAH   Idiopathic Pulmonary Arterial Hypertension
jetPEI® commercial polyethylenimine made by Polyplus, Strasbourg
KLF2   krüppel-like factor -2  (LKlf, lung krüppel-like factor)
LPS    lipopolysaccharide (endotoxin)
MCT    monocrotaline
MEF2A  myocyte-specific enhancer factor 2a
MMV    Moloney Murine Leukemia Virus
NO     nitric oxide
PA     pulmonary arteries
PAC    pro-angiogenic cell
PAH    Pulmonary Arterial Hypertension
PAI-1  plasminogen activator inhibitor -1
PAP    pulmonary arterial pressure
PASMC  pulmonary artery smooth muscle cell
PBS    phosphate-buffered saline
pCMV-X plasmid containing gene “X” under the control of cytomegalovirus enhancer/promotor
PCR    polymerase chain reaction
PE     phycoerythrin
PECAM-1 platelet endothelial cell adhesion molecule-1 (CD31)
PEI    polyethylenimine

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PFA  paraformaldehyde
PGI2  prostaglandin
PI  propidium iodide
PI3K  phosphatidylinositol 3'-kinase
pRS-X  plasmid containing gene “X” under the control of Rous Sarcoma virus promotor
PVR  pulmonary vascular resistance
RBC  red blood cell
RhoA  Ras homolog gene family member A
RLMVECs  rat lung microvascular endothelial cell
RTK  receptor tyrosine kinase
RV  right ventricle
RV/(LV+S)  weight ratio of right ventricle to left ventricle plus septum
RVSP  right ventricular systolic pressure
SEM  standard error of the mean
sh  short hairpin
SMC  smooth muscle cell
SNP  sodium nitroprusside
TF  tissue factor
Tie2  tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2 (TEK, tunica interna endothelial cell kinase)
TNF-α  tumor necrosis factor-α
VCAM-1  vascular cell adhesion molecule - 1 (CD106)
VEGF  vascular endothelial growth factor
VEGFR2  vascular endothelial growth factor receptor - 2
vWF  von Willebrand factor
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original abstracts, which are referred to in the text by their Roman numerals.


INTRODUCTION

The airways of the lung are in continual contact with the external environment; and the nearby pulmonary vasculature is nearly directly exposed to a wide variety of potential noxious agents that can produce lung microvascular endothelial damage. Endothelial injury and activation can set into motion a complex chain of events which leads to pulmonary arteriolar remodeling and ultimately to the obliteration and functional loss of a large proportion of the lung microcirculation [1]. The lung circulation is unique in the body since it needs to accommodate the entire cardiac output to oxygenate the blood and provide sustenance for the rest of the body. As such the lung is connected in series with the rest of the circulation and is the only vascular bed that must accept all the blood flow throughout the body, regardless of the level of physical activity. Remarkably, the lung vascular bed accomplishes this task at arterial pressures that are little more than venous (10-15 mmHg). This is because the normal lung represents the most vascular organ of the body, exhibiting an extensive microvascular cross sectional area, only a small proportion of which is necessary at rest. Therefore, as the cardiac output increases with exercise, the lung is able to recruit additional microvascular units, and thereby can maintain a very low arterial resistance (or impedance), even at peak exercise. However, in Pulmonary Arterial Hypertension (PAH) the ability of the lung to accommodate the cardiac output is compromised by a progressive loss of functional microcirculation, until ultimately, pulmonary vascular resistance increases so that, even at rest, the pulmonary arterial pressure rises [2], at which time a diagnosis of PAH is made [3, 4]. As increasingly more of the lung microcirculation becomes involved, eventually this exceeds the ability of the right ventricle to pump blood against an ever increasing resis-
tance, and right-sided heart failure ensues ultimately leading to limitation in cardiac output [5].

Pulmonary hypertension (PH) has been classified by the World Health Organization (WHO) based on the differing pathophysiologic mechanisms (Table 1.1). Group 1 categorizes PH that results from an abnormality of the arterial circulation, predominantly at the level of the precapillary bed, and thus is titled Pulmonary Arterial Hypertension. Other groups, 2 through 5, respectively include: Pulmonary Venous Hypertension, which contains one of the most common forms of PH, congestive heart failure; PH associated with respiratory diseases and hypoxemia; PH due to chronic thrombotic and/or embolic disease; and PH due to multi-factorial disorders such as Lymphangiomatosis. This study focused specifically on PAH and conducted experiments in two independent and well established rat models: chronic hypoxia (CH)-induced PH and monocrotaline (MCT)-induced PAH.

There are three pre-dominant subclassifications of PAH that are defined by the retro-perspective manifestation the disease [4]. One form, heritable PAH, was recognized in the mid-1980’s, and in 2000 was linked to mutations in the bone morphogenetic protein receptor type II (Bmpr2) gene and, more rarely, in the activin receptor-like kinase 1 (Alk1) gene [6-8]. However, only 20% of those genetically at risk, with Bmpr2 mutations, develop PAH [9, 10]. Two other forms of PAH are: Idiopathic PAH (IPAH), which develops from no known cause, and Associated PAH (APAH), which develops secondary to an existing ailment such as a connective tissue disease, congenital systemic-to-pulmonary shunts, portal hypertension and HIV infection [4].

The human lung is a complex organ and is host to a community of over forty different types of cells [11]. Each cell contains on average thirty thousand genes and additional non-coding gene regulating sequences which integrate and communicate together in health
and/or disease [11]. Research in the field PAH has produced a flood of sometimes conflicting information on a wide variety of factors and processes which contribute to the onset and progression of PAH. Therefore, it is perhaps remarkable that there is near universal agreement about the importance of pulmonary endothelial damage and dysfunction in this disease [12]. There is an emerging concept that is supported by an increasing body of evidence implicating endothelial apoptosis, resulting from environmental injury and/or genetic predisposition, as the central trigger of PAH regardless of its specific etiology. To elaborate, the pulmonary arterioles are in a strategically important location in determining the perfusion of the distal alveolar capillary units, and the pulmonary vascular resistance (PVR). Despite their importance, the pulmonary arteriole is perhaps a uniquely vulnerable vascular structure since it consists of little more than ‘naked’ endothelial tubes with little or no support from mural cells and minimal extracellular matrix. Thus, the pulmonary arteriole may be prone to degeneration in the event of endothelial injury and loss of endothelial continuity. For this reason, the health of the pulmonary vasculature may be particularly dependent on survival signaling at the level of the pre-capillary arteriole to protect against environmental insults which can occur as a part of everyday life. There is knowledge in the literature to support this. For example, the MCT model of PAH is known to involve early and sustained EC apoptosis in the small arterioles, and previous work in our laboratory demonstrated that pan-caspase inhibitor Z-Asp\(^{-}\)2,6-dichlorobenzoyloxymethylketone (Z-Asp) significantly blunted the increase in right ventricular systolic pressure (RVSP) [13-15]. Other studies have shown that gene transfer of survival factors vascular endothelial growth factor (VEgf) and angiopoietin-1 (Angpt-1), in MCT-induced PAH prevented endothelial apoptosis of small pulmonary arterioles and inhibited the development of pulmonary hypertension [15-17]. In a hypoxic PH model, treatment with SU5416, a semi selective inhibitor of VEGF
signaling was shown to trigger EC apoptosis which potentiated PH; and even induced pro-
iferative intimal lesions which appeared to contribute to the occlusion of intra-acinar arte-
rioles. Additionally, in models of hereditary PAH, the Bmpr2 pathway may play a critical 
role in preventing EC apoptosis and thus maintaining the integrity of the lung microvascular 
[18].

A more complete understanding of the fundamental mechanisms underlying the ini-
tiation and progression of PAH is critical to the development of better and more effective ther-
apies for this disease. Current treatments are based on pathophysiological concepts 
stemming from research in the 1980s and ’90s, that focused on endothelial dysfunction and 
an imbalance in endothelial vasodilator and vasoconstrictor factors. It was then newly 
known that the infusion of vasodilator prostaglandin E1 (PGE1) to pulmonary hypertensive 
patients resulted in a reduction of pulmonary arterial pressures and right ventricular end 
diastolic pressure [19]. Correspondingly, an imbalance between vasoconstrictor thrombox-
ane (TXA2) and vasodilator PGI2 was later reported in IPAH patients and elsewhere, it was 
reported that PAH patients had reduced prostacyclin synthesize expression [20, 21]. Similarly, 
other studies showed endothelial nitric oxide synthase (eNOS), a factor involved in generat-
ging vasodilator nitric oxide (NO), was reduced in the vascular endothelium of pulmonary 
arteries in PAH patients [22]; although this has been confuted by others [23]. Furthermore 
studies have shown that PAH patients have an increased expression of vasoconstriction fac-
tors. The arterio–venous ratio of plasma endothelin was shown to be elevated in patients 
with IPAH [24], and similarly, endothelin-1 (ET-1) was shown to be highly expressed in the 
endothelium of pulmonary arteries of PAH patients, positively correlating with vascular re-
sistance [25]. Moreover, there is increased plasma levels of vasoconstrictor serotonin in 
IPAH patients [26]. Results from experimental models of PAH have also supported that
the imbalance between vasoconstrictors and vasodilators exists in PAH likely contributing to endothelial dysfunction [27-30].

Unfortunately, the vast majority of patients with a recent diagnosis of PAH do not show a significant response to vasodilator testing during their diagnostic hemodynamic study, and only (6-12%) exhibit an acute improvement to inhaled NO or intravenous prostaglandin [31]. These patients, termed ‘responders’, represent a small subset that have a particularly good prognosis and can often be fully controlled by therapy with calcium channel blockers. However, for the rest, pulmonary vasoconstriction likely plays little role, and therapies for these patients need to target the mechanisms underlying pulmonary vascular remodeling [32, 33]. The concepts of endothelial dysfunction and disproportionate production of vasoactive factors such as NO, prostacyclin, ET-1, serotonin, and thromboxane lead to the introduction of drugs for PAH treatment: prostaglandins (PGs), endothelin receptor antagonists (ERAs), and phosphodiesterase type V inhibitors (PDEV inhibitors). The hope was that not only would these medications restore the balance between these vasoactive active mediators, but, since most of these factors affect the growth of the smooth muscle cells, that they would reverse pulmonary vascular hypertrophy and structural remodeling [12].

Although current PAH therapies have resulted in substantial symptomatic and functional benefits, many patients have suboptimal responses and many of those that do have an initial favourable response will show progression of their underlying disease despite continued treatment with one or multiple PAH medications. More-so, modern PAH therapies may appear to have improved the overall prognosis for patients with PAH, in particular the continuous infusion of prostacyclin derivatives [34, 35]. Yet the mean survival following the initial diagnosis is between 3-5 years, which is relatively grave considering that this disease often affects children and young adults. The only other option is lung transplantation,
which itself carries significant morbidity and mortality, and due limitations of donor organs, only 20% of PAH patients that require lung transplantation ultimately receive these before they succumb to the disease.

At the centre of the pulmonary vascular disease battlefield is the endothelium. Under physiological conditions, vascular endothelial cells are the source of many of vasodilator and anti-thrombotic factors, and regulate the traffic of plasma proteins and immune cells into the vessel wall. However, in vascular disease the endothelium becomes activated and actively contributes to vasoconstriction, vascular inflammation and remodeling. It then stands to reason that therapeutic strategies may be found by studying the “master switches” that control endothelial transcriptional regulation, particular those that promote a healthy non-activated endothelium which we define as a vasodilatory, anti-inflammatory, anti-thrombotic, and an overall quiescent phenotype.

Transcription factors turn on or off many different genes by promoting or inhibiting the initiation of gene transcription. Krüppel-like factor 2 (KLF2) is an endothelial-selective, shear-responsive transcription factor that is highly expressed in the pulmonary ECs under physiological conditions and thought to maintain endothelial cell homeostasis [36]. Thus, we hypothesized that reduced KLF2 expression would play a central role in experimental models of PAH, and that KLF2 overexpression would be beneficial, preventing and possibly reversing the functional and structural vascular changes characteristic of this disease; conversely, KLF2 down-regulation would induce pulmonary vascular endothelial dysfunction thus contributing to abnormal hemodynamics and the development of PAH. This study was undertaken to characterize and investigate the role of Krüppel-like factor 2, an endothelial transcriptional regulator, in experimental models of PAH.
Results from this research show that CH is associated with an early yet transient reduction in \( Klf2 \) mRNA expression (VI); whereas MCT-induced PAH was characterized by marked and persistent reductions in expression of this endothelial transcription factor (V). Interestingly, the pattern of down-regulation of \( Klf2 \) expression reflected the severity and persistence of disease in the two models; sustained down-regulation was seen in the progressive and irreversible MCT model, whereas there was a delayed recovery of \( Klf2 \) expression in the CH model that may represent an adaptive mechanism in this reversible model PH. To modulate \( Klf2 \) expression in vivo, we established a novel methodology using a commercial available linear polyethylenimine, jetPEI®, to deliver plasmids containing small hairpin (sh) or plasmid DNA selective to the lung microvasculature, to knockdown or overexpress \( Klf2 \) in the resident endothelium of the pulmonary microcirculation. \( Klf2 \) overexpression reduced RVSP and right ventricular hypertrophy in both experimental models of PH (I-III). Lung-targeted knockdown of \( Klf2 \) in healthy rats resulted in increased RVSP (I). In the MCT model, benefits of \( Klf2 \) overexpression were coupled with reduced arteriolar remodeling. Ex vivo functional experiments showed that \( Klf2 \) knockdown reduced endothelial-dependent vasodilation; and, further in vitro mechanistic experiments confirmed that \( Klf2 \) overexpression potently induced eNOS expression and reduced basal endothelial migration (IV). However, increasing \( Klf2 \) expression by gene transfer later in the course of the MCT model, after PAH and vascular remodeling was fully developed, was ineffective in reversing the structural and hemodynamic changes of the disease.

We conclude that down-regulation in lung \( Klf2 \) expression may be a critical event that facilitates EC activation, and may be involved in the initiation of PAH, and its transition to a progress and irreversible disease process. My data also suggests that an early therapeutic intervention to restore \( Klf2 \) expression can prevent the development of PAH.
However, once the full PAH phenotype is established, in particular with the presence of advanced arteriolar remodeling, it is not possible to reverse the disease using Klf2 gene-based transfer. Future studies trying to reverse established PAH, may require strategies that can promote the activation of the vascular repair mechanisms and induce regeneration or restoration of functional lung microcirculation. However, manipulation of Klf2 expression may be a valuable adjunctive therapy to maintain pulmonary endothelial homeostasis; thereby preventing the re-initiation of the disease and/or to prevent its development in the first place in individuals at high risk based on genetic or environmental factors. Finally, based on this work one could speculate that levels of Klf2 expression could provide a useful indicator of the health of the endothelium, and could potentially be used to better identify at risk populations, for example in carriers of Bmpr2 mutations among relatives of affected individuals.

Before describing the details of my study, I will first introduce some specific aspects of PAH, review the pulmonary circulation and various experimental models of PH, provide relevant background on Krüppel-like factor 2, and discuss pertinent issues related to gene therapy targeted to the lung vasculature.
RELEVANT BACKGROUND

1 CLINICAL PULMONARY HYPERTENSION (PH)

Pulmonary hypertension is a disease that develops as a result of widespread pulmonary arteriolar narrowing and occlusion. It is characterized by increased PVR and is clinically defined by a pulmonary arterial pressure (PAP) of more than 25 mmHg at rest or greater than 30 mmHg during exercise [37]. This hemodynamic definition was adopted in the mid-1980’s [38] and remains the standard. Although not as useful in the diagnosis of PH, there are a number of biomarkers which are strongly associated with PH such as: overproduction of ET-1 [25], increased incidence of pulmonary thrombosis [39, 40], high levels of inflammatory cytokines such as interleukin-1 (IL-1) and IL-6 [41-43], as well as structural changes to the pulmonary vasculature [33, 44]. Since patients only present with rather non-specific symptoms, such as dyspnea, fatigue, and dizziness, most are only diagnosed at the later stages of their disease. This has limited our ability to gain insights in the early events that trigger the onset of this devastating disease, and therefore our knowledge about the early development of PH is largely from experimental animal models.

1.1. EPIDEMIOLOGY

Idiopathic PAH is a rare disease and has an incidence of one to two cases per million in industrialized countries [45]. Statistics from the National Institute of Health (NIH) registry, 1987, show that the mean age of patients with IPAH is 36.4 years (Table 1.2) and although IPAH can occur in men and women of all ages and ethnicities, it develops more frequently in women than men by a ratio of 1.7 to 1 [46-48]. The severe form of PAH, IPAH, is uncommon, however, PAH which occurs secondary to a preexisting condition such as
HIV infection, congenital heart disease, anorexigen use, and lung diseases is presented by patients regularly in the cardiology clinic [4, 49].

1.1.1. PREVALENCE

The prevalence of severe pulmonary hypertension, including primary disease or PH associated with underlying conditions like connective tissue disease, congenital heart disease, chronic pulmonary thromboembolism, HIV infection, use of an appetite suppressant, or liver disease is reported to be 30 to 50 cases per million [47, 50]. This may be a conservative estimate of PH prevalence, since results from autopsy studies report that the prevalence is 1,300 cases per million [51]. Specifically, in cases of associated PH, such as patients with underlying sickle cell disease, the prevalence of PH is 20 to 40 percent [52]; and in patients with systemic sclerosis, the prevalence of PH is 16% [53].

1.2. PULMONARY ARTERIAL HYPERTENSION

In the last couple of decades, the classification of PH has been revised by an ongoing consensus process under the auspices of the World Health Organization (WHO). The nomenclature has been transformed from one which was based largely of pathological appearance, to one which is focused more of pathophysiologic processes and, is thereby more relevant for informing therapeutic decisions. This WHO classification (see Table 1.1) segregates PH into various groups, with Group 1 representing PH that results from an abnormality of the arterial circulation, predominantly at the level of the pre-capillary bed, and thus is termed Pulmonary Arterial Hypertension. Other groups include: Pulmonary Venous Hypertension (i.e. Group 2), which includes one of the most common forms of PH, congestive heart failure; PH due to lung diseases and hypoxemia (Group 3); PH due to chronic pulmonary thromboembolic disease (Group 4); and PH associated with a miscellaneous
conditions such as Lymphangiomatosis, Sarcoidosis, and Histiocytosis X. The idiopathic form of PAH (i.e. IPAH), previously called primary PH, is rare, with unknown etiology and mean life expectancy of 2.8 years after diagnosis [54]. However, PAH that develops secondary to other diseases such as collagen vascular (autoimmune) diseases, congenital heart disease with left to right shunts, or AIDS, is called Associated PAH (APAH), and is presented routinely to physicians by patients who suffer shortness of breath (Table 1.1). The familial form of PAH, hereditable PAH (FPAH), was recognized as showing an autosomal dominant pattern of inheritance with incomplete penetrance in first-order blood relatives of patients with PAH [46, 55]. In 2000, the gene causing hereditable PAH was discovered by two groups who independently described mutations in the bone morphogenetic protein receptor-II (Bmpr2) gene [6, 7]. Subsequently more than 200 separate mutations in the Bmpr2 gene have been reported [56], and mutations have been described in up to 80% of patients with familial and ~40% of patients with sporadic PAH in whom there is no evidence of a family history [57, 58]. Patients harbouring Bmpr2 mutations exhibit incomplete penetrance and PAH develops in as few as 20% of those at risk [10, 55]. More recently, PAH has been reported in families carrying mutations in the Activin-Receptor-Like Kinase-1 (Alk-1) gene, which is another member of the transforming growth factor β (TGF-β) receptor superfamily [59, 60]. Alk-1, along with endoglin, are better known as providing the genetic basis for another vascular disease, hereditary hemorrhagic telangiectasia (HHT), which is characterized by vascular malformation and arterial-venous fistulas through the pulmonary and systemic vascular beds [61, 62]. Mutations in the Bmpr2 gene were shown to result in increased SMC proliferation and dysregulated growth [63], whereas these same mutations in ECs may lead to endothelial loss [18]. Stewart and colleagues suggest that EC apoptosis is the initiating event in PAH and evidence from studies by Tudor et al. reported that plexi-
form lesions are formed following a sequence of events that begins with EC apoptosis, leading to intimal hyperplasia \[13, 64\]. Therefore, Bmpr2 loss in both SMCs and ECs, together in the pulmonary vasculature, could result in PAH development by facilitating apoptosis in ECs, initiating disease pathogenesis and plexiform formation, as well as inducing SMC proliferation which may contribute to medial hypertrophy and arteriolar narrowing.

1.3. PATHOGENESIS AND BASIS FOR CURRENT PAH THERAPY

No matter what the initial cause of PAH at the time when most patients present with symptomatic PAH, it is thought that they nearly universally exhibit pulmonary remodeling: medial hypertrophy in small pulmonary arteries and arterioles \[65, 66\]. The characteristic pathological features of PAH are largely confined to pulmonary arterioles, and include intimal fibrosis and intimal thickening \[67\]. The pulmonary vascular lumen can also reduced by intra-vascular thrombosis. The hallmark vascular pathology of PAH is the plexiform lesion \[65\]; although they are not necessarily present in all patients with this disease \[65, 68, 69\]. Plexiform lesions are complex structures composed of endothelial cells, often lining multiple small vascular channels intertwined with smooth muscle cells, myofibroblasts, and extracellular matrix \[66\]. They are commonly found at the site of branching of a small pulmonary arteriole from a higher order vessel, and often located distal to a region of arteriolar occlusion \[66, 70, 71\], and therefore, it has been suggested that these are the result of a dysfunctional vascular repair process in PAH \[72\]. Seminal work from the Voelkel and Tuder labs have suggested that these lesions arise by a dysregulated growth of ECs that exhibit an almost neoplastic phenotype with resistance to apoptosis and in some cases even “clonal” expansion \[73\]. These investigators have gone to show that plexiform-like lesions
can be produced experimentally in the rat by early and widespread endothelial apoptosis, induced by blockade of the VEGF type 2 receptor using SU5416 together with exposure to chronic hypoxia, which is followed by the emergence of hyperproliferative and apoptosis-resistant cells [64]. This model produces irreversible PAH that progresses well after returning the animal to normoxic conditions and is now becoming a new standard as an experimental model of PAH. A recent report shows that the severe PAH produced in the SU5416/hypoxia model persists for as long as 14 weeks, but that the plexiform lesions only occur after 8 weeks, long after the appearance of marked elevations in PAP [2]. Therefore, these authors concluded that these lesions were unlikely to be causing PAH, but rather were markers of the severe and persistent pulmonary hemodynamic abnormalities. Nonetheless, it is clear from these and other studies that damage to the endothelium and subsequent EC apoptosis is a fundamental trigger that underlies the development of PAH, and as in the MCT-induced PAH, the severe PAH phenotype in the model can be abrogated by inhibition of caspases, the final common pathway leading to apoptosis [13].

Results from early studies in rodent models of PH, as well as observations from patients with PAH, implicated endothelial dysfunction whereby there is increased vasoconstriction, abnormal SMC proliferation, and thrombosis [12, 39, 40, 44, 74]. Together this activity in the pulmonary vasculature was thought to contribute to medial hypertrophy, intimal thickening, fibrosis, adventitial remodeling and in situ thrombosis which results physiologically in increased pulmonary vascular resistance. Long standing increases in PVR and PAP results in enlargement on the major branches of the pulmonary artery, that can be appreciated on chest X-ray or more sophisticated imaging approaches such as CT and MRI. The right ventricle (RV) hypertrophies to compensate for the increased afterload, and its ability to maintain cardiac output in the face of increasing mechanical demands is a major
determinant of both the functional status and prognosis in patients with severe PAH. However, eventually the RV begins to fail, despite (or because of) sometimes massive hypertrophy, and as systolic function declines the ventricular chamber dilates with all the clinical consequences of rising venous pressures (peripheral edema, ascites) and reduced cardiac output (hypotension, syncope), and ultimately death.

Observations in patients with PAH in the early 1990’s have fueled much of the current PH research and targeted pharmaceutical drug development that lead to the introduction of the current standard pharmacological therapies for PAH. In 1991, Dr. Duncan Stewart was the principle investigator of a study which was the first to demonstrate high levels of ET-1 in the pulmonary circulation of patients with PAH, and subsequently his group was able to confirm increased expression of ET-1 in the lungs of patients with PH [24], localized mainly to the endothelium of diseased arteries and arterioles, especially in association with intimal and plexiform lesions [74]. As well, reduced eNOS expression was reported in lungs of PH patients soon afterwards [22], although this finding was subsequently challenged by others [23]. Specifically, decreased eNOS was observed in pulmonary arteries (PAs) of all sizes, with the greatest loss of eNOS characterized in the small PAs; which coincidently are the vessels involved in PH [22]. This observation has led to the procurement of knowledge that PH is associated with a defect in endothelial nitric oxide production which, among other dysfunctional consequences, results in impaired nitric oxide dependent vasodilation [75]. By 1999, prostacyclin synthase deficiency was shown in patients with severe IPAH where prostacyclin synthase expression, like eNOS, is reduced in medium and small PAs [21]. Moreover, measurements of altered ET-1, eNOS, and prostacyclin synthase expression in PH has inspired the availability of a great portion of the existing
drug target options: endothelin receptor antagonists, NO donors, and prostaglandins. Thus, knowledge of the pathogenesis of PH was key to treatment development.

Another understanding of the pathogenesis of PH, although not the focus of this study, is the involvement of serotonin and how anorexigens have caused many cases of PH [26]. Anorexigens, are appetite suppressants which act to increase serotonin levels (5-HT) by causing serotonin release from platelets and inhibiting its re-uptake; further inducing 5-HT receptor expression. Serotonin is a known mediator of vasoconstriction and smooth muscle cell proliferation and these actions contribute to the pathogenesis of PH. In patients with PH, the plasma 5-HT levels are known to be high and remain elevated following a heart-lung transplant; implicating 5-HT as primary in the disease process [26]. Additional evidence for the involvement of serotonin in PH, is the fact that 5-HT receptors are highly expressed in lung pulmonary arterial SMCs in patients with PH and are even increased on platelets in patients with IPAH [26, 76].

Other more recent findings in PH pathogenesis of particular interest for the context of KLF2’s postulated role in PH are: increased MCP-1, loss of caveolin in severe PH lesions, decreased HO-1, and reduced Tie2 signaling [77-80]. The relevance of these specific recent findings will be part of the discussion later in the thesis since KLF2 can modulate MCP-1, caveolin-1, HO-1, and Tie2 expression and/or function [81-84].
**Illustration 1.1** Summary of Pulmonary Arterial Hypertension

(Adapted from Benisty, J.I. Circulation 2002) [85]

*Normal Heart-Lung:*
PAP in humans at sea level is 12-16 mmHg.

*Pulmonary Arterial Hypertension:*
PAP is greater than 25 mmHg at rest and more than 30 mmHg with exercise.
Table 1.1 Clinical Classification of Pulmonary Arterial Hypertension from the Third World Symposium on Pulmonary Arterial Hypertension, Venice, 2003 [4].

<table>
<thead>
<tr>
<th>Group I.</th>
<th>Pulmonary arterial hypertension (PAH)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>• Idiopathic (IPA)</td>
</tr>
<tr>
<td></td>
<td>• Familial (FPAH)</td>
</tr>
<tr>
<td></td>
<td>• Associated with (APA):</td>
</tr>
<tr>
<td></td>
<td>◦ Connective tissue disease</td>
</tr>
<tr>
<td></td>
<td>◦ Congenital systemic-to-pulmonary shunts</td>
</tr>
<tr>
<td></td>
<td>◦ Portal hypertension</td>
</tr>
<tr>
<td></td>
<td>◦ HIV infection</td>
</tr>
<tr>
<td></td>
<td>◦ Drugs and toxins</td>
</tr>
<tr>
<td></td>
<td>◦ Other (thyroid disorders, glycogen storage disease, Gaucher's disease, hereditary haemorrhagic telangiectasia, haemoglobinopathies, myeloproliferative disorders, splenectomy)</td>
</tr>
<tr>
<td></td>
<td>• Associated with significant venous or capillary involvement</td>
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<tr>
<td></td>
<td>◦ Pulmonary veno-occlusive disease (PVOD)</td>
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<tr>
<td></td>
<td>◦ Pulmonary capillary haemangiomatosis (PCH)</td>
</tr>
<tr>
<td></td>
<td>• Persistent pulmonary hypertension of the newborn (PPHN)</td>
</tr>
</tbody>
</table>

| Group II. | Pulmonary hypertension associated with left heart diseases |

| Group III. | Pulmonary hypertension associated with respiratory diseases and/or hypoxemia (including chronic obstructive pulmonary disease) |

| Group IV. | Pulmonary hypertension due to chronic thrombotic and/or embolic disease |

<table>
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<tr>
<th>Group V.</th>
<th>Miscellaneous group</th>
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<tbody>
<tr>
<td></td>
<td>• Eg. sarcoidosis, histiocytosis X and lymphangiomatosis</td>
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</tbody>
</table>
Table 1.2 Distribution of Patients with Idiopathic Pulmonary Arterial Hypertension, According to Symptom Onset, in the NIH Database (1987) [38]
1.4. CURRENT TREATMENTS

In the 1950’s Dr. Paul Wood, a cardiologist, first identified vasoconstriction in PH which spurred early work on the vaso-reactivity of the peripheral pulmonary vasculature [86]. However, with little knowledge of endothelial biology and decades before the discovery of endothelial derived relaxing factor (EDFR), research efforts shifted focus to the vaso-occlusive pathology in PH. One thought was that anticoagulation therapy is important in PAH where there is increased intravascular thrombosis [87]. This concept is based largely on postmortem evidence of thrombosis in situ in patients with PAH [68] and from a study, primarily focused on calcium-channel blockers, published in 1992 in the New England Journal of Medicine that lacked placebo controls; using instead retrospective patient data and post hoc analyzing of warfarin as a concomitant therapy [88]. Although controversial in its effectiveness, Warfarin, a vitamin K antagonist, is used in long-term anticoagulation treatment in patients since its use was associated with reduce mortality by inhibiting the incidence of in situ intravascular thrombosis [89].

As earlier discussed in the introduction, current treatments are based on pathophysiological concepts stemming from research twenty to thirty years ago, which then focused on endothelial dysfunction and an imbalance in endothelial vasodilator and vasoconstrictor factors. At that time, initial reports of disproportional levels of vasoconstrictor TXA2 and vasodilator PGI2 in IPAH patients, as well as evidence elsewhere that PAH patients had reduced prostacyclin synthase expression [20, 21], preceded the discovery that the infusion of vasodilator PGE1 to pulmonary hypertensive patients reduced pulmonary arterial pressures and right ventricular end diastolic pressure [19]. Other studies in IPAH and/or PAH patients showed concurring results, for example: reduced eNOS [22] (although this was confuted by others [23]), and increased expression of vasoconstriction factor ET-1.
which correlated with increased vascular resistance [24, 25]. In addition, results from experimental models of PH supported that the imbalance between vasoconstrictors and vasodilators exists in PAH likely contributing to endothelial dysfunction [27-30]. Therefore, four classes of drugs were developed to reestablish a balance of vasodilators and vasoconstrictors in the pulmonary vasculature: prostaglandins (prostacyclin analogues), phosphodiesterase-5 (PDEV) inhibitors, calcium channel blockers (CCBs), and endothelin antagonists.

Prostaglandins are a class of drugs that work by increasing cyclic AMP which causes vasodilation and are known to prevent smooth muscle cell proliferation, as well as reduce thrombi formation by inhibiting platelet activation. Patients with PAH have low levels of prostacyclin which, in theory, contributes to pulmonary vasoconstriction, smooth muscle cell proliferation, and platelet activation associated pulmonary thrombi formation. Synthetic forms of prostacyclin are used in PAH patients as an attempt to correct this prostacyclin deficiency.

Nitric oxide (NO) was originally described as the endothelium-derived relaxing factor. As its namesake suggests, NO is biosynthesized in the vascular endothelium. NO is produced from the conversion of the amino acid L-Arginine, together with oxygen and NADPH, into L-Citrulline by nitric oxide synthase (NOS) enzymes: eNOS, iNOS, and nNOS. Once NO is produced, NO signals the proximal smooth muscle cells to relax by first stimulating the soluble guanylate cyclase, a heterodimeric enzyme, which in turn forms cyclic GMP. Cyclic GMP then activates protein kinase G which phosphorylates the myosin light-chain (MLC) phosphatase, inactivating the MLC kinase, which dephosphorylates the MLC leading to SMC relaxation. The result is vasodilation and increased blood flow. Populations living at high altitudes are known to have higher NO levels which prevents hypoxia
by increasing pulmonary dilation. Additionally, NO is able to contribute to homeostasis by inhibiting vascular SMC growth, platelet aggregation, and leukocyte-endothelial cell adhesion. Phosphodiesterase-5 inhibitors are a class of drugs that inhibit the PDEV enzyme which would otherwise function to hydolyze cGMP. Thus, by inhibiting PDE5, cGMP is able to accumulate and the result is increased NO-dependent pulmonary vasodilation. The phosphodiesterase-5 enzyme is found in the pulmonary arterial smooth muscle cells of the lung, making PDEV inhibition an attractive therapy since it is a pulmonary selective vasodilator and it is also know to increase the contractility of the RV and thus may be important for long-term cardiac function. However, the long-term effect of PDEV inhibitors on PAH patients is unknown.

Calcium channel blockers are a class of drugs that cause vasodilation in both the systemic and pulmonary circulations. These drugs work by blocking voltage-gated calcium channels in blood vessels which causes decreased intracellular calcium levels and results in a reduction of vascular SMC contraction. Only 6.8% of IPAH patients respond to CCB therapy [31]. Still, to see benefits in the pulmonary circulation often high doses of CCBs are required, for example: Nifedipine up to 300 mg per day or Diltiazem up to 720 mg per day. These drugs are also dangerous for those PH patients with overt RV failure and/or systemic hypotension because of the associated negative inotropic effects.

Endothelin antagonists are another class of drugs commonly used in the treatment of PH; a disease known to involve an overproduction of ET-1 [24]. Endothelin-1 stimulates the ET\textsubscript{A} receptor on smooth muscle cells (SMCs) to cause potent vasoconstriction and SMC proliferation. The other ET-1 receptor, ET\textsubscript{B}, is on both SMCs and ECs. Localized to SMCs ET\textsubscript{B} stimulation induces vasoconstriction, however when ET-1 acts on the endothelial ET\textsubscript{B} receptor nitric oxide is released, inducing vasodilation.
There are a small subset of PAH patients (6-12%) that respond to vasodilator testing, exhibiting acute improvement to inhaled NO or intravenous prostaglandin. These patients have a particularly good prognosis and can often be fully controlled by therapy with calcium channel blockers (Table 1.3). However, for the rest in this disease that often affects children and young adults (Table 1.2), pulmonary vasoconstriction likely plays little role and therapies for these patients need to target the mechanisms underlying pulmonary vascular remodeling [32, 33].

The only other modern option is lung transplantation, which itself carries significant morbidity and mortality, and due to limitations of donor organs, only 20% of PAH patients that require lung transplantation ultimately receive these before they succumb to the disease.
### Table 1.3 Pulmonary Arterial Hypertension Treatment Algorithm
(Adapted from Badesch, D.B. Chest 2007) [329, 330]

<table>
<thead>
<tr>
<th>Symptomatic PAH</th>
<th>General treatment measures: oral anticoagulants, diuretic, oxygen</th>
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<tbody>
<tr>
<td></td>
<td>Acute vasoreactivity testing</td>
</tr>
<tr>
<td></td>
<td><strong>Oral CCB</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Sustained response?</strong></td>
</tr>
<tr>
<td><strong>Yes</strong></td>
<td><strong>Continued CCB</strong></td>
</tr>
<tr>
<td><strong>No</strong></td>
<td><strong>PDEV inhibitors, Prostacyclin analogues</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Functional Class II:</strong> Slight limitation of physical activity. Comfortable at rest. Ordinary physical activity causes undue dyspnea or fatigue, chest pain, or near syncope.</td>
</tr>
<tr>
<td></td>
<td><strong>Functional Class III:</strong> Marked limitation of physical activity. Comfortable at rest. Less than ordinary activity causes undue dyspnea or fatigue, chest pain, or near syncope.</td>
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<tr>
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<td><strong>Functional Class IV:</strong> Inability to carry out any physical activity without symptoms. Signs of right-heart failure. Dyspnea and/or fatigue may even be present at rest. Discomfort is increased by any physical activity.</td>
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<tr>
<td></td>
<td><strong>Combination therapy:</strong></td>
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<td>PDEIV inhibitor</td>
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<td>Prostacyclin analogue</td>
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<tr>
<td></td>
<td>Endothelin receptor antagonist</td>
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<tr>
<td></td>
<td><strong>Endothelin receptor antagonists, PDEIV inhibitors, Prostacyclin analogues</strong></td>
</tr>
<tr>
<td></td>
<td><strong>No improvement or deterioration</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Atrioseptostomy and/or lung transplantation</strong></td>
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23
Lung transplantation is only considered as a last possible course of action for patients that have end-stage lung disease where all other available treatments have failed. Severe IPAH patients are considered for transplant as long as there is no concurrent chronic illness such as: congestive heart failure, liver disease, or kidney disease. There are three options for lung transplants: single lung transplant, bilateral lung transplant, or heart-lung transplant. Besides the challenge of there being very few lung transplant donors, there are numerous other obstacles that hinder graft and patient survival. For example, primary graft dysfunction may occur as a result of the surgically induced ischemia-reperfusion injury, which causes increased permeability and pulmonary edema, and may result in acute respiratory distress syndrome [90, 91]. Later, or delayed, graft dysfunction may occur as a result of anastomotic complications such as bronchial anastomosis narrowing [92]. Also, despite immunosuppressive therapy, there is an acute transplant rejection rate of 55% to 75% within the 1st year caused by alloreactive T-lymphocytes [93, 94]. Within the first 2 years, two-thirds of transplant recipients develop chronic rejection where a progressive and irreversible fibro-proliferative process obliterates the pulmonary airways (bronchiolitis obliterans) [95]. Bronchiolitis obliterans is associated with 40% mortality rate within two years of onset [95].

Transplant recipients may also develop serious lung infections. Five percent of transplant recipients are expected to acquire the most threatening infection from invasive aspergillus which is associated with a 60% mortality rate [96, 97]. Even for lung transplant recipients that are considered success stories, chronic use of immuno-suppressants may lead to other medical problems which include: osteoporosis, hypertension, hypercholesterolemia, renal insufficiency, gastropareses and reflux disease [98].

New understandings of the pathogenesis of PAH have elucidated the importance of targeting the underlying vascular remodeling for future PAH therapies. Plexiform lesions,
although not always present in PAH, are mixed structures of ECs, SMCs, myofibroblasts, and extracellular matrix [66], found within a small pulmonary arteriole at the branch-point from a higher order vessel [67, 70]. Spatially, plexiform lesions follow sites of arteriolar occlusion [66, 70, 71], which begged researchers to consider that plexiforms developed as a result of a dysfunctional vascular repair process in PAH [72]. Groundbreaking work from the Voelkel and Tuder labs provided evidence that these lesions arise by a dysregulated growth of ECs [73]. The ECs within plexiforms exhibited a neoplastic-like phenotype which resisted apoptosis and had the ability to undergo “clonal” expansion [73]. These investigators have since demonstrated that early and widespread endothelial apoptosis in rats, induced by blockade of the VEGF type 2 receptor using SU5416 together with exposure to chronic hypoxia, induced plexiform-like lesions which contained hyper-proliferative and apoptosis-resistant cells [64]. Since these experimentally induced plexiform lesions occurred long after PAP were first elevated [2], these results elucidated that plexiform lesions do not induce increased PAP but rather are formed following a sequence of events that begins with EC apoptosis, leading to intimal hyperplasia as a consequence of severe and sustained increased PAP. Still these findings support EC damage and apoptosis is a fundamental step in the development of PAH, and as in the MCT-induced PAH, the severe PAH phenotype can be abrogated by inhibition of caspases, the final common pathway leading to apoptosis [13]. Thus, Stewart and colleagues suggest that EC apoptosis is the initiating event in PAH which leads to either the degeneration of pre-capillary arterioles or to the selection of hyper-proliferative, apoptosis-resistant ECs that, in part, form plexiform lesions [13]. In addition, Bmpr2 mutations, known to cause hereditary PAH [6, 7], have been shown to result in EC loss [18], as well as increased SMC proliferation and dysregulated growth [63]. Together, these results suggest that Bmpr2 loss may lead to the development of PAH through
EC apoptosis, initiating disease pathogenesis and plexiform formation, as well as inducing SMC proliferation which may contribute to medial hypertrophy and arteriolar narrowing.

In summary, despite a boom in PAH therapies over the past two decades, there are little benefits to IPAH patient mortality [99-101]. We suggest that the greatest limitation of current therapies is that they target end-stage symptoms of PAH rather than focus on the underlying cause of the disease: endothelial dysfunction, leading to apoptosis and pulmonary arteriolar remodeling. We further propose that a relevant strategy to improve PAH therapy would be to explore factors that may regulate the underlying EC dysfunction.
2 EXPERIMENTAL MODELS OF PH

2.1. MONOCROTALINE (MCT) MODEL OF PAH

The monocrotaline (MCT) model of pulmonary arterial hypertension (PAH) was established in 1961 by Lalich and Merkow after they observed that weanling rats which were fed *Crotalaria spectabilis* developed acute pulmonary hemorrhage and arteritis. The *Crotalaria spectabilis* seed contains the toxic pyrrolizidine alkaloid, monocrotaline, which caused toxic in vivo effects. Soon after their discovery, there were reports in monocrotaline-treated rodents of cor pulmonale, and increased permeability of the pulmonary vasculature [102, 103]. As early as 1967, research groups were using MCT to study pulmonary hypertension in experimental animal models [102, 104-107].

The monocrotaline model of PAH is mostly limited to a rat model of PAH, in which a one-time intraperitoneal or subcutaneous injection of MCT is given, and rats develop established PAH three weeks later. There are some variations on dosing in this model since some researchers give a subcutaneous injection of a higher concentration of MCT than what is given by intraperitoneal injection.

2.1.1. MECHANISM

The MCT model of PAH is considered a pneumotoxicity model. In vivo, MCT is activated to a reactive metabolite by the liver through a dehydration reaction where MCT is converted to monocrotaline pyrrole (MCTP) [108]. This reactive hepatic metabolite, MCTP, then accumulates on red blood cells (RBCs) and is transported to the lung [108]. The initial action of MCTP is pulmonary endothelial cell injury, in the absence of alveolar capillary damage [104, 108]. After injuring the pulmonary endothelium, the MCTP is
quickly excreted and acutely there is increasing pulmonary permeability without EC necrosis [104]. In response to the EC injury, the host inflammatory response is triggered and there is platelet activation; but the endothelial injury is irreversible resulting in MCT-induced EC apoptosis [109]. In the MCT model of PAH there is an abundance of pulmonary perivascular inflammation, megalocytosis of type II alveolar epithelial cells, and enlarged alveolar macrophages accumulate [108]. Unexpectedly, attempts to alter immune response using steroids did not affect MCT toxicity [110, 111]. However platelets were shown to play a role in action of MCT-induced PAH; independent of 5-HT or thromboxane [112].

While the MCT model of PAH reproduces some of the features of the human disease such as medial hypertrophy, extracellular matrix secretion, as well as increased and de novo SMCs within the pulmonary arteries and arterioles [113-116], it fails to reproduce the pulmonary plexiform lesions that may exist in end-stage human PH.

2.1.2. RELEVANCE

The MCT model, although criticized for being a chemical toxicity model, it is one of the main models of PAH, and, until very recently, arguably the best model. This disease model develops following pulmonary endothelial-specific injury as is hypothesized in idiopathic PAH [13, 117]. Rats that develop PAH this way present with pulmonary arterial remodeling (medial hypertrophy and hyperplasia), right ventricle hypertrophy, and vast pulmonary inflammation, all like is seen in end-stage human disease. The severity of the disease model itself is in line with clinical PAH, since MCT-induced PH is also severe and lethal.
The shortcomings of this model are that plexiform lesions are not present, whereas they may be found in human diseased lungs. Also, although MCT is primarily pneumotoxic, it may also cause hepatotoxicity and renal damage [108, 118, 119], which may explain part of the high mortality seen in this model [119].

2.2. CHRONIC HYPOXIA MODEL OF PH

Hypoxia-induced PH can be studied at high altitudes which is done in Colorado, or an hypoxic environment can be artificially created by using hypoxic chambers for animal housing. Typically in the CH model, animals are housed at approximately 10% oxygen acutely (days to weeks) or chronically (three or more weeks). The benefit of the CH model is that it provides a murine model of PH, Group III (Table 1.1), which allows for the use of genetically modified mice.

2.2.1. MECHANISM

In 1946 hypoxia-induced pulmonary vasoconstriction was first observed [120], and is an important mechanism in which the small muscular resistance arteries, primarily, constrict within local poorly ventilated areas of the lung to divert blood flow to oxygen-rich areas to maximize pulmonary gas exchange [121, 122]. Shortly after, the pulmonary artery catheter by Swan and Ganz was developed to measure pulmonary hemodynamics, and, by the 1960's, measurement of increased PAPs in patients, together with the knowledge of hypoxic pulmonary vasoconstriction, led to the hypothesis that pathological global hypoxia was the cause of PH [123, 124]. In the acute phase of CH there is sustained vasoconstriction which precedes the vascular remodeling that dominates during the chronic phase [125, 126]. The result of CH is a model of PH that presents with inward remodeling of the small pulmonary arteries and arterioles from smooth muscle cell hypertrophy and hyperplasia that alter ho-
mogeneously the vascular structure of the lung and obliterates the pulmonary microvasculature [41, 126, 127].

Early investigations into the mechanisms of hypoxic pulmonary vasoconstriction elucidated chief roles for voltage-gate K+ channels (Kv) and hypoxia-inducible factor-1 (HIF-1) during hypoxia. Now known, one sensor controlling hypoxic vasoconstriction is the Kv1.5 channel (Kv voltage gated K+ channels). A hypoxic environment is also known to affect Ca+ channels either directly or indirectly by closing K+ channels that are exclusively found in smooth muscle resistance vessels [128]. The critical regulator of the physiological response to hypoxia, however, is transcription factor HIF-1. HIF-1 regulates hypoxia-induced glycolysis, angiogenesis, and erythropoiesis. All of which processes are quintessential hallmarks of the hypoxic response. Even partial deficiency in HIF-1 will hinder the development of hypoxia-induced hypertension by lessening the development of polycythemia, right ventricular hypertrophy, hypoxia-induced pulmonary artery smooth muscle cell depolarization and increased calcium levels [129].

Other proteins shown to have a strong role in the physiologic response to hypoxia are nitric oxide (NO) and heme-oxygenase-1 (HO-1). Models of CH-induced PH, have demonstrated a role for the potent vasodilator NO in PH development. For example, eNOS overexpressing mice were protected from hypoxia-induced increases in RVSP and vascular remodeling [130]. Additional results from experiments in homozygous eNOS-null mice demonstrated that after weeks of hypoxia, eNOS deficient mice developed more severe pulmonary hypertension than in hypoxic wild type controls [131]. Additional evidence for eNOS having a role in CH-induced PH is that hypoxic eNOS-null mice had a two-fold increase in vessel muscularization compared to hypoxic controls [131]. Since eNOS has been demonstrated to have a robust protective role in PH and KLF2 is known to potently regu-
late eNOS gene expression [132], KLF2-targeted therapy is likely to be beneficial in PH. Other experiments in human pulmonary endothelial cells (HPAECs), showed that heat shock response proteins HO-1 and HO-2 are expressed, acting to generate carbon monoxide (CO) [133]. Similar to NO, CO can also elicit vasodilation. Under conditions of CH, hypoxia exposure increases HO-1 protein expression, which in turn increases CO production through a chemical reaction where HO-1 breaks down heme in the blood into CO and biliverdin (biliverdin is subsequently converted into bilirubin by biliverdin reductase). Increased production of CO is known to not only have vascular dilation effects but also to reduce pulmonary smooth muscle cell proliferation and thus prevent hypoxia-induced pulmonary vascular remodeling [134, 135]. Interesting, KLF2 regulates genes that contribute to a vasodilatory and vascular quiescent phenotype similar to the actions of CO; and both shear stress as well as KLF2 are known elevate HO-1 expression [83]. This adds to the evidence, that will be later discussed, supporting that KLF2 acts in multiple, redundant and at the same concerted pathways to promote healthy vascular quiescence.

2.2.2. RELEVANCE

The CH model is a model of hypoxic PH. Although commonly used to study PH, hypoxic PH is a model of Group III PH, and not IPAH (previously called primary PH) which is clinically the most challenging problem. Also, this model is not progressive and irreversible, as in IPAH, but is reversible over a period of days to weeks after reintroduction of the animals to room air [136, 137]. Yet, unlike the MCT model which only produces PAH in rats, the CH model can be used in murine experiments, enabling the ability investigate the role of different genotypes; in particular the role of BMPR2.
2.3. OTHER MODELS OF PH

In a CH model of PAH, treatment early-on with SU5416 (SUGEN), a semi selective inhibitor of vascular endothelial growth factor signaling was shown to trigger EC apoptosis which potentiated PAH, and even induced proliferative intimal lesions which appeared to contribute to the occlusion of intra-acinar arterioles [2, 64]. This SUGEN-Hypoxia model is rapidly becoming the preferred PAH model; as it highlights some of the mechanistic importance of EC apoptosis and activation in this disease, while producing plexiform lesions that are indistinguishable from those in human PAH (Group 1 PH).
2.4. KNOWLEDGE OF PH FROM BASIC SCIENCE RESEARCH IN ANIMAL MODELS

2.4.1. Vasoconstrictors and Vasodilators

In both the MCT and CH models of PH there is reduced endothelium-dependent relaxation [29, 138-140]. Two of the most potent manipulators of vasomotor tone are ET-1 and eNOS. These vasoactive proteins have well established roles in the progression of PH: ET-1 as a potent vasoconstrictor, known to be overproduced in PH [24]; and eNOS as a potent vasodilator, found reduced in PH [22, 29]. Moreover knowledge of human PAH, and from experimental models, show that the pathobiology of PH involves a predominance of vasoconstrictors and impaired vascular function [22, 24, 29].

2.4.2. Thrombosis

In situ thrombosis is observed in human PH, and, in models of PH, thrombi form in the lungs of animals and are established contributors to disease pathogenesis [141-143]. Events leading to thrombotic obliteration of vascular lumen are known to begin as early as three days following MCT injection. At this early time point, lung fibrinolytic activity is decreased, which directly results in increased thrombi formation in the blood [142, 144]. From models of CH-induced PH, it is understood that the early hypoxic response factor, HIF-1 or HIF-2, transcriptionally induces pro-thrombotic plasminogen activator inhibitor-1 (PAI-1) [145]. PAI-1 not only regulates thrombosis, but also inflammation and angiogenesis, thus having an influence on multiple pathways involved in the pathogenesis of PH. Other experiments inhibiting tissue factor (TF) in the CH model of PH demonstrated a role for the coagulation pathway in the pathogenesis of PH beyond lumen obstruction. TF is known for acting as a lead component in the extrinsic coagulation pathway, ushering the release of thrombin. However, in CH murine experiments, up-regulation of the TF inhibition path-
way resulted in improved hemodynamics and reduced pulmonary vascular remodeling \[146\]. Interestingly, KLF2 is known to inhibit these key coagulation factors, \(PAI-1\) and \(TF\), which already have established roles in PH \[147, 148\]. Also, KLF2 acts to up-regulates thrombomodulin which is expressed on the endothelial cell surface and has powerful anticoagulation effects \[147\].

2.4.3. **Pulmonary Vascular Remodeling**

**Endothelial Cells**

Pulmonary hypertension is believed to initiate from endothelial injury that triggers endothelial dysfunction and a non-quiescent (activated) EC phenotype \[13, 44, 117, 149\]. In IPAH, and in models of PH, there are glaring vascular structural changes that include alterations in cell composition within pulmonary arteries through to small pre-capillary pulmonary arterioles. In patients with severe PAH, the plexiform lesions, made up of hyper-proliferative endothelial cells, functionally obstruct pulmonary arterioles. In the early 2000’s, the expression of angiogenesis-associated markers such as VEGFR2 in these plexiform lesions was the first evidence for the postulated role of disordered angiogenesis in PAH \[150\].

**Smooth Muscle Cells**

Not only is the endothelium impaired in PH, but there is also evidence that smooth muscle cells (SMCs) have reduced responsiveness compared to control rats in both MCT and CH models of PH \[75\]. Histological analysis of pulmonary arterioles in MCT and CH treated rats show increased muscular wall thickness that is specific to the pulmonary circulation \[116, 126\]. Together this supports that the SMC phenotype in PH is proliferative and
less contractile. Since changes to pulmonary artery (PA)-SMCs is a central feature in not only PAH animal models but also human disease [151, 152], recent research has focused on using different mechanisms (such as: elastase inhibitors, epidermal growth factor receptor inhibitors, simvastatin, and dichloroacetate) to induce apoptosis specifically in pulmonary artery (PA)-SMCs as a strategy to reverse established PAH in animal models and results have been successful [153-156]. In animal models of PH, and human PAH, PASMC Kv channels are down-regulated [157-160], resulting in an increase in intracellular K+ in PASMCs [161]. This increased intracellular K+ is known to inhibit caspases and thus may explain, in part, the anti-apoptotic phenotype of intimal SMCs in PH [161]. Others have built on this theory by investigating the remodeled mitochondria in PASMCs in PH as a possible cause for apoptosis-resistant SMCs; since healthy mitochondria have been shown to act as oxygen sensors [162], and can regulate vascular tone by producing activated oxygen species which act on K+ channels and thereby induce apoptosis [163].

2.4.4 INFLAMMATION

There is also evidence that inflammation, and related increased levels of inflammatory cytokines are implicated in the development of pulmonary vascular remodeling in PH [164]. More-so, PH is often associated with autoimmune diseases such systemic sclerosis, suggesting that the immune system has a principal role in PH [165, 166]. In both IPAH and APAH the presence of macrophages and other mononuclear cells predominate the lungs [167, 168], which is in accordance with the recruitment of macrophages and neutrophils that is evident in murine and rat lungs in the CH and MCT models of PH [169, 170]. In addition, T and B lymphocytes as well macrophages are present in plexiform lesions in human PH [167] and have also been found in peripheral blood of patients [171]. There are conflict-
ing results in animal studies that implicate lymphocytes in the pathogenesis of PAH. One study in athymic rats demonstrated a protective role of T-lymphocytes in PAH and pulmonary vascular remodeling [172], whereas another study showed that mice depleted of CD4+ T-cells did not develop severe pulmonary arterial muscularization in response to *A. pergillus fumigatus* antigen, known to induce severe thickening of the walls in PAs [173].

From analysis of human serum, proinflammatory cytokines: interleukin-1 (IL-1) and IL-6, are known to be elevated in PH [43, 171]. IL-1, an established mitogen in human and rat VSMCs, has the potential to contribute to SMC hyperplasia and vascular remodeling [41, 42]. Overexpression of Il-6 in mice was sufficient to cause the development of severe PH with vascular remodeling [174], and, in a murine model of CH-induced PH, IL-6-deficient mice had reduced RVSP, RV hypertrophy, and pulmonary arterial remodeling compared to wild-type controls [175]. Taken together, these inflammatory cells within the lung, are postulated to contribute to the pathogenesis of PAH by releasing cytokines, chemokines, and growth factors which lead to vascular remodeling in PAH by matrix remodeling, collagen deposition, as well as SMC proliferation and migration [167, 176, 177].

### 2.4.5 Genetics

Since the discovery of mutations in the *Bmpr2* gene in families with HPAH in 2000 [6, 7], the PH research field anticipated the results of experiments in BMPR2-deficient mice. The homozygous *Bmpr2* knockout mice died early-on in development because BMPR2 is required for epiblast differentiation and mesoderm induction, however the *Bmpr2* gene has been studied in mature heterozygous knockout mice. These *Bmpr2*+/− mice had no phenotype compared to wild-type mice [178, 179], but showed modest PH in response to hypoxia combined with serotonin infusion as stressors [178]. However, results from these
animal experiments that took care to produce mutations in the Bmpr2 tail domain specifically in smooth muscle cells, not only demonstrated increased RVSP in response to a vascular stressor (hypoxia), but also showed a priori changes in pulmonary vascular muscular organization and function, as well as altered gene expression in proliferation, apoptosis, and developmental pathways [180].

2.4.6. Commonalities and Distinct Differences Between CH and MCT Models of PH

The differences between the MCT and CH models of PH predominantly lie in the manifestation of the disease model itself. The MCT model begins with a host reaction to an initial toxic pulmonary endothelial injury. As a result, there is irreversible endothelial injury, a heterogenous pattern of remodeled vasculature, and a widespread inflammatory response. Whereas in the CH model, sustained hypoxic stimulus gives rise to a homogenous pattern of altered smooth muscle cell hypertrophy with a much lesser degree of inflammation. Plexiform lesions, a hallmark of human PH, are not present in the MCT or CH models in this study. However, as earlier-mentioned, plexiform lesions can be developed in these models by a single administration of SUGEN 5416, a receptor tyrosine kinase (KDR/VEGFR2) inhibitor [2]. Despite the differences between the CH and MCT models of PH, the similar remodeling of the pulmonary vasculature in both disease presentations suggests that in PH there is a stereotypical response to vascular injury [181].
3 PULMONARY CIRCULATION

3.1. STRUCTURE AND FUNCTION

The pulmonary circulation is connected in series with the rest of the circulation and thus accepts the entire cardiac output in its function to oxygenate the blood to provide oxygen to the rest of the body, and to move carbon dioxide out of the body. Additionally, the pulmonary circulation filters anything larger than a circulating cell (5-10 µm) and acts as a blood reservoir [182]. Beginning at the pulmonary artery, mixed venous blood from the right ventricle travels at pulmonary arterial pressures normally a little more than venous pressure (10-15 mmHg), and much lower than systemic arterial blood pressure, through a hierarchy of branching arteries to numerous smaller pre-capillary arterioles, to capillaries, and back through post-capillary venules, to pulmonary veins. The normal lung is the most vascular organ of the body, exhibiting an extensive microvascular cross sectional area, but only requires small proportion of the vasculature at rest. At rest, some pulmonary capillaries are “closed” or “open without blood flow”, however, as the cardiac output increases with exercise, the blood pressure rises and these previously non-perfused capillaries conduct blood which is termed: recruitment (Illustration 3.1) [182]. Also, often occurring together with recruitment, at higher pressures capillaries widen by changing their shape from flattened to more circular, termed distension (Illustration 3.1). Therefore, this ability of the lung to recruit microvascular space allows the pulmonary circulation to maintain a very low arterial resistance (or impedance), even at peak exercise [182]. In response to hypoxia, however, active recruitment occurs whereby blood flow is reduced, through active vasoconstriction, to low oxygenated areas of the lung, termed: hypoxic vasoconstriction.
Walls of pulmonary arteries are composed of a three layered structure: intima, media, and adventitia. The intima is the internal layer of the vessel wall which is made up of a single layer of ECs that rest on a collagen-containing vascular basement membrane and together they are surrounded by the media. The media is composed of concentrically arranged SMCs that rest on a smooth muscle cell basement membrane, and is sandwiched between the internal elastic lamina and the external elastic lamina. In this way, the internal elastic lamina defines the border between the intima and the media, whereas the external elastic lamina separates the medial and the adventitial layers. The adventitia is outermost layer of the vessel wall and is formed by longitudinally arranged collagen fibres and fibroblast cells. All three layers contain endothelial cell matrix (ECM) which is made up of varying amounts of collagen, elastin, laminin, glycosaminoglycan and fibronectin, to maintain vessel elasticity or rigidity [183]. Importantly, the structure of the arteries in the pulmonary circulation is different from other arteries in the body's circulation in that the medial vascular smooth muscle layer is smaller, containing fewer layers and less numbers of SMCs, small pulmonary arteries are partially muscularized; and SMCs are even absent in the pre-capillary pulmonary arterioles (pericytes provide vessel stabilization in place of the smooth muscle layer) [184].

3.2. Mechanisms of Pulmonary Vascular Remodeling

There are physiological consequences to the remodeling of the pulmonary vasculature, however the degree to which the remodeled vasculature is responsible for increased PA pressures in PH remains a matter of debate. Pulmonary vascular resistance increases if there is vasoconstriction, vascular obstruction from thromboembolism and occluded vessels, or as a consequence of obliterative and degenerative changes from capillary bed destruction.
or “dropout” [13]. In PAH two mechanisms of pulmonary vascular remodeling are: 1) muscular changes, and 2) obliteration and degenerative.

3.2.1. MUSCULAR CHANGES

In human PAH there is nearly universal pronounced medial SMC changes, including SMC hypertrophy and hyperplasia in the small pulmonary arteries, and neo-muscularization of the pre-capillary pulmonary arterioles [65, 66]. Proliferation of SMCs in the vascular wall, thickening and peripheral extension of smooth muscle in the media, also occurs in the CH model of PH and MCT model of IPAH [113-116, 126]. Other changes to the pulmonary arterials in PH include: adventitial thickening, increased ECM proteins such as collagen and fibronectin, and intimal proliferation [69, 185, 186]. Together these changes affect the structure of the pulmonary vasculature which can result in changes to pulmonary vascular function such as increased PVR. Neo-muscularization of the small pre-capillary pulmonary arterioles and increases to medial smooth muscle area in muscular arteries are known to result as a hyper-responsiveness to vasoconstrictors and hypoxia and thus increase PVR [187-189]. More obvious, hypertrophy and hyperplasia of the SMCs in small pulmonary arteries and arterioles will also narrow the vascular lumen size [190, 191]. Poiseuille’s law, dictates that even small decreases in arterial luminal diameter will result in marked increases in PVR (Illustration 3.2). Consistent with this, in PH, where the pulmonary vasculature is remodeled, the pulmonary artery pressures are high, even under basal conditions. Furthermore, vasoconstrictors are predicted to cause a larger increase in PVR in vessels with a lower lumen-to-wall ratio compared to the same degree of smooth muscle shortening in a similar vessel with a larger lumen (Illustration 3.3; mathematical model by Folkow). Resistance is also increased
in PH because of vascular remodeling of the extracellular matrix, such as increased collagen. This additional collagen reduces pulmonary vascular distensibility [192].

3.2.2. Obliteration and Degenerative Changes

While intimal fibrosis and concentric intimal thickening can cause obliteration of the vessel lumen [67, 184], degenerative loss of pulmonary vascular units triggered by EC apoptosis in the pulmonary arterioles and proliferative plexiform lesions obliterating the vessel lumen are thought to be pivotal in PH development [2, 64]. Stewart and colleagues reasoned that pulmonary arterioles are in a strategically important location in determining the perfusion of the distal alveolar capillary units and PVR, and yet these arterioles are particularly vulnerable to EC injury and apoptosis [13]. The pulmonary arteriole may be prone to degeneration in the event of endothelial injury and loss of endothelial continuity since its structure essentially consists endothelial tubes with little support from mural cells and ECM. Therefore, loss of ECs in the pre-capillary arteriole would result in the “dropout” of the capillary pulmonary vascular reserve and a devastating loss in cross-sectional pulmonary vascular area, leading to increased PVR (Illustration 3.3). In other words, if the distal pulmonary artery is either obstructed or obliterated then recruitment of capillaries at this location cannot take place. It follows that the lung’s ability to maintain low PVR is further strained in situations of exercise and hypoxic pulmonary vasoconstriction. In support of this theory, in the MCT model of PAH, known to involved early and sustained EC apoptosis in the small arterioles, increases in RVSP were significantly reduced with treatment using pan-caspase inhibitor Z-Asp-2,6-dichlorobenzooyloxymethylketone (Z-Asp) [64]. Other studies have demonstrated that gene transfer of survival factors such as Vegf and Angpt1, in MCT-induced PAH prevented EC apoptosis in small pulmonary arterioles and inhibited the
development of PAH [15-17]. In a hypoxic PH model, treatment with SU5416, a semi selective inhibitor of VEGF signaling trigged EC apoptosis, enhancing PH development, and even inducing proliferative and intimal lesions which appeared to obliterate the arteriole lumen [2, 64]. Furthermore, the Bmpr2 pathway may be involved in preventing EC apoptosis and these degenerative changes to the lung microvascular [18].
**Illustration 3.1**

Pulmonary Vascular Reserve: Recruitment & Distension to maintain low pulmonary vascular resistance (Adapted from James West, Respiratory Physiology -the essentials, 2006) [182]
**Illustration 3.2**

*POISEUILLE’S LAW*

Resistance is particularly sensitive to pulmonary vascular radius assuming blood viscosity is constant.

Pulmonary vascular resistance (PVR) = (Pressure inflow - Pressure outflow) ÷ flow, where flow is cardiac output.

For a particular vascular segment:

\[ \text{PVR (mmHg/L/min)} = \frac{(8 \times l \times n)}{(\pi \times r^4)} \]

(mmHg/L/min) is a Wood unit

Wood unit x 80 converts to dynes/sec/cm^5

l = length of tube

n = viscosity coefficient

r = radius of tube

*OHM’S LAW*

\[ \Delta P = CO \times TPR \]

TPR = total peripheral resistance

CO = cardiac output = stroke volume × heart rate

Resistance is particularly sensitive to pulmonary vascular radius assuming blood viscosity is constant.
Illustration 3.3
Folkow Model: PVR and Vessel Lumen to Wall Ratio [184]
4 TRANSCRIPTION FACTORS REGULATING ENDOTHELIAL GENE EXPRESSION

4.1 KRÜPPEL GENE

DROSOPHILA DEVELOPMENT

The Krüppel gene (Kr) was first discovered in *Drosophila*. “Krüppel” is German for “cripple”. When the Kr gene was knocked-out, the thoracic and anterior abdominal segments did not form properly, resulting in deformities of leg formation or “crippled” flies [193]. Developmental genes in drosophila genetics are commonly found to be highly conserved and exist in mammalian genetics. More than 17 krüppel-like transcription factors (KLFs) are known to exist in mammals (Table 4.1). The KLFs are a subclass of zinc-finger family of transcription factors, all of which are involved in cellular growth, differentiation, and tissue development. For example, KLF1/erythroid-KLF is needed for red blood cell maturation; KLF4/gut-KLF regulates differentiation and maturation of dermal and gastrointestinal epithelial cells [194]. This family of transcription factors is highly conserved in mammalian genetics. Each KLF is made up of three zinc-fingers, composed of 2 histeine and 2 cysteine residues, forming an alpha helices and two beta-pleated sheets that are held together by a zinc atom. Every KLF is a CACCC-box binding protein.
**Table 4.1** Summary of the Krüppel-like factors

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<th><strong>Gene Symbol</strong></th>
<th><strong>Gene Name</strong></th>
<th><strong>Previous Alias</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>KLF1</td>
<td>Krüppel-like factor 1 (erythroid)</td>
<td>EKLF</td>
</tr>
<tr>
<td>KLF2</td>
<td>Krüppel-like factor 2 (lung)</td>
<td>LKLF</td>
</tr>
<tr>
<td>KLF3</td>
<td>Krüppel-like factor 3 (basic)</td>
<td>BKLF</td>
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<td>Krüppel-like factor 4 (gut)</td>
<td>EZF, GKLFL</td>
</tr>
<tr>
<td>KLF5</td>
<td>Krüppel-like factor 5 (intestinal)</td>
<td>BTEB2, IKLF, CKLF</td>
</tr>
<tr>
<td>KLF6</td>
<td>Krüppel-like factor 6</td>
<td>BCD1, ST12, COPEB, CPBP, GBF, Zf9, PAC1</td>
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<tr>
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<td>Krüppel-like factor 7 (ubiquitous)</td>
<td>UKLF</td>
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4.2. ENDOTHELIAL KRÜPPEL-LIKE FACTORS

There are three known KLFs that are found in vascular endothelial cells: KLF2, KLF4, and KLF6.

4.2.1. KLF2

INTRODUCTION

In 1995 the cDNA for *Klf2*, then called Lung Krüppel-like factor, was first isolated and characterized by the Lingrel laboratory at the Department of Molecular Genetics, University of Cincinnati, Ohio [195]. It was shown to be highly expressed in the lung, and was first called Lung Klf (LKLF). In rat, the relative RNA expression in different tissues is: uterus, 1; skeletal muscle, 1.4; heart, 1.7; spleen, 4.4; and lung, 5.2; (by contrast, in mice Klf2 expression in the spleen is very low) [195]. *Lklf* was observed to be expressed at in the embryo, at day 7, during the early primitive streak, or gastrulation stage, expression disappears at day 11, and is turned-on at day 15 through day 17 in the embryo [195]. Three years later the same laboratory reported that KLF2 is essential for murine embryonic development since they observe that between embryonic day 12.5 (E12.5) and E14.5 *Klf2* knock-out mice die from hemorrhage. Visualization of the blood vessels in these developing embryos revealed that the hemorrhage was due to a failure to recruit pericytes during the final stage of blood vessel development: vessel stabilization [196]. Soon afterwards experiments which introduced *Klf2* deficient embryonic stem cells into blastocysts showed that KLF2 played an essential role in the late stages of lung development [197]. Another early sign that *Klf2* had a developmental regulatory role was its temporal pattern of expression in murine embryonic life: expressed at day 7, decreased by day 11, and expressed at day 15 throughout adult life [197]. In humans, *Klf2* was shown to be expressed in ECs at aortic bifurcations, but not
SMCs, with highest expression in areas which were predicted to have the highest levels of shear stress [198]. *Klf2* is also expressed in other cell types, notably some subsets of mononuclear cells. Apart from its role in lung development and EC function, KLF2 is important in T-cell quiescence, monocyte quiescence, erythropoiesis, and adipogenic differentiation [81, 199-206]. Essentially, KLF2 functions to direct cell activity towards a state of maturation and homeostasis.

The segment of the promoter region of *Klf2* between bases -138 and -111 is required for its transcription [207, 208]. Proteins known to bind to this region, and thus early identified KLF2 activation regulators, include: heterogenous nuclear (hn) RNP-U, hnRNP-D, PCAF, and P-300 [209]. While hnRNP-proteins are known for binding to pre-mRNA to aid in shuttling from the nucleus to the cytoplasm, PCAF and P-300 are histone transfe-
rases. In 2005, Kush Parmar fueled further interest in KLF2 in vascular biology when he published that *Klf2* was up-regulated 8 hours following statin treatment, and was both necessary and sufficient to modulate the atheroprotective transcriptional responses attributed to statin therapy in human endothelial cells [210]. Below I will further describe what is presently known about endothelial KLF2 function, implicating it as a master switch controlling healthy vascular homeostasis.

**Flow-mediated induction**

In 2002 it was discovered that endothelial *Klf2* was exquisitely induced by vascular shear stress [211]. Using *in vitro* flow chamber experiments, endothelial *Klf2* expression was shown to be highly up-regulated by unidirectional shear stress (25 dynes/cm²) and even more highly increased by healthy physiological pulsatile flow (12 ± 7 dynes/cm²) [211]. These findings led to considerations that KLF2 may be responsible for vascular health associated in
vessel regions with non-turbulent flow. Further inquiry into the relationship of shear stress and Klf2 expression, looked at the spatial localization of Klf2 expression in human aortas. It was observed that Klf2 was highly expressed in aorta segments experiencing normal healthy shear and there was low expression in areas of turbulent flow such as bifurcations [198]; which are also areas prone to atherosclerosis [212]. Experiments in murine microvascular endothelial cells using a luciferase reporter gene and sequence deletion analysis showed that the region from -157 to -95 base pairs (bp) from the start site of transcription is responsible for Klf2 induction by pulsatile shear stress [213]. This shear stress response region is known to be highly conserved within mouse and human gene homologs [214, 215].

Much about the signaling pathway for shear-induced Klf2 up-regulation has been elucidated in the current published literature. In particular, vascular shear stress is known to induce Klf2 by signaling through the pathway: MEK5, ERK5, and lastly to MEF2a which directly binds to the Klf2 promoter [82, 216, 217]. In a separate study, Klf2 induction by shear stress was shown to signal through a phosphatidylinositol 3-kinase (PI3K) dependent and Akt independent pathway, and that inhibition of PI3K alone is enough to prevent Klf2’s ability to induce eNOS [213]. Other factors shown to be involved in the regulation of the Klf2 promoter are: p300/CBP-associated factor (PCAF), heterogeneous nuclear ribonucleoprotein D (hnRNP D), and nucleolin [209, 218].

**Vascular Tone Regulation**

Studies in endothelial cell cultures that overexpressed and silenced Klf2 in the context of flow revealed that KLF2 was responsible for flow-mediated induction of vascular tone regulating genes. For instance, Klf2 silencing inhibits shear-stress associated regulation of ET-1, adrenomedullin, and eNOS [82, 198]. Additionally, Klf2 induction alone can alter
the expression of angiotensin-converting enzyme, ET-1, adrenomedullin, and eNOS similar to the levels produced by prolonged shear stress [82, 198]. \( Klf2 \) is also known to increase transcriptional expression of C-type natriuretic peptide (CNP), another vasodilatory factor [82, 219]. Thus, in the presence of high levels of \( Klf2 \), multiple genes controlling vasodilation are upregulated, potent vasoconstrictors are downregulated, and the vascular endothelium, in turn, exhibits a strong vasodilatory phenotype. More evidence for KLF2’s role in regulating vascular tone has been elucidated during embryonic development. In the developing embryo, \( Klf2 \) was shown to be highly expressed at areas of highest blood flow velocity (correlated with fluid shear stress) that were also associated with low ET-1 and increased enos expression [220, 221]. Interestingly, statin-mediated induction of protective eNOS was shown to be \( Klf2 \)-dependent [222]. There are at least three ways that KLF2 can regulate eNOS expression or activity: by acting directly on the eNOS promoter [132]; through strong transcriptional activation of argininosuccinate synthase (ASS), since ASS increases NO bioavailability [210, 223]; and by down-regulating caveolin-1, a cell membrane protein that potently acts to reduces eNOS activity, and thereby increase eNOS activity and NO production [82].

**Cytokine-mediated Down-regulation of KLF2**

The pro-inflammatory cytokines, tumour necrosis factor-alpha (TNF-\( \alpha \)) and interleukin-1\( \beta \) (IL-1\( \beta \)), both have been shown to reduce \( Klf2 \) expression [147, 211]. IL-1\( \beta \) and TNF-\( \alpha \) reduce \( Klf2 \) expression by inhibiting the recruitment of its transcriptional co-activator, cyclic AMP response element binding protein (CBP/p300). As well, cytokine inducer p65, which is a protein component of a chief inflammatory signaling factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB), inhibits \( Klf2 \) gene expression [224]. Transcription factor p65 acts together with histone deacetylase 4 (HDAC4) to
inhibit Klf2 transcription by inhibiting the inducing actions of MEF2 factors [224]. Since Klf2 is inhibited by NF-κB, it is tempting to speculate that the prevention MCT-induced PAH by an NF-κB decoy, reported in an earlier study, [225] supports our hypothesis that KLF2 has a protective role in PAH. In other words, downregulation of Klf2 inhibition may be involved in the development of PAH. More so, KLF2 inhibition may be a requirement for PH development.

**ANTI-INFLAMMATORY**

There is evidence in the literature that KLF2 has potent anti-inflammatory actions. For instance, KLF2 is known to inhibit lipopolysaccharide (Endotoxin; LPS)-mediated pro-inflammatory cytokines and chemokines including: CD40L, MCP-1, MIP-1 α, MIP-1β, IL-8, as well as IL-1β and TNF-α [81]. The overexpression of KLF2 is known to reduce the pro-inflammatory gene expression induced by IL-1β and to lessen inflammatory activation of pro-inflammatory NF-κB [81, 132]. There is evidence that IL-1 contributes to PAH development in both experimental models and humans patients, thus inhibition of IL-1 is yet another potential way in which KLF2 may protect against this disease process [43, 226, 227]. Additionally, Angpt-1/Tie-2 signaling is known to be protective in PAH, and both TNF-α and IL-1β inhibit Angpt-1; thus KLF2 mediated inhibition of these cytokines may be another way in which to inhibit PAH [79, 80, 228].

Klf2 also inhibits vascular adhesion molecule-1 (VCAM-1) and E-selectin expression [132]. These adhesion molecules are expressed on the endothelial cell surface in response to pro-inflammatory cytokines and encourage leukocyte rolling and firm adhesion as well as T-cell binding [229, 230]. Consistent with Klf2’s ability to inhibit VCAM-1 and E-selectin expression, *in vitro* flow assays demonstrated that T cell attachment and rolling are attenuated
in HUVECs with forced Klf2 overexpression [132]. Competitive recruitment of CBP/p300 by Klf2, which would otherwise function as a co-activator for NF-KB, represents another anti-inflammatory mechanism [132, 231]. While at the same time, CBP synergizes with KLF2 and together they result greater induction of eNOS than either factor alone [132, 231].

**Anti-Thrombotic**

Through regulation of relevant genes KLF2 has been shown to indirectly inhibit coagulation, platelet aggregation and overall thrombotic function as well as pro-inflammatory thrombin effects. Specifically KLF2 induces thrombomodulin expression and thrombomodulin elicits anticoagulant effects by increasing the rate of thrombin-catalyzed protein C activation [147, 222, 232]. Also KLF2 acts to inhibit the expression of plasminogen-activator inhibitor (PAI-1) and TNF-α mediated induction of tissue factor expression [147]. Thus, KLF2 overexpression increased clotting time and flow rates in an *in vitro* blood flow assay where TNF-α treated HUVECs were exposed to whole human blood in a flow chamber [147]. Conversely clotting time decreased when Klf2 was silenced under both basal and inflammatory conditions [147]. Statins, a class of cholesterol-lowering drugs, are known to provide beneficial effects in part through upregulation of thrombomodulin and interestingly this gene regulatory effect has been shown to be KLF2-dependent [222].

KLF2 is also known to regulate genes that have pivotal roles in pathways that inhibit pro-inflammatory thrombin effects; for example, by KLF2 inhibiting protease-activated receptor-1 (PAR-1) expression, PAR-1 thrombin-mediated NF-KB nuclear translocation and subsequent messaging for a pro-inflammatory environment is prevented [233]. As well, Klf2 overexpression was shown to inhibit thrombin-mediated increases in tissue factor, CD40L,
plasminogen activator inhibitor-1, monocyte chemotactic protein-1 (MCP-1), IL-6, IL-8, as well as matrix metalloproteinases 1, 2, and 9 [233].


In addition to KLF2 turning on a profile of gene expression that defines a vasodilatory, anti-inflammatory, and anti-thrombotic phenotype, KLF2 also supports homeostasis in other ways. Under normal conditions endothelial cells are quiescent, but when exposed to stimulators like vascular endothelial growth factor (VEGF) the endothelial layer loses its normal barrier function and become more permeable and the cells begin to proliferate and migrate. In the context of angiogenesis, this is necessary to establish new blood vessels [234], but in a physiological context, this could lead to loss some of the normal protective role of the endothelium. Targeted overexpression of Klf2 to the dermis inhibited VEGF-induced angiogenesis and edema of the ear in a nude mouse model [235]. This was suggested to result in part through the repression of transcription VEGFR2 (a receptor for VEGF) [235]. Results from other studies suggest that KLF2 may exert anti-migratory actions through transcriptional upregulation of a known potent anti-migratory factor: semaphorin-3F (SEMA-3F) [36]. In line with these findings, cultured primary endothelial cells derived from Klf2 deficient [Klf2 (+/-)] mice had higher levels of thrombin and increased H2O2-induced permeability compared to ECs derived from wild type mice [236]. Altogether the data supports KLF2 having an important role in endothelial barrier integrity.

Even though the results in the literature may on first glance suggest that KLF2 is anti-angiogenic, it is important to keep in mind that KLF2 does have an angiogenic role in the late stages of vessel stabilization in embryonic angiogenesis [196]. In many ways KLF2 functions similarly to the Angpt-1 system, which acts later in angiogenesis during blood ves-
sel stabilization and maturation [237]. Other evidence of KLF2’s angiogenic abilities was demonstrated in an aged, murine hind-limb ischemia model, where lentiviral overexpression of *Klf2* in bone-marrow-derived mononuclear “proangiogenic cells” (PACs) resulted in increased cell numbers and improved neovascularization abilities of the PACs [238].
Illustration 4.1 Summary of KLF2

A Profile for Endothelial Cell Quiescence: Illustration of shear induced expression of KLF2 and KLF2's known regulation of endothelial gene expression downstream.
KLF4 was at first named gut-enriched Krüppel-like factor (GKlf) or epithelial zinc-finger protein (EZF) based on where it was initially found to be expressed [239]. Later it was renamed KLF4 to signify it being the fourth KLF identified. Like other KLFs, KLF4 is involved in cell differentiation; specifically, Klf4 is highly expressed in during differentiation of the epidermal layers [240, 241]. Homozygous Klf4-deficient mice die within 15 hours following birth due to the loss of the skin-barrier function [242, 243]. In the gastrointestinal epithelium alone, absence of Klf4 results in a loss of increased proliferation and altered differentiation of gastric epithelia [240]. This suggests that KLF4 is critical for normal gastric epithelial homeostasis. In the eye, conditional deletion of Klf4 in the surface ectoderm-derived structures, where Klf4 is highly expressed, resulted in a fragile corneal epithelium, stromal edema, and loss of conjunctival goblet cells. In cancer biology, Klf4 was shown to be a cell growth inhibitor. Though KLF4 research has discovered the above-mentioned critical roles KLF4, KLF4 has garnered most of its fame for being important in maintaining the pluripotent state of stem cells [244]. As one of the four Yamanaka factors (Oct3/4, Sox2, Klf4, c-Myc), Klf4 is highly expressed in embryonic stem cells and by overexpressing it together with the other three factors, pluriepotency can be induced in both human and murine somatic cells [244].

Important results from studies investigating KLF4 have shown that KLF4 and KLF2 have numerous overlapping functions. For instance, both Klf2 and Klf4 are induced by laminar flow [211, 245]. Also, like KLF2, it was demonstrated that KLF4 regulates endothelial activation to inflammatory stimuli [246]. Similarly to KLF2 overexpression, KLF4 overexpression induces several anti-inflammatory and anti-thrombotic factors such as eNOS and
TM [247, 248]. In addition, KLF4 acts to decrease TNF-induced VCAM-1 and TF expression [249]. Unlike Klf2, Klf4 is expressed in VSMCs [250].

4.2.3. KLF6

KLF6 is known by several other names: core promoter element binding protein (COPEB), GC-rich sites binding factor (GBF), or Zf9). Klf6 is highly expressed in the liver, and is also expressed in the vascular endothelium. In both the liver and the vasculature KLF6 is known to have a principle role in injury and repair. For example, in the liver, Klf6 is induced upon hepatic stellate cell activation. The hepatic stellate cells are essential to liver injury and repair, and within these cells KLF6 is responsible for trans-activating genes involved in injury response such as: collagen I, TGF-β1, and types I and II TGF-β receptors.

In endothelial cells KLF6 also activates TGF-β1 indirectly by transcriptionally activating urokinase plasminogen activator. Urokinase plasminogen activator has a role in tissue remodeling, tumor metastasis, and apoptosis. At the moment of vascular endothelial injury KLF6 cooperatively binds along with Sp1 to transactivate the endoglin promoter which is known to have a role in vascular remodeling [251]. Giving further support to Klf6 having an important role in vascular remodeling, KLF6 is involved in EC motility, which is an essential step in the vascular repair and remodeling processes. Mechanistically, disruption of the SP2/KLF6 repression complex on the matrix metalloproteinase-9 (MMP9) promoter via SHP (small heterodimeric partner), results in increased MMP9 and EC migration.
5 LUNG-TARGETED GENE TRANSFER

Transgenes are most frequently delivered to lung through the airways because this is a convenient route that can achieve lung-specific transfection. However, generally this results in transfection of epithelial cells and alveolar macrophages which are in direct contact with the airspace [252]. Since ECs are adjacent to the airway epithelium, it is possible to achieve some degree of gene transfer to the resident pulmonary ECs via the airway [253]. But gene transfer to the vascular endothelium through the airways is inefficient and will undoubtedly result in transfection of airway residents (epithelium and macrophages). In contrast, gene transfer by way of the vasculature has the advantage of being the most direct route to pulmonary ECs transfection, however, it suffers from lack a specificity since it may also result in gene transfer to other systemic beds such liver [254, 255].

In general there are two main approaches to gene therapy: viral and non-viral gene transfer. Below I will discuss different methods of lung-targeted gene therapy as a background to provide a rationale for using the commercially available polyethylenimine, jetPEI (Polyplus-transfection™), to transfect the resident pulmonary ECs in our experiments.

5.1. VIRAL

Viral gene therapy is an advantageous option for non-human experiments because of the aggressive infectiousness of viruses themselves and resultant ease of transduction of DNA to target cells. Viruses transfer DNA through specific receptors on the cell surface however, the DNA can also enter the cell through non-selective uptake such as phagocytosis. Such is the case in our experiments which use an adenovirus to overexpress Klf2 in cultured ECs. Yet, in an in vivo context this method can be tricky for two reasons: 1) there is a high likelihood that non-gene-targeted cells (liver vascular bed) will be transfected, and 2)
viral gene therapy may cause a serious host immune response [256-258]. Even so, viral-mediated gene transfer to the lung has been accomplished using either an adenovirus, ACE-targeted adenovirus, or adeno-associated viruses; almost exclusively delivered through the airways. Overall the adenovirus is not an ideal vehicle to mediate gene transfer to the endothelium since the endothelium does not have the CAR receptor on its surface to facilitate transfection and gene transfer. There is however a way to modify the adenovirus in an attempt to increase the likelihood of adenoviral gene transfer by modifying or coating the existing CAR ligand so that it is ACE-targeted [259, 260]. Less specific for the endothelium than the ACE-targeted adenovirus, the adeno-associated virus is another option for targeting gene therapy to the lung. Unlike the adenovirus, the lentivirus can achieve stable gene transfer, however this brings about other safety concerns in the context of translation to human therapy [258].

5.2. NON-VIRAL METHODS OF GENE TRANSFER

There are various non-viral approaches to lung-targeted gene therapy which include the use of small molecule activators, cell-based gene therapy, and non-cellularized biomaterials. To modulate transcription factor Klf2 expression in the resident pulmonary ECs the use of non-cellularized biomaterials were considered.

5.2.1. LIPOSOMES

Liposomes are cationic lipids that form a complex with plasmid DNA and intravenous delivery is known to selectively target the lung vascular endothelium [261]. Compared to viral delivery systems, liposomes are advantageous because repeated administrations can be performed in vivo without adverse consequences due to its low immunogenicity, they are low cost, the absent risk of generating an infectious virus and that the use of lipids in the
clinic is approved by the Food and Drug Association [254]. Although liposomes have been used successfully for lung-targeted gene transfer in both human and animal models, these lipid-DNA complexes have been shown to induce pulmonary inflammation [262]. Other shortcomings of the liposome delivery system are low levels of delivery and gene expression compared to viral delivery methods, however, DNA-liposome complexes have successfully been modified by positively charging liposomes and increasing retention time to improve transfection efficiency in the lung [263-265]. The positively charged liposomes complex with the negatively charged DNA plasmids into stable particles and after tail vein injection the cationic complexes use the filtration capacity of the lungs and the cationic liposome complexes interact though charge with the EC surface proteoglycans [266]. Song et al. demonstrated in an elegant study using sequential delivery of liposomes, then plasmid DNA, that prolonging the exposure time of DNA is an important strategy to achieve a lipoplex-mediated transfection in vivo; and that large liposomes could be best used to sludge through the pulmonary microvasculature targeting gene transfer to the lung endothelium [267].

5.2.2. POLYETHYLENIMINE

Cationic polyethylenimine is commercially available as jetPEI (Polyplus-transfection™), which has been used to achieve efficient gene transfer in vivo at doses that are well tolerated in, for example, rat endothelial cells and a human study of superficial bladder Cancer [268]. In vivo jetPEI, forms complexes with nucleic acids that are 50 nm nanoparticles in size, which are small enough to easily enter the cells by endocytosis [269, 270]. It has also been demonstrated that jetPEI-DNA complexes, when administered by intravenous injection, will selectively target the pulmonary vasculature [269, 270]. Of additional importance, polyethylenimine-mediated gene delivery is believed to produce much
less immunological reactions than gene transfer with cationic lipids and viral vectors [271].

In fact, the immune and toxic response described in response polyethylenimine-DNA complexes have been attributed to the bacterial elements plasmid DNA itself and can be avoided by removing the immune-stimulating CpG motifs [271].
GENERAL HYPOTHESIS

KLF2 represents a homeostatic vascular signaling pathway, up-regulating a vasodilatory, anti-thrombotic and anti-inflammatory program of endothelial gene expression. We therefore hypothesize that Klf2 will play a critical role in pulmonary hypertension, orchestrating mechanisms that protect endothelial cells from injury and maintain normal pulmonary microvascular structure and function.
AIMS OF THE PRESENT STUDY

This study was undertaken to obtain information on Klf2 in the context of experimental pulmonary hypertension. The four specific aims of this body of work are:

1. Characterize Klf2 expression in established experimental models of pulmonary hypertension (PH): specifically, the Chronic Hypoxia Model and the Monocrotaline Model.

   Hypothesis 1.1. Klf2 mRNA expression is reduced early-on in the temporal development of PH in both the MCT and CH models.

2. Elucidate the functional role of Klf2 in the context of experimental PH by developing working methodology to manipulate lung Klf2 gene expression in vivo: specifically, using the jetPEI system for use in delivering shKlf2 for gene knockdown, or the Klf2 gene to increase endogenous expression, to the resident pulmonary arteriolar endothelial cells.

   Hypothesis 2.1. Klf2 knockdown in the pulmonary vasculature will increase right ventricular systolic pressure even in healthy animals, and will exacerbate the development of PH in experimental models.

   Hypothesis 2.2. Klf2 overexpression will prevent the development of experimental PH in experimental models of this disease.

3. Provide insight into the mechanisms which underlie the protective effects of Klf2 and how it affects experimental PH models.

   Hypothesis 3.1. Klf2 prevents PH by: protecting against endothelial apoptosis, maintaining pulmonary arteriolar endothelial dilatory function, and inhibiting vascular remodeling in experimental models of PH.
4. Determine if overexpression of *Klf2* can reverse established experimental PAH in a treatment model.

*Hypothesis 4.1. Although Klf2 overexpression is effective in preventing PH, alone it cannot reverse established MCT-induced PAH once endothelial injury and activation have already occurred.*
MATERIALS AND METHODS

6 EXPERIMENTAL MODELS

6.1 IN VIVO

All animal procedures were approved by the Animal Care Committee of St. Michael’s Hospital, Toronto, Canada.

6.1.1 MONOCROTALINE-INDUCED PAH

Male Fisher-344 rats (Charles River, Canada) weighing 150-200g were randomly assigned to either one of the experimental or control groups. Experimental rats were given a one-time bolus intra-peritoneal injection of monocrotaline (MCT; 70 mg/kg, {Vehicle= 0.9% NaCl, 20mg/mL of MCT in saline in each injection}; Aldrich Chemical, Milwaukee, WI) and control rats were given a bolus injection of the comparable volume of saline. For all groups, normal rat chow and water were provided ad libitum. Unless otherwise stated, gene transfer procedures to alter Klf2 expression in the pulmonary vasculature were performed 7 days after MCT administration, and right ventricular systolic pressure (RVSP) and the right to left ventricular plus septal weight ratio, RV/(LV+S), were assessed 14 days afterwards (or 21 days following MCT administration) (Illustration 6.1).

In the reversal experiment, gene transfer of Klf2 was performed at 21 days after MCT administration, and RVSP and RV/(LV+S) were assessed 14 days afterwards, at day 35 following MCT injection (Illustration 6.1). All MCT-treated rats between days 22 and 34 were: given Transgel (Charles River, Canada) mixed with yogurt and cereal as treats to stimulate appetite, weighed daily, and those whose weights had decreased compared to their surgical
weight (weight measured on day 21) were given 3 mL of saline by sub-cutaneous injection to promote study survival. In extreme cases where weight loss neared a 20% reduction from surgical weight, rats were given up to 5 mL of saline by sub-cutaneous injection. RVSP measurements that were assessed at day 35 were excluded from animals who had lost more than 25 grams body weight within the final two experimental weeks (day 21 compared to day 35), since their low blood volumes caused artificially low RVSP in PAH afflicted rats.
Illustration 6.1 Timelines for MCT Experiments

1. Characterization of KLF2 expression in the MCT model of PAH:

2. Can KLF2 overexpression in the MCT model prevent PAH?

3. Does KLF2 knockdown in MCT-induced PAH worsen the disease?

4. Can KLF2 overexpression in established PAH reverse disease progression?
Experimental male Fisher-344 rats weighing 150-200g were housed in hypoxic chambers maintained at ~10% oxygen (mixture of compressed air and nitrogen, or a custom order tank: 10% O₂ – 500ppm CO₂ – balance nitrogen; Praxair; Illustration 6.2). Control rats were housed in identical chambers containing normal air and considered normoxic. For both groups of rats, normal rat chow and water were provided ad libitum. Unless otherwise stated, gene transfer procedures to induce Klf2 expression in the pulmonary vasculature were performed after 3 days of CH exposure, gene transfer procedures to knockdown Klf2 expression was performed after 3 days of CH exposure, and RVSP and RV/(LV+S) were assessed after 21 days of CH exposure (Illustration 6.3).
Illustration 6.2 Chronic Hypoxia Apparatus

Hypoxia Chamber Setup
Illustration 6.3 Timelines for CH Experiments

1. Characterization of KLF2 expression in the CH model of PH:

2. Can KLF2 overexpression in the CH model prevent PH?

3. Does KLF2 knockdown in CH-induced PH worsen the disease?
6.2. IN VITRO

*In vitro* experiments were carried out in various endothelial cells. All cells were cultured according to manufacturers’ protocols: human umbilical vein endothelial cells (HU-VECs; Cambrex Corp., East Rutherford, New Jersey) were grown in Clonetics® EGM (Lonza, Basel, Switzerland), pulmonary artery endothelial cells (PAECs; Cambrex) were grown in Clonetics® EGM (Lonza), and rat lung microvascular endothelial cells (RLMVECs; VEC Technologies, Rensselaer, New York) were grown on fibronectin-coated plasticware (BD bioCoat™) in MCDB-131C (VEC Technologies).

6.2.1. HYPOXIA CHAMBER APPARATUS

For *in vitro* hypoxia experiments, ECs were grown to approximately 70-80% confluence and placed into a sealed and humidified hypoxia chamber which was housed inside a standard cell culture incubator. A regulated compressed gas tank supplied the hypoxia chamber with 1% oxygen, 5% carbon dioxide and balance nitrogen (Praxair).

6.3. TRANSFECTION

6.3.1. PLASMIDS

The 6.8-kb plasmid vector pCMV-KLF2 (Illustration 6.4) was generated by inserting the 1056-nucleotide long full-length coding sequence of the rat *Klf2* cDNA (Accession Number NM_001007684.1) into the polylinker sequence of the expression vector (Illustration 6.5) pCMV6-AC, (Origene Technologies, Rockville, MD). The plasmid vector pCMV-empty was the expression vector pCMV6-AC (Origene Technologies, Rockville, MD) without a nucleotide insert.
The DNA sequence:

ATGACGACCTCAACAAACGTTCATTCAGAGAAGCGTTGTTGAGGTTCGTCAT, encoding a short hairpin RNA structure that was demonstrated by Wick et al., 2005 [272] to be processed to produce a mature siRNA against rat Klf2 expression, was cloned into the pRS shRNA expression vector, 5.430kb (Origene; Illustration 6.6) to generate the plasmid pRS-shKLF2. The expression vector pRS shRNA (Origene Technologies, Rockville, MD) without a nucleotide insert was plasmid vector pRS-null. CopyCutter™ EPI400™ E. coli cells (Epicentre Biotechnologies, Madison, WI) were transformed and induced into high copy number as per vendor’s protocol. Cells were preserved at -80°C in cryogenic tubes containing 20% sterile glycerol. Plasmids were mass produced, purified, and quantitated by Aldevron using the E. coli glycerol stock (Fargo, North Dakota).
**Illustration 6.4** pCMV-KLF2 plasmid details

5’ restriction site: Sgfl
3’ restriction site: MluI
Final vector: pCMV6-AC (Origene)
Full insert sequence:

```
vector-(SgfI)GCGATCGCC
ATGGCGCTCAGCGAGCCCTATCTTTGCCGCTCATTGGCCGAGCCCTCTGGCGAGCGGCGGCCT
CCAGGAGCGCTGGCGCGAGGCGCGGAGCGAGTAGGAGACCTAAACAGCGTGC
TGGACTTTCACCTGTCCCATGGGACTGGAGCGCTTGCGGCGGAGCAGCGCAGCTTACGGATAGAAGCGGTCTACAG
GCCGCTTCCTCTCAGCGCTTCCGGGCGAGGCGGTGTGAAGGCCGAGCCCCCGGAGGGTGGACGGCGGC
GGCTACGGGTCGCCAGCCCGCTTTGGCTGCGGAGCCGGCGGGCTGAAGCGTCTATCGCGAGGGCGCCCTTGGG
AGCGACAGGTGCATGCAGCGGCTGCTGCGCTGCCCTCGCCCTCCCCACCTTGCCACAGCGGCGCCGC
TCAGCCCCGACGGCCCGCCCGCTCCCGGCGCCCGGTCCCCAGCCACGACGGCCGCCCTCGTCCCCGCTGGAGCTGTTGGAGGCCAAGCCCAAGCGCGGCCGCCGCTCCTGGCCCCGCAAGCGCGCCGCCACGCACACTTGCAGCTACACCAACTGCGGCAAG (natural stop codon included)
```

There are no internal SgfI sites or internal MluI sites.
Illustration 6.5 pCMV-AC expression vector (Origene)
Illustration 6.6 pRS-shKLF2 plasmid and pRS shRNA expression vector

ATGACGACCTCAACAACGTTTCAAGAGAACGTTGTTGAGGTCGTCAT

Biochem. J. (2005) 387, 239-246 (Printed in Great Britain) Lung Krüppel-like factor (LKlf) is a transcriptional activator of the cytosolic phospholipase A2 \( \alpha \) promoter. Marilee J. WICK, Stacy BLAINE, Vicki VAN PUTTEN, Milene SAAVEDRA and Raphael A. NEMENOFF [272]

“The exact shRNA as was published is what was made.”
... “It is in the pRS vector.”
Karl
[Karl Kovacs, PhD; correspondent at Origene Technologies Inc.]
6.3.2. Preliminary Work Involving Electroporation

Early preliminary evaluation of pCMV-KLF2, prior to work with jetPEI, was conducted in rat lung microvascular endothelial cells (RLMVECs) using electroporation technique. The electroporation technique was validated using AMAXA™ Nucleofector™ (Lonza, USA) and deemed effective after visualization GFP fluorescence following GFP plasmid transfection. The conditions were: “S05” program with 5µg of plasmid DNA for 500,000 cells per well (a 6-well plate was used).

6.3.3. In Vivo Gene Transfer to the Lung Vasculature

In vivo transfections were carried out using the in vivo-jetPEI cationic polymer transfection reagent (Polyplus-transfection SA, Illkirch, France) delivered by tail-vein injection to target the intravascular lung endothelial cells. Effective transfection, assessed by both increased KLF2 and eNOS mRNA expression, was achieved by delivering 150µg of DNA complexed with jetPEI to obtain a PEI nitrogen/DNA phosphate (N/P) ratio of 5 in a total volume of 1 ml of 5% glucose.

To induce Klf2 gene expression in the pulmonary endothelium, rats were given an intravenous injection of either pCMV-KLF2/jetPEI or pCMV-empty/jetPEI complexes. For knockdown experiments, rats were given an intravenous injection of either pRS-shKLF2/jetPEI or pRS-null/jetPEI complexes at day 3 of CH or day 7 post-MCT administration. In healthy animals, Klf2 knockdown treatment was delivered in two injections, 7 days apart, and RVSP and RV/(LV+S) was measured 7 days after the second injection (Illustration 6.4).
Illustration 6.7 Timeline for Experiment in Healthy Normoxic Animals

1. What is the effect of KLF2 Knockdown alone on RVSP?
6.3.4. IN VITRO

In order for results from the *in vitro* transfections to be useful as preliminary proof of principle data *in vivo*, transfections were performed using the *in vivo*-jetPEI cationic polymer transfection reagent (Polyplus) in cultured RLMVECs. Some adjustments were made based on experimentation and conversations with Polyplus technicians:

A 1× working solution was made by adding 50µL of *in vivo* jetPEI into 450µL of sterile water. In line with manufacturer’s recommendations, for a 6-well plate, 3µg of DNA was added to 100µL sterile 150mM NaCl (vortex; spin down) and, in a separate epindorf, 3µL of 1× solution was added to 100µL sterile 150mM NaCl (vortex; spin down). The jetPEI mixed solution was then added to the DNA mix solution (vortex; spin down). Transfection of the RLMVECs were performed in serum-free MCDB-131C medium and incubated for 6 hours. Media was then aspirated, replaced with serum-containing media. Cells were lysed for RNA extraction 20–24 hours later.
7 PHYSIOLOGICAL AND ANATOMICAL MEASUREMENTS IN IN VIVO MODELS

7.1. RIGHT VENTRICULAR SYSTOLIC PRESSURE

The pressure in the right ventricle is measured as an estimate of luminal systolic blood pressure in the pulmonary artery which leads to the lungs. In normal rats, right ventricular systolic pressure (RVSP), a close correlate to mean PAP, is measured between 21-29 mmHg, whereas in rat models of PH the RVSP is often two or three fold higher [15, 273]. Since the normal rat PAP (and mouse) is the same as the PAP in humans [274], some variation in measured normal rat RVSP may be technical.

RVSP was measured in rats anesthetized with an intra-peritoneal injection of xylazine (10 mg/kg) and ketamine (100 mg/kg). A 3F Millar microtip catheter inserted through the right external jugular vein and into the right ventricle (Biopac System and AcqKnowledge software, Goleta, CA).

7.2. HEMATOLOGY

After RVSP was measured and the catheter was removed, a laparotomy was performed and approximately 3 mL of venous blood was taken from the hepatic portal vein for processing of full blood differentials (CBC). A heparinized syringe was used and venous blood was stored in purple vacutainers, containing EDTA.
7.3. RIGHT VENTRICULAR HYPERTROPHY (RIGHT VENTRICLE/LEFT VENTRICLE PLUS SEPTUM WEIGHTS)

Following RVSP measurement, laparotomy and venous blood sampling, rats were exsanguinated by severing the abdominal aorta. The chest was then opened by cutting through the diaphragm to make vertical inline incisions along both sides of the sternum. The heart was excised and micro-dissected for the assessment of the right ventricular hypertrophic response by right to left ventricle plus septal weight ratio, RV/(LV+S). The micro-dissection in detail: the pulmonary artery, vena cava, and aortic tissue were carefully removed from the heart, the heart was rinsed in saline to remove any blood clots, then the outer right ventricle wall was removed from the left ventricle and septal wall using fine surgical scissors and wet weights of RV and (LV+S) were separately measured.

7.4. HISTOLOGY

The right-side of the lungs were clamped off using a hemostat at the level of the right bronchi (right-lung is fast-frozen in liquid nitrogen for molecular analyses). In a careful manner so that the lung was not overly distended, a 18 gauge needle and syringe was used to inflate the left lung through the trachea with a bubble-free solution of half 4% paraformaldehyde (PFA) and half Tissue Tek O.C.T. Compound (Fisher Scientific, Nepean, ON). Once inflated, the left-lung was excised and cut laterally into five even pieces and the resultant midsections of the top and bottom of the right-lung (piece two and four taken in a linear sequence) were placed in 4% PFA at 4°C. After forty-eight hours, the lung sections were washed with PBS and immersed in PBS at 4°C for approximately 12 hours. Afterwards the tissue was washed and stored in 70% ethanol (EtOH).
For staining with hematoxylin and eosin, or elastic Von Geison, the tissue was later embedded into paraffin and cut into 5µm thick sections. Lung sections were imaged using a Nikon Eclipse E800 microscope at 40× magnification.

For assessment of arterial remodeling, ImageJ (NIH website) was used in a blinded analysis to measure the inner and outer elastic lamina circumference in cross-sections of all vessels with a perceptible media. The medial area was determined for each vessel, and for each rat an average medial area was obtained for vessels with an external diameter between 30 and 50 µm.

7.5. FLUORESCENT MICROANGIOGRAPHY

To prepare rats for fluorescent microangiography (FMA), a catheter was inserted into the pulmonary artery and the lungs were flushed with warmed heparinized phosphate buffered saline (PBS). The lungs were inflated with room air through the trachea using a modified blunted catheter and syringe while simultaneously being perfused through the pulmonary artery with 45°C solution of 0.2% of 0.2µm yellow-green fluorescent microangiography spheres (505 nm/515nm peak excitation and emission; Molecular Probes) in 1% low-melting-point agarose (Sigma-Aldrich, Oakville, ON). Once the agarose solidified, the lungs were fixed in 4% PFA in PBS for 48 hours at 4°C. Then, 200µm-thick sections were prepared using a microtome (Leica, Wetzlar, Germany).

Sections were counterstained with the nuclear marker ToPro3 iodide, 642 nm/661 nm peak excitation/emission (Invitrogen, USA) and smooth muscle alpha-actin-cy3 antibody (Sigma-Aldrich, Canada) (Appendix 2). Confocal optical sectioning (BioRad Radiance, Hercules, USA) was used to produce projections of Z stack of images spanning 150µm.
7.6. CASPASE-3 STAINING

MCT-induced PAH experiments, 7 days following MCT injections rats were given either an intravenous injection of either pCMV-KLF2/jetPEI or pCMV-null/jetPEI complexes. Seventy-two hours later, rats were anesthetized with an intra-peritoneal injection of xylazine (10 mg/kg) and ketamine (100 mg/kg) and the right-lung was excised then cut laterally into 5 even pieces. Midsections of the top and bottom of the right-lung (piece 2 and 4 taken in a linear sequence) were placed in 4% PFA at 4°C. After 48 hours, the lung sections were washed with PBS and immersed in PBS at 4°C for approximately 12 hours. Next, the tissue was washed and stored in 70% ethanol (EtOH). The tissue was later processed and embedded into paraffin. Slides were made using 4µm thick sections and Caspase-3 staining was carried out by the Centre for Modeling Human Disease (CMHD), Toronto.
8 PHYSIOLOGICAL IN VITRO AND EX VIVO ASSAYS

8.1 MIGRATION ASSAY

Endothelial cell migration was quantified using modified Boyden chambers with an 8µm pore size. Boyden chambers were prepared prior to the migration assay by coating them with human fibronectin. HUVECs were serum-starved for 4 hours before trypsination and seeding 100,000 cells in a 500-µL volume (0.5% BSA in EBM; Cambrex Bioscience) to each prepared Boyden chamber membrane. Cells were then incubated in the presence or absence of VEGF (50ng/mL) and allowed to migrate across the membrane over 4 hours. Afterwards cells were stained using Diff-Quik (VWR International, Mississauga, ON) and all migrated cells were image-captured in 5 different fields of view at 20× magnification (Nikon Eclipse E800 microscope) and later migrated cells were counted using ImageJ software.

ADENOVIRUS TRANSFECTION

HUVECs grown in EGM (Lonza) at a density of 1 x 10^6 cells/75cm² flask were infected either with an adenovirus encoding green fluorescent protein (Ad-GFP) or Ad-GFP-KLF2 for 24 hours at 10 MOI. (Both adenoviruses were generous gifts from Dr. Mukesh Jain and Dr. Zhiyong Lin, Case Western Reserve University, Cleveland, OH, USA [235].) Ad-GFP-KLF2 contained the human KLF2 sequence.

8.2 ENDOTHELIAL FUNCTION EXPERIMENT

Twenty-four hours following in vivo-jetPEI-mediated transfection of shKLF2 or a control plasmid in 300-350g rats, lungs were excised and prepared for ex vivo analysis.
Systolic pulmonary artery pressures were measured, as an indicator of dose-dependent dilation, in isolated perfused rat lungs to assess the endothelium-dependent vasorelaxant response to acetylcholine (ACh; 10^{-8} - 10^{-5} mol/L) and/or the endothelium-independent response to sodium nitroprusside (SNP; 10^{-9} - 10^{-6} mol/L). All isolated perfused lungs were pre-constricted with endoperoxide analog U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α; 50 pmol/min; Sigma-Aldrich, Oakville, ON).
MOLECULAR ANALYSIS

9.1. RNA

ISOLATION

RNA was isolated from exsanguinated lung samples that were snap-frozen. In CH rats only, RNA was isolated from snap-frozen samples from euthanol-treated rats that did not undergo any other manipulations after being removed from the CH environment. RNA was purified using Trizol extraction (Invitrogen). Samples were later treated with Turbo DNAase according to manufacturer’s recommendations to remove any possible genomic DNA contamination (Ambion). RNA yield (A260) and purity (A260/A280, 1.7-2.0 deemed high quality; A260/A230, greater than 1.0 and close to 2.0) was assessed using the Nanodrop spectrophotometer (Thermal Scientific).

REVERSE TRANSCRIPTION

RNA, 2 µg, from each animal was reverse-transcribed using the Moloney Murine Leukemia Virus reverse-transcriptase (Invitrogen) with random hexamers (Invitrogen) in a 20 µL solution. The program on the Eppendorf Mastercycler gradient thermocycler was: lid 80°C; T=37°C × 1:30:00; T=95°C × 0:02:00; hold 10°C.

QUANTITATIVE REAL-TIME PCR

Real-time PCR was carried out using the Eppendorf Mastercycler gradient machine. For the analysis of rat Klf2 mRNA, the RT² Primer Assay for Rat Klf2 (SA Biosciences) primer system was used with a 60°C annealing temperature in 25 µL reactions. For all other reactions SYBR Green PCR master mix (Applied Biosystems) was used in 30 µL reactions. All reactions followed manufacturer’s recommendations and were conducted in ninety-six
well plates, each contained a dilution curve and other 'no template control' wells. Relative quantification of messenger RNA expression was calculated using a relative expression software tool (REST; Pfaffl MW et al. Nucleic Acids Research. 30: 2002). Details of all the primers used in this study are in Table 9.1.

Table 9.1 - Primers

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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</thead>
<tbody>
<tr>
<td>rat</td>
<td>CycA</td>
<td>5' – ATG GTG ATC TTC TTG C – 3'</td>
<td>5' – ATG GTT CCC AGT TTT TTA TCT GCA C – 3'</td>
</tr>
<tr>
<td>rat</td>
<td>eNOS</td>
<td>5' – CTA CGA AGA ATG GAA GTG GTT CC – 3'</td>
<td>5' – GTG CTG AGC TGA CAG AGT CGT ACC – 3'</td>
</tr>
<tr>
<td>rat</td>
<td>KLF2</td>
<td>5’ – CAC GCA CAC TTG CAG CTA CA – 3' and</td>
<td>5’ – TGC AAT GAT AAG GCT TCT CA – 3' and</td>
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<td></td>
<td></td>
<td>SA Biosciences Rat KLF2 primers</td>
<td>SA Biosciences Rat KLF2 primers</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Accession # NM_001007684.1; reference positions 106-126; band size 123 bp)</td>
<td>(Accession # NM_001007684.1; reference positions 106-126; band size 123 bp)</td>
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<td>5’ – TCT ACG ATG GCT TTT CGG TTC TTA G– 3’</td>
</tr>
<tr>
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<td>TM</td>
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<td>5’ – TCT GGA GTA AAT CCC TCA GAA CTT C – 3’</td>
</tr>
<tr>
<td>human</td>
<td>CycA</td>
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<td>5’ – CTA GGC ATG GGA GGG AAC A – 3’</td>
</tr>
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<td>5’ – AGC ACG GCC ACG TTG ATT TC – 3’</td>
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<td>5’ – CTG TTG CCT TTG TGG GAA GT – 3’</td>
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<td>5’ – CCC ATG GAC AGG ATG AAG TC – 3’</td>
</tr>
<tr>
<td>human</td>
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<td>5’ – TCT ACG ATG GCT TTG CGG TTC TTG</td>
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<td>5’ – GTT GAC TCT AGC TCG GAC CAC – 3’</td>
</tr>
<tr>
<td>human</td>
<td>TM</td>
<td>5’ – CTT CAG AGC CAA CTG CGA GTA C – 3’</td>
<td>5’ – CAG GCA GTC TGG TTG CAA AAC – 3’</td>
</tr>
</tbody>
</table>
9.2. PROTEIN ISOLATION

Protein from cell cultures was isolated using the PARIS™ Kit (Ambion) as described in manufacturer’s protocol.

Frozen rat lung tissue samples were manually crushed using a ceramic mortar and pestle chilled with liquid nitrogen. Pulverized samples were transferred into eppendorf tubes each containing approximately 600 µL of RIPA lysis buffer [supplemented with 100-200 µL of proteinase inhibitor cocktail (Sigma) per 10mL RIPA lysis buffer], and were kept on ice for twenty minutes before being transferred to storage at -80°C.

QUANTIFICATION

Protein content in each sample was quantified using Bio-Rad’s ABS reagents, BSA standard, and optical densities were measured at 750 nm. Prior to sampling, all samples underwent a one-minute centrifugation to pellet and remove insoluble debris. Protein abundance between treatment groups was assessed by Western blotting performed in triplicate and β-actin was assessed as a loading control. Proteins were resolved by electrophoresis on Novex® Tris-glycine 4-12% or 4-20% gels (Invitrogen) as appropriate. SeeBlue® Plus ladder (Invitrogen) was used for molecular sizing in each gel.

ANTIBODIES

Antibodies used are described in Appendices J and Q.
9.3. FLOW CYTOMETRY

TRANSFECTION EFFICIENCY

Endothelial cells were plated as 375,000 cells per 25cm² flask, and 24 hours later were infected either with an adenovirus encoding green fluorescent protein (Ad-GFP) or Ad-GFP-KLF2 for 24 hours at 10 MOI. (The adenovirus was a generous gift from Dr. Mukesh Jain and Dr. Zhiyong Lin, Case Western Reserve University, USA). Fluorescence was induced with the 488-nm argon laser and assessed using a Beckman Coulter Cytomics FC500 analyzer.

APOPTOSIS ASSAY

In the apoptosis assay, 24 hours following transduction of either KLF2 or control virus, rat lung microvascular endothelial cells (RLMVECs) were treated with 500µM hydrogen peroxide (H₂O₂) to induce apoptosis. After another 24 hours, cells were suspended by a brief trypsinization (0.05% with EDTA) followed by trypsin neutralization. Cells were then resuspended in 100 µL of binding buffer with 5 µL of AnnexinV–phycoerythrin (Annexin-PE, BD Biosciences) and 10 µL of propidium iodide (PI). The cells were gently mixed and incubated for 15 minutes in the dark at room temperature before adding 400 µL of binding buffer. Compensations were set up using: cells unstained, cells with GFP only, cells with Annexin-PE, cells with PI only, and cells with GFP + PI + Annexin-PE. Fluorescence was induced with the 488-nm argon laser and assessed using a Beckman Coulter Cytomics FC500 analyzer.
10 STATISTICAL ANALYSIS

All data is presented as means ± SEM. Statistical analyses consisted of a one-way analysis of variance ANOVA, followed by a post hoc Tukey’s, non-parametric Mann-Whitney test or t-test where appropriate. A value of $P<0.05$ was considered statistically significant.
RESULTS

II WHAT ARE THE CHANGES IN KLF2 EXPRESSION IN PH?

11.1 IN VIVO

11.1.1 MONOCROTALINE-INDUCED PULMONARY ARTERIAL HYPERTENSION

To demonstrate a working model of MCT-induced PAH, right ventricular systolic pressures were measured in rats 21 days after intraperitoneal MCT injection (70mg/kg) and compared to pressures in saline-treated controls. Animals that received MCT had significantly increased RVSP after 21 days (56.4 ± 6.9 mmHg versus control, 25.6 ± 0.9; P<0.01) (Figure 11.1). Another benchmark for PAH progression is right ventricle (RV) hypertrophy and was evaluated as a ratio of RV weight to left ventricle plus septal weight (LV+S). At 21 days after MCT treatment rats RV/(LV+S) ratio was significantly greater than saline-treated controls (0.40 ± 0.03 versus 0.21 ± 0.01; P<0.01) (Figure 11.2).

Further characterization of our MCT model and disease progression included microscopy and histology using hematoxylin and eosin (H&E) staining. Tissue visualization confirmed that MCT treated animals had the hallmark features of PAH: medial hypertrophy from smooth muscle cell hyperplasia and hypertrophy along with increased cellularity and increased septal thickness at 21 days (Figure 11.3). Fluorescent microangiography verified that there was evident functional vascular loss (green fluorescence in intra-vascular space) and increased distal pulmonary vascular muscularization (red fluorescence, α-actin) in the MCT versus the sham control group (Figure 11.4). Further structural comparisons sug-
gest that at day 21 that lung alveolar structure was maintained in MCT-treated animals as compared to controls (Figures 11.3 and 11.4); suggesting that there was no overall loss of lung endothelial cells, in contrast to emphysema models which are known to exhibit endothelial loss [275-277]. Therefore, the lack of perfusion of the distal capillary bed that is evident on the FMA studies likely reflects a functional abnormality in the low-pressure pulmonary circulation involving the loss of efficient continuity in the distal arteriolar bed. In the MCT model, which lacks in the proliferative intimal and more complex (plexiform) vascular lesions, this is likely due to a discrete degeneration at the level of the pre-capillary arteriole which represents the critical junction between the arteriole and the capillary beds.

Other observations in MCT-induced PAH include loss in body weight, and changes to hematology (Supplement Figure 10.1 and Supplement Figures 11.2-11.8 respectively).

Seven days after MCT treatment, before any changes in RVSP or RV/(LV+S), between saline-treated or MCT-treated groups, KLF2 mRNA in the lung was significantly decreased, compared to normalized saline-treated controls (0.65 ± 0.10 versus 1.0; P<0.01) (Figure 11.5). This decrease was sustained after 21 days (0.42 ± 0.07; P<0.01 versus normalized controls) (Figure 11.5).
Figure 11.1. Right ventricular systolic pressure (RVSP) for sham rats and for rats 7 days, and 21 days, after injection of pulmonary endothelial toxin monocrotaline (MCT). RVSP was significantly increased 21 days post-MCT administration compared to saline-treated sham rats. *P<0.01 vs. control. Data are mean ± SEM.
Figure 11.2. Ratio of right ventricle weight to left ventricle plus septum weight, RV/(LV+S), as a measurement of right ventricular hypertrophy, in sham animals and for animals 7 days and 21 days after MCT injection. RV hypertrophy was evident 21 days following MCT administration in comparison to saline-injected sham controls. RV hypertrophy was not evident early-on at 7 days post-MCT injection. *P<0.01 vs. control. Data are mean ± SEM.
Figure 11.3. Hemotoxylin and eosin stained pulmonary cross-sections at 21 days following either saline or MCT administration. Visually, there was evident increases in cellularity, vascular muscularization, and septal thickness in lungs at 21 days post-MCT in comparison to saline-injected sham controls. Bronchiole (Br) small PAs (▲).
**Figure 11.4.** Fluorescent microangiography of the pulmonary arterial microcirculation (green fluorescence) at 21 days in animals given either saline or MCT injection. Lung sections were counterstained with smooth muscle α-actin (red fluorescence) and cell nuclei stain ToPro3 (blue fluorescence). Compared to saline-treated sham controls, MCT-treated rats had increased distal pulmonary arterial muscularization (Δ) and a heterogeneous pattern of loss of functional vasculature (green fluorescence). Bronchiole (Br).
Figure 11.5. Pulmonary KLF2 mRNA expression was assessed in whole exsanguinated rat lung using real-time semi-quantitative PCR and calculated relative to the Cyclophilin A reference gene expression which remained at consistent levels between groups. Fold change in MCT-treated animals after 7 or 21 days is corresponding to normalized sham values. MCT-induction of PAH resulted in a sustained reduction of KLF2 mRNA expression at 7 and 21 days post-MCT injection. *P<0.05 versus saline-treated shams. Data are mean ± SEM.
11.1.2. CHRONIC-HYPOXIA-INDUCED PULMONARY HYPERTENSION

To characterize a working model of CH-induced PH, RVSP was measured in rats 1, 3, 7, and 21 days after being housed in a hypoxic environment (10% oxygen) and compared to control animals housed in normal air. Animals housed in CH conditions had significantly increased RVSP after 3 days compared to control (39.5 ± 3.2 mmHg versus 21.7 ± 0.4; P<0.05), which continued to be increased at 7 and 21 days (Figure 11.6). Right ventricle weight ratio RV/(LV+S), an indicator of right ventricular hypertrophy, was significantly increased after 3 days of CH exposure to 0.47 ± 0.02 versus 0.23 ± 0.01 weight ratio in controls at 21 days (Figure 11.7).

Qualitative observation of H&E stained lung tissue sections confirmed the presence of smooth muscle cell hypertrophy in small pulmonary arterioles after 21 days of CH (Figure 11.8). Also, there was no observed difference in immune cell infiltrate in the lungs of normoxic versus hypoxic animals (Figure 11.8). Confocal images using fluorescent microangiography showed that following 21 days of CH there was a homogenous pattern of functional vascular loss (green fluorescence in intra-vascular space) together with an even spread increased in distal pulmonary vascular muscularization (red fluorescence, α-actin) (Figure 11.9). This is different from the MCT model of PAH where there was heterogenous loss of perfusion in distal pulmonary arteries and arterioles.

Other observations in CH-induced PH included loss in body weight, and changes to hematology (Appendix M and Appendix C respectively).

There is an early decrease in KLF2 mRNA expression three days after CH initiation to 0.34 ± 0.09 fold of normoxic control, coinciding with CH-associated increased RVSP and
RV/(LV+S) (Figures 11.10, 11.6 and 11.7 respectively). This KLF2 reduction was transient; re-turning to and sustaining normoxic sham levels at day 7 (0.92 ± 0.12 fold) through day 21 (Figure 11.10).
Figure 11.6. Right ventricular systolic pressure (RVSP) was measured in vivo using a Millar catheter for normoxic sham rats and for rats after 1, 3, 7 and 21 days of chronic hypoxia (CH; 10% O₂). In comparison to normoxic sham animals (27.7 ± 0.4 mmHg), RVSP was significantly increased 3 days following CH and continued to increase over 7 and 21 days when it reached a mean RVSP of 61.9 ± 3.0 mmHg. *P<0.05 vs. sham controls. Data are mean ± SEM.
Figure 11.7. Ratio of right ventricle weight to left ventricle plus septum weight, RV/(LV+S), in normoxic sham animals and for hypoxic (10% O₂) animals after 1, 3, 7 or 21 days of CH. There was significantly increased RV weight ratio in rats after 3 days of CH and RV weight continued to be increased after 21 days of CH compared to sham (0.29 ± 0.01, 0.47 ± 0.02 versus 0.23 ± 0.01, respectively). *P<0.05 vs. sham controls. Data are mean ± SEM.
**Figure 11.8.** Hemotoxylin and eosin stained pulmonary cross-sections at 3, 7, and 21 days following CH. Visually, there was no evident increases in cellularity, and vascular muscularization of small PAs. Bronchiole (Br), small PAs (▲).
**Figure 11.9.** Fluorescent microangiography of the pulmonary microcirculation (green fluorescence) in animals given housed in normal air (sham) and animals in 10% O$_2$ (CH) after 7 and 21 days. Lung sections were counterstained with smooth muscle α-actin (red fluorescence) and cell nuclei stain ToPro3 (blue fluorescence). Compared to saline-treated sham controls, CH-housed rats after 21 days had increased distal pulmonary arterial muscularization and a homogenous pattern of loss of functional vasculature (green fluorescence).
**Figure 11.10.** Real-time PCR assessment of KLF2 mRNA in whole exsanguinated rat lung in the CH model of PH, using Cyclophilin A reference gene (which remained at consistent levels between groups) and normalized to levels in control animals housed in normal air. Pulmonary KLF2 mRNA expression was transiently reduced after 3 days of CH (0.34 ± 0.09 fold), returning to normal levels by 7 days of CH (0.92 ± 0.12 fold). Data are mean ± SEM. *P<0.05 versus normoxic shams.
11.2. IN VITRO

11.2.1. EFFECT OF HYPOXIA ON ENDOTHELIAL KLF2 EXPRESSION

In vitro experiments using HUVECs showed that KLF2 mRNA expression is reduced following 24 hours of exposure to 1% hypoxia to 0.5 fold the expression in normoxic control endothelial cells (Figure 11.11).
Figure 11.11. HUVECs were placed into a humidified hypoxia chamber with its own air supply of: 1% oxygen, 5% carbon dioxide, and nitrogen gas. Twenty-four hours later KLF2 mRNA expression was assessed using real-time PCR and Cyclophilin A (known not to be responsive to Hypoxia) was used as a reference gene. Hypoxia significantly reduced KLF2 mRNA expression in comparison to sham cells exposed to normoxic cell culture air. *P<0.01. Data are mean ± SEM of 5 experiments in triplicate.
To establish jetPEI as a feasible delivery system for modulating KLF2 expression in rat lung endothelial cells in vivo, first in vitro experiments were conducted. Preliminary studies in cultured rat lung microvascular endothelial cells (RLMVECs) demonstrated successful jetPEI delivery of at first GFP plasmids, confirmed by visualized green fluorescence, and then of KLF2 plasmids, confirmed by increased RNA expression (data not shown). With this known, early in vivo studies used a tetramethylrhodamine-conjugated jet PEI (excitation at 555 nm; emission at 580 nm) to show that the jetPEI-DNA construct was interacting with the lung endothelium and had the potential to deliver the plasmid to the targeted endothelium (Figure 12.1). Since the jetPEI is known to breakdown and be excreted by the kidneys within ~6 hours (Polyplus Technical Services; Appendix A), these lung samples were obtained approximately three and a half hours following tetramethylrhodamine-conjugated jetPEI-DNA injection. Observations in these lung samples also showed evidence of the PEI-DNA complex present within vessels of the pulmonary microcirculation (Figure 12.1).

To provide further support that jetPEI was targeting the lung endothelium jetPEI was used to deliver a plasmid containing GFP and twenty-four hours later, lungs were harvested for immunostaining for GFP protein (GFP could not be visualized using fluorescence since lung tissue is highly green auto-fluorescent). In these samples the immunostaining for GFP was positive (brown staining) (Figure 12.2). KLF2 mRNA expression was increased by 1.8 ± 0.2 fold (P<0.05; n=5) twenty-four hours after in vivo jetPEI-mediated transfection (Figure 12.3). KLF2 is known to act directly on the eNOS promoter and therefore increased
eNOS mRNA expression to 1.3 ± 0.1 fold (P<0.05; n=5), following in vivo KLF2 transfection, suggests that an active KLF2 protein was produced (Figure 12.4).
Figure 12.1. Confocal microscopy of rat lung 3.5 hours following injection of tetramethylrhodamine-conjugated jet PEI-DNA complexes (red fluorescence). Lungs were counterstained with ToPro3 (blue fluorescence). Red fluorescence was observed in ring like patterns tracing pulmonary arterioles and in line with pulmonary capillaries. Jet PEI-DNA complexes were adhered to the pulmonary microcirculation, allowing for opportunity to transfer DNA to the resident pulmonary ECs.
Figure 12.2. Twenty-four hours following delivery of jetPEI-pGFP complexes, containing a total of 150µg pGFP, lungs were harvested for immunostaining for green fluorescent protein (GFP). Since lung tissue is highly green auto-fluorescing, GFP presence was assessed using immunohistochemistry. In lung samples from animals receiving the GFP plasmid, shown above right, immunostaining for GFP protein was positive (brown staining; using only a biotin-conjugated primary antibody); control lung sections, above left, stained negative.
**Figure 12.3.** Real-time PCR assessment of KLF2 mRNA expression in whole ex-sanguinated rat lung 24 hours following jetPEI mediated delivery of 150µg of either pCMV-KLF2 or control (pCMV-empty). Rats receiving the pCMV-KLF2 plasmid directed to the pulmonary ECs had increased KLF2 mRNA expression to 1.8 fold that of empty plasmid transfected controls. *P<0.05 versus pCMV-empty; n=5. Data are mean ± SEM.
I N C R E A S E D  e N O S  m R N A  E x p r e s s i o n

**Figure 12.4.** Real-time PCR assessment eNOS mRNA expression in whole exsanguinated rat lungs 24 hours following jetPEI mediated delivery of 150µg of either pCMV-KLF2 or control (pCMV-empty). KLF2 is known to promote eNOS transcription by acting directly on its promoter and therefore increased eNOS mRNA would suggest that there was increased functional KLF2 protein. Not having good anti-KLF2 antibodies, eNOS mRNA expression is a workable surrogate to indicate changes in KLF2 protein production. Rats transfected with PEI-pCMV-KLF2 had significantly increased lung eNOS mRNA compared to sham-transfected controls. *P<0.05 versus pCMV-empty; n=5. Data are mean ± SEM.
Figure 12.5. Real-time PCR assessment KLF2 mRNA expression in whole exsanguinated rat lungs 24 hours following jetPEI mediated delivery of 150µg of either pRS-shKLF2 or control (pRS-null). In vivo transfection of PEI-pRS-shKLF2 significantly decreased KLF2 mRNA expression to approximately 0.37 fold that of PEI-pRS-null transfected shams. *P<0.01 versus pRS-null; n=4. Data are mean ± SEM.
**Figure 12.6.** Real-time PCR assessment eNOS mRNA expression in whole exsanguinated rat lungs 24 hours following jetPEI mediated delivery of 150µg of either pRS-shKLF2 or control (pRS-null). KLF2 is known to promote eNOS transcription. So, without good anti-KLF2 antibodies, eNOS expression is a surrogate to indicate acute changes to KLF2 protein levels. Rats transfected with PEI-pRS-shKLF2 had significantly decreased lung eNOS mRNA versus sham-transfected controls. *P<0.05 versus pCMV-empty; n=4. Data are mean ± SEM.
13 WHAT IS THE ROLE OF KLF2 IN PH?

13.1. PHYSIOLOGICAL

13.1.1. EFFECT OF KLF2 OVEREXPRESSION ON ENDOTHELIAL CELL MIGRATION

The role of KLF2 in EC migration was assessed using a Boyden chamber assay. Using this approach HUVECs were transduced with KLF2 using an adenovirus and twenty-four hours later cells the migration assay was conducted over five hours. KLF2 overexpression was confirmed and associated with increased eNOS expression (Appendix I). Cells that overexpressed KLF2 showed reduced migration in both the presence, and absence, of VEGF (50ng/mL) (30 ± 8 and 17 ± 5 cells, respectively; P<0.01) in comparison with their appropriately transfected null controls (50 ± 6 and 67 ± 4 cells, respectively; P<0.01; Figure 13.1). Infection alone (Ad-empty) had no effect on EC migration in comparison to non-transduced control cells with or without VEGF stimulus (68 ± 6 and 50 ± 5 cells, respectively; Figure 13.1).
Figure 13.1. Migration over four hours was assessed using fibronectin-coated modified-Boyden chamber assay in HUVECs that were transduced 24 hours earlier by an adenovirus containing the KLF2 cDNA (Ad-KLF2; 10 MOI) and compared to Ad-empty transduced controls. Endothelial migration was reduced by KLF2 overexpression in both unstimulated (left), and in the presence of VEGF (right; 50ng/mL). *P<0.01 versus Ad-empty unstimulated. #P<0.01 versus non-transduced unstimulated control. %P<0.01 versus Ad-empty + VEGF. #P<0.01 versus non-transduced + VEGF control. (non-transduced unstimulated control versus non-transduced + VEGF, P=0.057; Ad-empty unstimulated versus Ad-empty + VEGF, P=0.057; Ad-KLF2 unstimulated versus Ad-KLF2 + VEGF, P=0.2). Data are mean ± SEM of 4 experiments each performed in triplicate.
Delivery of PEI-pRS-shKLF2 to knockdown KLF2 mRNA levels, resulted in decreased responsiveness to acetylcholine-induced dilation as compared to sham-transfected controls (RS) (Figure 13.2). The pulmonary dilatory response to sodium nitroprusside was no different between treatment groups (Figure 13.2).
Figure 13.2. Ex vivo pulmonary functional analyses twenty-four hours following PEI-pRS-shKLF2 delivery to the pulmonary endothelium in vivo measured responsiveness as a change in pulmonary arterial pressure, to acetylcholine (ACh, upper) and sodium nitroprusside (SNP, lower). KLF2 repression significantly inhibited response to ACh-induced dilation (P=0.004, n=6; F-ratio is 4.47, Numerator degrees of freedom is 4, Denominator degrees of freedom is 40), and had no affect on SNP-induced dilation (n=4) compared to sham-transfected controls (RS). Data are mean ± SEM. Significance was determined using nonlinear regression modeling as described in Appendix G.
13.1.3. Can KLF2 Inhibition In Vivo In Healthy Normoxic Rattus Induce Pulmonary Hypertension?

Krüppel-like factor 2 mRNA expression was inhibited in the resident pulmonary ECs of healthy normoxic rats to assess the physiological role of KLF2 in health. Sustained KLF2 knockdown was achieved by administering jetPEI-pRS-shKLF2 as two bolus injections given seven days apart and assessed seven days later. The seven day interval was selected based on results from early preliminary data which showed that Klf2 expression is not significantly reduced at 7 days after gene delivery. RVSP was significantly increased to 39.3 ± 5.3 mmHg as a result of pulmonary EC targeted KLF2 knockdown in comparison to transfected controls, 27.1 ± 0.8 mmHg, and naive control animals 26.5 ± 0.4 mmHg (n=8-11; P<0.05; Figure 13.3). Right ventricle hypertrophy was not present at this early time-point in rats where KLF2 expression was knocked down (Figure 13.4).
Figure 13.3. Right ventricular systolic pressure (RVSP) was measured in rats seven days (day 14) following transfection (150µg plasmids) with KLF2 shRNA or sham transfection (pRS-null) using jetPEI at day 0 and again on day 7. RVSP was significantly increased to 39.3 ± 5.3 mmHg in rats where KLF2 was knockdown versus both naive (26.5 ± 0.4 mmHg) and sham-transfected controls (27.1 ± 0.8 mmHg). #P<0.05 versus pRS-null. $P<0.05 versus naive control. Data are mean ± SEM; n=8-11.
Figure 13.4. Ratio of right ventricular weight to left ventricle plus septum weight [RV/(LV+S)] in rats seven days after transfection of either 150µg of pRS-shKLF2 or pRS-null on day 0 and again on day 7. Right ventricle weight ratios were not significantly different (N.S.) between groups. Data are mean ± SEM; n=8-11. Means are not significantly different (ANOVA; P=0.15).
13.2. PATHOLOGICAL

13.2.1. IN VIVO

13.2.1.1. CAN KLF2 OVEREXPRESSION IN THE MCT MODEL PREVENT PAH DEVELOPMENT?

In order to increase KLF2 expression in the resident pulmonary endothelial cells, gene transfer was directed using intravenous injection of a commercial polyethylenimine, jetPEI (Polyplus) complexed with the KLF2 gene. Transfections took place 7 days following MCT injection to not interfere with the initial MCT toxic response and at the same time to up-regulate KLF2 at a time where expression was low (Figure 11.5). KLF2 gene transfer significantly reduced RVSP (41.2 ± 1.7 mmHg versus 61.3 ± 4.0; p<0.05; n=14-19) and RV/(LV+S) weight ratios (0.26 ± 0.01, n=17 versus 0.34 ± 0.02, n=12; p<0.05) compared with sham transfection (Figures 13.5 and 13.6). Right ventricular systolic pressure and RV hypertrophy in the group receiving the empty plasmid following MCT treatment was no different from MCT alone. Quantitative analysis of pulmonary arterial muscularization in small arteries and arterioles 30 to 50 micrometers in external diameter showed that KLF2 gene therapy in the MCT model (n=5) significantly reduced medial area to 682 ± 39 µm² compared to both sham-transfection (MCT+pCMV-null; 884 ± 51 µm², n=7) and MCT alone (858 ± 52µm², n=7; Figure 13.7). The mean PA medial area was not significantly different in saline-injected sham (615 ± 59 µm²) versus MCT plus KLF2 overexpression group (Figure 13.7). In MCT treated animals, early-on at 72 hours following gene therapy there was reduced Caspase-3 staining in the KLF2 overexpression group compared to the sham-transfected controls (Figure 13.8).
Figure 13.5. Right ventricular systolic pressure (RVSP) for sham rats and for rats 21 days after injection of pulmonary endothelial toxin monocrotaline (MCT). In MCT treatment groups, rats were transfected with jetPEI-pCMV-KLF2 or an empty plasmid (pCMV-null). KLF2 overexpression prevented the increase in RVSP seen in MCT alone (57.6 ± 5.5 mmHg, n=7) and MCT plus sham transfection (61.3 ± 4.0 mmHg, n=14) groups in comparison to sham rats (26.4 ± 1.1 mmHg, n=5). RVSP was significantly decreased in the pCMV-KLF2 group (41.2 ± 1.7 mmHg, n=19) compared to MCT control groups. *P<0.05 versus sham. #P<0.01 versus MCT. $P<0.01 versus MCT+pCMV-null. Data are mean ± SEM.
Figure 13.6. Ratio of right ventricular weight to left ventricle plus septum weight [RV/(LV+S)] in rats 21 days after saline or MCT injection. Treatment rats were either transfected with 150µg pCMV-KLF2 or sham gene transfer (pCMV-null). Right ventricular weight ratio was significantly increased in both MCT alone and MCT + pCMV-null groups (0.36 ± 0.03; n=5 and 0.34 ± 0.02; n=12) compared to saline treated sham animals (0.21 ± 0.00; n=7). Lung KLF2 overexpression significantly reduced RV hypertrophy (0.26 ± 0.01; n=17) compared to MCT treated control groups. *P<0.05 versus saline-injected sham. #P<0.01 versus MCT alone. $P<0.01 versus MCT+pCMV-null. Data are mean ± SEM.
Figure 13.7. On experimental day twenty-one lungs were excised and prepared for histology. All vessels found in two medial pulmonary cross-sections in each rat were imaged on a Nikon Eclipse E800 microscope at 40X magnification. After which Image J software was used for blinded measurement of medial area. Shown on the previous page are representative images of hemotoxylin and eosin stained cross-sections of rat lung depicting representative pulmonary arterioles. The graph above depicts the average medial thickness in cross-section of pulmonary arterioles with an external diameter of 30-50 µm, for saline-treated sham animals (n=5) and for MCT-treated animals that were given either pCMV-null (n=7), pCMV-KLF2 (n=5) or none (n=7). *P<0.01 vs. saline-treated control, $P<0.01 versus MCT alone, #P<0.01 versus MCT+pCMV-null. Data are mean ± SEM.
**Figure 13.8.** Rats were either administered endothelial toxin monocrotaline (MCT) or saline (sham). Seven days later, rats were treated by transfection to overexpress KLF2 or given a sham treatment (pCMV-null). After 72 hours lungs were harvested and prepared for histological analysis of apoptosis by Caspase-3 staining. There was evidence of reduced MCT-induced apoptosis (brown staining ↑) in rats treated with KLF2 gene therapy (MCT + pCMV-KLF2; n=4) compared to sham-transfected controls (MCT + pCMV-null; n=4). Bronchiole (Br).
13.2.1.2. Can KLF2 overexpression in CH model prevent PH development?

Since previous data characterizing the CH model of PH showed that at day three of CH exposure there was a transient reduction of Klf2 mRNA expression to 0.3 ± 0.1 fold of sham levels (P<0.05; Figure 11.9), we delivered KLF2 gene therapy in the CH model at day three. Klf2 overexpression was targeted in the resident pulmonary ECs by intravenous injection of jetPEI-DNA complexes. Right ventricular systolic pressures (RVSP) were significantly increased (p<0.01) after twenty-one days of CH in all groups compared to pressures in normoxic control rats (26.8 ± 1.3 mmHg). Compared with control transfection plus CH, Klf2 overexpression early-on in CH resulted in significantly reduced RVSP (84.4 ± 9.3 versus 54.7 ± 2.8 mmHg, respectively; P<0.01; n=5-6). In parallel, KLF2 gene therapy significantly reduced the right ventricle (RV) weight ratio to 0.31 ± 0.02 compared to the CH plus sham-transfection and CH alone groups (0.41 ± 0.02, and 0.35 ± 0.04, respectively). Measurement of the medial area in pulmonary arterioles between 30 to 50 micrometers showed that Klf2 overexpression early-on did not alter pulmonary arterial remodeling in the CH model compared to sham-transfected control (771 ± 73µm² versus 770 ± 48µm² respectively, n=5-7). There were also no significant differences of medial areas between treatment groups when medial areas of larger or smaller pulmonary arteries and arterioles were assessed (Appendix F).
**Figure 13.9.** Rats were housed in either 10% oxygen (chronic hypoxia, CH) or normal room air for twenty-one days. In CH groups, after three days of hypoxia rats were either given KLF2 gene therapy (pCMV-KLF2), sham-transfection of an empty plasmid (pCMV-null) or no gene therapy. On day twenty-one, right ventricular systolic pressure (RVSP) in all groups of CH rats was significantly higher than in normoxic sham rats (26.8 ± 1.3 mmHg, n=4). RVSP in the CH alone group (74.0 ± 5.6 mmHg, n=4) was no different than in the CH plus sham-transfection group (CH+pCMV-null: 84.4 ± 9.3 mmHg, n=5). KLF2 gene therapy early-on in CH (n=6) significantly blunted the CH-induced increase in RVSP to 54.7 ± 2.8 mmHg compared to both CH plus pCMV-null and CH alone groups (P<0.01). *P<0.01 versus normoxic sham. $P<0.01 versus CH alone. #P<0.01 versus CH plus sham-transfection (CH + pCMV-null). Data are mean ± SEM.
Figure 13.10. After three days of being housed in 10% hypoxia (CH) rats were given either KLF2 gene therapy (pCMV-KLF2), sham-transfection (pCMV-null) or no gene therapy. At day twenty-one of CH exposure, right ventricular (RV) hypertrophy was assessed as a ratio of RV weight to left ventricle plus septum (LV+S) weight. Each CH group had significantly increased RV weight ratios compared to the normoxic sham control group (0.22 ± 0.00, n=4). Right ventricle weight to LV+S weight in the CH alone group (0.35 ± 0.04, n=6) was no different than in the CH plus sham-transfection group (CH+pCMV-null; 0.41 ± 0.02, n=5). KLF2 gene therapy early-on in CH (n=6) significantly reduced the RV weight ratio to 0.31 ± 0.02 (compared to both CH+pCMV-null and CH alone groups). *P<0.05 versus normoxic sham. $P<0.05 versus CH alone. #P<0.01 versus CH plus sham-transfection (CH + pCMV-null). Data are mean ± SEM.
Figure 13.11. After twenty-one days of CH rat lungs were excised and prepared for histological evaluation. All vessels found in two medial pulmonary cross-sections in each rat were imaged on a Nikon Eclipse E800 microscope at 40X magnification and later Image J software was used to measure pulmonary arterial medial area (representative images shown on left). In pulmonary arterioles with an external vessel diameter of 30 to 50 µm, the medial area in the KLF2 gene therapy treatment group (771 ± 23 µm², n=7) was not significantly different than that of the sham-transfected control group (CH+pCMV-null; 770 ± 48 µm², n=5). Means are not significantly different between groups (N.S., P=0.97). Data are mean ± SEM.
13.2.1.3. Does KLF2 knockdown in CH-induced PH worsen the disease?

Using the jetPEI delivery system, optimized for DNA transport, a plasmid for the expression of short-hairpin RNA against KLF2 achieved KLF2 knockdown in the resident pulmonary ECs (Figure 12.5). Gene knockdown at day three of CH exposure, when KLF2 levels are known from previously mentioned data to already be reduced to 0.3 ± 0.1 fold of sham levels (P<0.05; Figure 11.9), resulted in a significant worsening of RVSP (91.6 ± 7.0 mmHg, n=9) compared to the hypoxic sham-transfected control group (71.0 ± 6.4 mmHg, n=9) at day twenty-one (Figure 13.12). As well, there was a significantly higher RV weight ratio in the KLF2 knockdown group (0.47 ± 0.02, n=9) versus the transfected controls (0.39 ± 0.02, n=9) after twenty-one days of CH exposure (Figure 13.13). Data from quantitative analysis of medial area in pulmonary arterioles with an external diameter between 30 and 50 micrometers showed that further decreases in KLF2 expression early on in CH resulted in increased vessel medial area (867 ± 39 µm², n=6) significantly more so than in sham-transfected hypoxic animals (728 ± 21 µm², n=6; Figure 13.14). Results of comparisons of medial area in larger and smaller pulmonary arterioles between treatment groups are shown in Appendix G.
Figure 13.12. Rats were housed in either 10% oxygen (chronic hypoxia, CH) or normal room air for twenty-one days. In CH groups, after three days of hypoxia rats were either given plasmids encoding short hairpin RNA for KLF2 knockdown (pRS-shKLF2), sham-transfection of an empty plasmid (pRS-null) or no gene expression modifiers. On day twenty-one, right ventricular systolic pressure (RVSP) in all groups of CH rats was significantly higher than in normoxic sham rats (27.0 ± 0.9 mmHg). RVSP in the CH alone group (73.4 ± 3.7 mmHg, n=8) was no different than in the CH plus sham-transfection group (CH+pRS-null: 71.0 ± 6.4 mmHg, n=9). Exogenous KLF2 gene knockdown early-on in CH significantly exacerbated CH-induced increase in RVSP to 91.6 ± 7.0 mmHg (n=9) compared to both CH plus pRS-null and CH alone groups (P<0.01). *P<0.01 versus normoxic sham. Data are mean ± SEM.
Figure 13.13. After three days of being housed in 10% hypoxia (CH) rats were given treatment to knockdown KLF2 gene expression (pRS-shKLF2), sham-transfection (pRS-null) or no gene knockdown. At day twenty-one of CH exposure, right ventricular (RV) hypertrophy was assessed as a ratio of RV weight to left ventricle plus septum (LV+S) weight. Each CH group had significantly increased RV weight ratios compared to the normoxic sham control group (0.22 ± 0.00). Right ventricle weight to LV+S weight in the CH alone group (0.35 ± 0.04) was no different than in the CH plus sham-transfection group (CH+pRS-null; 0.39 ± 0.02, n=9). Forced KLF2 gene knockdown early-on at day 3 of CH significantly increased the RV weight ratio to 0.47 ± 0.02; compared to both CH+pRS-null (n=9) and CH alone groups (n=6). *P<0.05 versus normoxic sham. $P<0.05$ versus CH alone. #P<0.01 versus CH plus sham-transfection (CH + pRS-null). Data are mean ± SEM.
Figure 13.14. After twenty-one days of CH, rat lungs from all treatment groups were excised and prepared for histological evaluation. All vessels found in two medial pulmonary cross-sections in each rat were imaged on a Nikon Eclipse E800 microscope at 40X magnification. Image J software was used to measure pulmonary arterial medial area in vessels with an external diameter of at least 30µm and no greater than 50µm. Shown on the following page are representative images of hemotoxylin and eosin stained cross-sections of rat lung depicting representative pulmonary arterioles. All CH groups had increased medial area within pulmonary arterioles compared to the normoxic sham group (451 ± 34 µm$^2$, n=5). KLF2 knockdown, at day 3 of CH exposure, shown above, resulted in a further increased in medial area (867 ± 39 µm$^2$, n=6) beyond that of both CH alone (727 ± 34 µm$^2$, n=6) and sham-transfected + CH (728 ± 21 µm$^2$, n=6). *P<0.05 versus normoxic sham. $P<0.05$ versus CH alone. #P<0.01 versus CH plus sham-transfection (CH + pRS-null). Data are mean ± SEM.
Figure 13.14.
13.2.1.4. Can Increasing KLF2 in Established PAH Reverse Disease Progression?

In Figure 13.15, a single dose of KLF2 gene therapy at day twenty-one was unable to decrease RVSP (80.3 ± 5.6 mmHg) compared to MCT alone (84.5 ± 12.4 mmHg) and MCT plus sham transfection (83.8 ± 8.7 mmHg) at day thirty-five. In all MCT treatment groups RVSP increased from day twenty-one to day thirty-five (Appendix G). Also in MCT groups, there were no benefits to right ventricular hypertrophy in the animals receiving KLF2 treatment (0.49 ± 0.02) compared to sham-transfected controls (0.52 ± 0.04) or saline-injected sham controls (0.46 ± 0.03) (Figure 13.16). Klf2 overexpression at twenty-one days post-MCT, in established PAH, did not effect the medial area in PAs having an external diameter between 30 to 50 micrometers compared to either sham transfected controls (646 ± 48 µm² versus 668 ± 14 µm² respectively, n=; Figure 13.17). Klf2 treated rats, on average, lost half as much weight from day 21 to day 35 compared both MCT+pCMV-null and MCT+vehicle groups (Figure 13.18), however, there were no differences in mortality in any of the MCT groups (Figure 13.19).
Figure 13.15. Twenty-one days following MCT injection, animals were given either KLF2 gene therapy (pCMV-KLF2), sham transfection (pCMV-null) or saline injection. Two weeks later, at day thirty-five, RVSP was assessed. KLF2 gene transfer did not improve RVSP 14 days later, at 35 days (80.3 ± 5.6 mmHg, n=8) versus MCT+ vehicle (84.5 ± 12.4 mmHg, n=4) and MCT+pCMV-null (83.8 ± 8.7 mmHg, n=6). *P<0.01 versus saline-treated control. Data are mean ± SEM.
**Figure 13.16.** Twenty-one days following MCT injection, animals were given either KLF2 gene therapy (pCMV-KLF2), sham transfection (pCMV-null) or saline injection. Two weeks later, at day thirty-five, RV hypertrophy was assessed as a ratio of RV weight to left ventricle plum septum (LV+S) weight. KLF2 gene transfer did not improve RV/(LV+S) weight ratio 14 days later, at 35 days (0.49 ± 0.02, n=10) versus MCT+saline (0.46 ± 0.03, n=4) and MCT+pCMV-null (0.52 ± 0.04, n=9). *P<0.01 versus saline-treated control. Not significantly different (N.S.); P=0.33. Data are mean ± SEM.
Figure 13.17. Thirty-five days following MCT administration, rat lungs were excised and prepared for histological evaluation. All vessels found in two medial pulmonary cross-sections in each rat were photographed using a camera and a Nikon Eclipse E800 microscope at 40X magnification. Later, Image J software was used to measure pulmonary arterial medial area in vessels with an external diameter of at between 30µm and 50µm. KLF2 gene transfer at 21 days post-MCT treatment, when severe PH is established in this model, did not lessen the vascular remodeling seen at day 35 (668 ± 14 µm² versus 646 ± 48 µm², MCT + pCMV-null and MCT + pCMV-KLF2 respectively, n=4-5, P=0.71). Not significantly different (N.S.). Data are mean ± SEM.
Body Weight

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<thead>
<tr>
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<th>MCT + VEHICLE (5% GLUCOSE)</th>
<th>MCT + PCMVT-NULL</th>
<th>MCT + PCMVT-KLF2</th>
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<tbody>
<tr>
<td>SHAM</td>
<td>(24 ± 4 g, 268 ± 6 g)</td>
<td>(224 ± 4 g, 204 ± 20 g)</td>
<td>(197 ± 11 g, 176 ± 11 g)</td>
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**Figure 13.18.** Average body weight (Day 21, Day 35). Data are mean ± SEM.
### Mortality

<table>
<thead>
<tr>
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<th>Sham</th>
<th>MCT + Vehicle (5% Glucose)</th>
<th>MCT + PCMV-Null</th>
<th>MCT + PCMV-KLF2</th>
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<tr>
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<td>0 (10)</td>
<td>1 (5)</td>
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**Figure 13.19.** Absolute mortality (total number of experiments at Day 21). Data are mean ± SEM.
DISCUSSION

It is generally understood that pulmonary EC injury underlies the development of PAH. Increasingly, it has been recognized that EC apoptosis in response to injury is the initiating event which leads to PA remodeling and functional loss of small pre-capillary arteries [1, 12, 117, 278]. Current PAH treatments attempt to improve pulmonary EC dilatory function in the remodeled pulmonary arteries and arterioles by using vasodilatory therapies such as CCBs, prostacyclin analogues, ET receptor antagonists and PDEV inhibitors. Yet, these therapies are grossly deficient since all patients with PAH will show progression of the underlying disease despite therapy and that the 87% majority of PAH patients do not respond acutely to vasodilatory testing using inhaled NO or intravenous prostaglandin [31]; which suggests that pulmonary vasoconstriction may only have a small role in PAH in symptomatic patients. It has recently been recognized that angiogenic proteins may be protective in PAH, possibly by preventing apoptosis of endothelium as well as promoting the repair and regeneration of the lung microcirculation [79, 80, 119]. Previous studies and results from PH models in Bmpr2 deficient mice corroborate that the pulmonary endothelium is central to PAH initiation, pathogenesis, and disease progression, where ECs are activated and actively contributing to vasoconstriction, vascular inflammation, and PA remodeling.

My study is the first to look into the role of endothelial transcription factors that promote a healthy, quiescent endothelium in models of PH. KLF2 is a shear stress induced zinc finger transcription factor that is highly expressed in the vascular endothelium of the lung [195], known to contribute to angiogenesis by stabilizing newly formed blood vessels, and acts as a “master switch” turning on a profile of gene expression consistent with a healthy, quiescent endothelial phenotype [36, 82, 132, 147, 195, 198, 211, 279, 280]. In this
study I report for the first time to my knowledge that endothelial transcription factor KLF2 preserves EC function; and inhibits PA remodeling in the MCT model of PAH, but not in experimental CH-induced PH; reducing PH development in both models of PH. Beyond uncovering mechanisms related to improving hemodynamics and reducing RV hypertrophy, my study also provides evidence that an early decrease in lung Klf2 expression may be a critical event that gives rise to a permissive environment for PH development.

14 ENDOTHELIAL APOPTOSIS & VASCULAR REMODELING

Work from a number of groups, including ours, has lead to the realization that EC apoptosis represents a critical link between vascular injury and vascular remodeling in development of PAH and eventually to the obliterative and degenerative changes to the pulmonary microvasculature that characterize the advanced stages of this disease [15, 33, 44, 117, 149, 278]. Reports from the Voelkel and Tuder labs provided evidence that obliterative plexiform lesions, containing hyper-proliferative and apoptosis-resistant cells are formed following widespread EC apoptosis in rats in the Sugen-CH model of PH. In addition, results from previous work demonstrated that Bmpr2 mutations, known to cause hereditable PAH [6, 7], result in EC apoptosis [28], as well as SMC proliferation and dysregulated growth [63]. Thus, Stewart and colleagues suggested that EC apoptosis is the initiating event in PAH which leads to the degeneration of pre-capillary arterioles and the selection of hyper-proliferative, apoptosis-resistant ECs which contribute to plexiform lesion formation [278]. The role of plexiform lesions in the progression of PAH is still a matter of debate as they are not universally seen in end-stage disease. However, loss of ECs at the level of the fragile pre-capillary arteriole could result in “dropout” and loss of efficient perfusion of the distal pulmonary arterial-capillary unit bed. If widespread, a devastating loss in cross-
sectional pulmonary vascular area alone could lead to increased PVR. Here, I provide additional evidence that inhibiting EC apoptosis by Klf2 gene transfer at the early stages (i.e. 10 days) of the MCT model prevents the development of PAH, improving RVSP and RV hypertrophy at 21 days post-MCT. This initial reduction in MCT-induced EC apoptosis was associated with reduced underlying vascular remodeling. Anti-apoptotic actions of KLF2 have also been reported by Wang et al., demonstrating that siRNA inhibition of KLF2 sensitized HUVECs to oxidized LDL-induced apoptosis, but had no effect on basal apoptosis in non-stimulated control cells [214]. It is also possible that the anti-proliferative actions of increased eNOS expression, a gene up-regulated by KLF2 [281], contributed to reductions in PA remodeling in my study. In addition, new evidence suggests that KLF2 may increase the expression of EC microRNAs (miRNAs) such as miR-143/145, which can then be transported in extracellular vesicles to regulate SMC gene expression; thereby altering the function and phenotype of co-cultured SMCs [282].

15 ENDOTHELIAL FUNCTION

My study showed that Klf2 knockdown in CH-induced PH model was associated with both worsening RVSP and increased PA remodeling; however, Klf2 gene transfer protection in this same model were not associated with reduced PA remodeling. This latter result suggests that PA remodeling does not necessarily have a role in reductions to PH development that were afforded by Klf2 gene transfer. Besides pulmonary vascular structural changes, KLF2 has the ability to induce a strong vasodilatory EC phenotype by altering the expression of eNOS, ACE, ET-1, and CNP, as well as indirectly increasing the production of CO by increasing HO-1 expression [81, 82, 198, 219]. ENOS gene transfer as well as the use of PDEV inhibitors to increase NO, have been shown to be beneficial in PAH by restoring...
EC dilatory function and, in theory, may act to suppress cellular proliferation as well as induce PA SMC apoptosis to reverse PA remodeling in human disease [119, 273, 283-285]. My study suggests that the prevention of CH-induced PH by KLF2 may be in large part be mediated by up-regulation of eNOS-dependent pathways and maintenance of EC dilatory function. KLF2 can enhance NO production by directly up-regulating enos expression, decreasing caveolin-1 expression [82], and transcriptionally activating argininosuccinate synthase [210, 223], thus sustaining NO synthesis and bioavailability. Previous studies have also reported that pulmonary vascular remodeling may be a consequence, rather than the cause, of increased PVR. For instance, James West et al. showed that increased RVSP in a transgenic mouse model overexpressing a “dominant negative” Bmpr2 mutation targeted to VSMCs occurred even in the absence of any evidence of arterial remodeling and muscularization [286]. They suggested that this could be due to enhanced vasoconstriction [286]. Our group and others have shown that ET-1 is overproduced in human disease and animal models of PH [24, 287-289], thus an alternative mechanism by which restoration of KLF2 levels in experimental PH acts to improve EC function, may be through transcriptional inhibition of ET-1. It is also possible that KLF2 elevated HO-1 expression which has been shown in other studies to prevent CH-induced PH, potentially through vasodilatory and anti-proliferative effects of CO produced from the enzymatic breakdown of heme by HO-1 [134, 135]. Interestingly, there is evidence in severe PH models and in human disease that KLF2 transcriptional targets HO-1 and caveolin-1 are expressed in healthy lung but are absent or have reduced expression in plexiform lesions and muscularized pulmonary arterioles [78].
Previous work on angiogenic factors has focused on inducing microvascular angiogenesis with the aim of regenerating and restoring the obliterated and degenerated pulmonary arteriolar bed. Our laboratory has shown that cell-based gene therapy with angiogenic factors ANGPT1 and VEGF can attenuate MCT-induced PAH, possibly by inducing angiogenesis in the microvasculature [15, 16]. Landmark studies using enos-transfected endothelial progenitor cells demonstrated disease reversal in the MCT model which was linked to restoration of the continuity of the distal arterial bed presumably afforded by the angiogenic actions of NO [119, 290, 291]. Sphingosine-1-phosphate (S1P) is a platelet-derived lipid known to regulate EC migration, adherens junction assembly, and shown to have a role in regulating angiogenesis by signaling through the endothelial sphingosine-1-phosphate receptor-1 (S1P1/Edg) [292, 293]. Additionally, there are reports that S1P1 signaling is protective against EC apoptosis and promotes vasodilation by maintaining eNOS activation [292, 294, 295]. It is therefore interesting that the phenotype of embryonic lethality and abnormal blood vessel formation in Klf2 knockout mice overlaps with both those of the Angpt1-Tie2 and S1P1/Edg angiogenic pathways; lethality occurs at E12.5-E14.5 [196], E12.5 [237], and E13.5-E14.5 [296], respectively. Absence of either the Klf2, Angpt1, or S1P1 gene in mice results in similar incomplete vascular maturation from absence of recruitment of pericytes which is required for blood vessel stabilization, suggesting that these genes are part of complementary angiogenic pathways [196, 237, 296]. Consistent with this idea of crosstalk are the reports that KLF2 transcriptionally up-regulates S1P1 in T-cells [206], inhibits hypoxia-mediated secretion of angiopoietin-2 (ANGPT2, a context-dependent ANGPT1-TIE2 antagonist) [297, 298], and in the presence of cell-cell contact, ANGPT1-TIE2 signaling induces KLF2 expression [84]. Furthermore ANGPT1 alone was shown to induce Klf2 ex-
pression by stimulating the Klf2 promoter [84]. Results from studies in models of hypoxic PH have provided evidence that decreases in the ANGPT1-TIE2 pathway contribute to PAH and can be overcome by Angpt1 gene transfer [79, 80]. Also, in the MCT model of PAH, increasing ANGPT1 by cell-based gene transfer inhibited EC apoptosis and protected the pulmonary microvasculature, resulting in increased survival and improved RVSP and RV hypertrophy [15]. Thus KLF2 may act to increase the expression of angiogenic factors in complementary angiogenic pathways to inhibit the pathogenesis of PAH. Boon et al. published exciting results that Klf2 overexpression increased both the numbers and neovascularization abilities of bone-marrow-derived mononuclear PACs in a mouse hind-limb ischemia model [238]. Therefore, it is possible that cell-based gene therapy using KLF2 in PACs might improve the neo-vascularization beyond the current limits of existing angiogenic therapies; however, it remains to be determined how to best make use of KLF2’s ability to indirectly induce microvascular angiogenesis in PAH through the modulation of angiogenic gene expression.

17 TRANSLATIONAL PERSPECTIVE

Given the wide variety of factors and processes which have been shown to contribute to the onset and progression of PAH, and the complexity involved in trying to reverse established changes in pulmonary arterial structure and function, it is not surprising that strategies focusing on a single pathway are likely to be insufficient. It may arguably be more promising to use a therapeutic strategy targeting a master gene, like KLF2 which regulates 1039 endothelial genes (600 induced, 439 repressed) [36], rather than picking one target. Overexpression of Klf2, a known master switch for a profile of genes that result in endothelial homeostasis, could have multiple effects that together are more powerful in restoring...
normal endothelial function and pulmonary vascular structure in PAH. My study demonstrates that a 0.37 fold knockdown of Klf2 expression significantly reduced EC dilatory function, suggesting that early decreases in Klf2 expression may contribute to PH development in both CH and the MCT models by resulting in EC dysfunction. In support of the physiological importance of even a 50% reduction in Klf2 expression, heterozygous KLF2 knock-out mice showed increased diet-induced atherosclerosis in apolipoprotein E-deficient background [299]. Also, in another study, KLF2 hemizygous mice developed a more severe methylated-BSA and IL-1β-induced arthritis compared to littermate wild type controls [300].

However, my experiments showed that Klf2 gene transfer at day 21 failed to reduce RVSP and RV hypertrophy at day 35, suggesting that this strategy may not be effective in the treatment of establish PAH. Since patients invariably present with relatively fairly advanced disease, this may mean that the translational potential of Klf2 gene therapy may be limited. This is consistent with KLF2's role as an EC quiescent factor, which predominantly acts to maintain vascular homeostasis. Future investigations attempting to reverse the established PAH may require strategies directed vascular repair mechanisms and induce regeneration or restoration of functional pulmonary microcirculation. It is still possible that KLF2 may be useful as an adjunctive strategy to restore a quiescent EC phenotype in the context of such an angiogenic therapeutic strategy. As well, it may able to reverse or inhibit the progression of established PAH in other models of PAH, in particular the angioproliferative model induced by Sugen and CH, or even human disease where there are proliferative and oblitative plexiform lesions. If so, KLF2 may provide the fine tuning necessary to protect against site and cell state specific apoptosis in fragile and functional pulmonary arterioles while decreasing cell growth in angioproliferative lesions. Consistent
with this idea, Wang et al. showed that KLF2 overexpression in cells with aberrant growth (ovarian cancer cell lines) reduced cell growth and sensitized cells to DNA damage-induced apoptosis [301]; in a separate study, another group showed that KLF2 may act to protect against apoptosis in normal ECs [214]. If not, a direct therapeutic transgene in established PAH, Klf2 gene transfer may be a valuable adjunctive therapy to maintain pulmonary EC homeostasis; thereby preventing the re-initiation of the disease or to inhibit its development in the first place in individuals at high risk based on genetic or environmental factors. This may be particularly useful in pediatric congenital heart disease patients after corrective surgery or carriers of Bmpr2 mutations. Furthermore, one may consider levels of Klf2 expression as an important indicator of pulmonary microvascular health and therefore could be used as identifier biomarker of individuals at risk of PH development; in particular among carriers of the Bmpr2 mutations. KLF2 may also have an indirect role in maintaining BMPR2 signaling, since KLF2 is known to up-regulate BMPR1A [36], a transmembrane protein that is required for BMPR2 signaling [302, 303]. To the best of my knowledge KLF2 has not been reported to regulate Bmpr2 expression, however, it is interesting that upstream SMURF1 ubiquitin ligase is known to degrade KLF2 as well as down-regulate BMPR1 and 2 receptors [304, 305]; and SMURF1 is reportedly induced in both MCT and CH models of PH [304]. Thus, inhibition of SMURF1 may be an alternative target to maintain both KLF2 expression and BMPR2 signaling.

18 IN VITRO: CELL MOTILITY

*In vitro, Klf2* overexpression reduced basal EC migration; and even though KLF2 is known to downregulate Vegfr-2 expression, the anti-migratory effects of increased Klf2 were not dependent on VEGF stimulus. Thus, this lack of involvement of VEGF suggests that
KLF2 did not alter EC chemotaxis but rather reduced chemokinesis (undirected cell motility). Studies by Boon et al. demonstrated that overexpression of Klf2 in ECs resulted in dephosphorylation of focal adhesion kinase and activation of the small guanosine triphosphate (GTP-ase) Ras homolog gene family member A (RhoA), altering the organization of the actin cytoskeleton which would reduce EC motility [306]. Others have speculated similarly, suggesting that HDAC4 promotes random cell motility through the down-regulation of Klf2 transcription [307]. In a standard wounding assay, viral transduction of Klf2 also reduced EC migration compared to non-transduced controls and did not alter VEGFR2 phosphorylation (necessary for VEGF-mediated EC migration) [36]. As an alternative mechanism for KLF2-associated decreased EC migration, Dekker et al. suggests that KLF2 may act through transcriptionally down-regulating EC chemorepulsant SEMA3F [36, 308].

**19 HYPOXIA & THE PROTECTIVE ROLE FOR HIGH LEVELS OF LUNG KLF2 EXPRESSION**

Results from *in vitro* experiments showed that endothelial Klf2 expression is reduced after 24 hours of hypoxia (1% O2). This observation suggests that hypoxia may be one mechanism of the early reduction in Klf2 expression in the CH model. Recently, certain microRNAs (miRNAs), such as miR-92a, which have been implicated in the pathogenesis of PH [309], have been shown to reduce KLF2 expression [310]. Therefore, this provides another possible mechanism for reduced Klf2 expression in hypoxia. Interestingly, miR-92a was demonstrated to be transiently induced at day 7 of CH in mice [311], which is accordant with the transient decreased Klf2 expression observed in the CH rat model in this study.
Elevated KLF2 levels are known to turn on a profile of endothelial gene expression that is consistent with EC health, and results from this study add to the recent reports that high levels of KLF2 may be protective in cardiovascular disease [36, 299]. Uniquely, my study provides evidence that lung EC-targeted KLF2 gene transfer is protective in pulmonary vascular disease.

The effects of CH can also be studied in animal models at high altitudes. The Fawn-Hooded rat (FHR) spontaneously develops PH and is particularly sensitive to high altitudes; in Denver developing PH at only 1 month after birth (elevation 1600 metres) [312], whereas PH takes 4–10 months to develop at a lower altitude in Edmonton, Alberta (668 metres) [313]. The increased sensitivity of the FHR for PH is thought to be in part caused by reduced eNOS expression and increased ET-1 expression in response to mild hypoxia [314]. Thus, I speculate that there is a role for KLF2 in adaptation to high altitude by acting to upregulate enos and downregulate Et-1. In addition FHR-Brown Norway 1 (FHR-BN1) consomic rats are identical to FHR except that they have a chromosome 1 substitution which renders them hypoxia-resistant [315]. Interestingly, FHR-BN1 rats, unlike the FHR, do not have activation HIF-1α under normoxic conditions [313]. This observation presents a concomitant mechanism for KLF2 in adapting to high altitudes through its ability to inhibit hypoxia-induced expression of HIF-1α [297]. Moreover, there is evidence in the literature that KLF2 is important for cell viability under hypoxia and early angiogenic conditions, whereby after exposure to hypoxia, decreases in KLF2 expression of more than 80% increased EC death [297]. This finding supports our hypothesis that the delayed recovery of Klf2 expression in the CH model may represent an adaptive mechanism in this reversible model of PH.
LIMITATIONS

ENDOTHELIAL CELL LOSS

Although this study suggests that the early down-regulation of Klf2 may be an important mechanism contributing to PH development, I cannot fully exclude the possibility that the decreased in lung KLF2 mRNA expression may be partly explained by EC loss. To address this possibility, Klf2 expression could be assessed in lung tissue sections using in situ hybridization and compared to sham controls. A more definitive measurement of EC loss in our models would be beneficial to not only my study but future work implicating reduced EC gene expression in MCT-induced PAH where there is known EC injury. One way this could be done is by enzymatic digestion of whole lung tissue followed by flow cytometry, using fluorescent-labelled antibodies against EC markers such as platelet endothelial cell adhesion molecule-1 (PECAM-1) and/or VCAM-1 to quantify the overall number of ECs in the lung [316]. This was done in another study from the Stewart lab, in which it was shown that reduced lung expression of Tie2, another endothelial-selective gene, was not related to an overall decrease in the proportion of ECs after endotoxin lung injury (unpublished data from Sarah McCarter; [317]).

PULMONARY HYPERTENSION MODELS

The rat models of PH used in this study are models of the WHO’s categorization of group 1 and 3 PH. Group 1 PH, PAH, is modeled in this study by MCT-induced PAH in rats, and the group 3 PH, hypoxic PH, is modeled by exposure of rats to chronic hypoxia for 3 weeks. Our laboratory is primarily interested in PAH, wherein which the ideal model would spontaneously develop; have a female predominance; right ventricular hypertrophy;
show medial hypertrophy, intimal hyperplasia and adventitial thickening of small PAs; plexiform lesions; increased inflammatory cells infiltrate; and mortality would ensue from RV heart failure [274]. In the CH model, by contrast, PH eventually occurs over prolonged exposure to hypoxia, and is ultimately reversible. Other PH experts agree that this model presents modest PH that does not recapitulate the extent of abnormalities in the small PAs, pulmonary haemodynamics or survival in humans [274]. The MCT model is limited by the absence of intimal and plexiform lesions, and lethality that may be a result of hepato- and renal toxicity [108, 118, 119]; although MCT is though to be primarily toxic to the lung endothelium. Importantly, the MCT model does reproduce abnormalities in PASMCs: increased proliferation, decreased apoptosis and hypertrophy; and reflects the increased apoptosis of ECs seen in group 1 PH patients [274, 318]. In my study, Fischer 344 (F344) rats were chosen because this in bred strain exhibits a predictable response to MCT compared to Sprague-Dawley and Wistar rats. For instance, all F344 rats in my study developed PAH, RV hypertrophy and remodeling of PAs, whereas reportedly only 75% of MCT-treated Sprague-Dawley rats present these same changes [319]. Also in Wistar rats, weighing less than 230g, MCT caused 100% death within 3 weeks [319]. Some of these differences can be overcome by changing the dose of MCT; however, it was particularly important for my study to have consistent survival at 3 weeks post-MCT to conduct experiments in established PAH at day 21, assessing the effect of gene transfer at day 35. Another limitation is that both models used in my study generally use male rats, whereas human idiopathic PAH is predominantly a female disease. Unfortunately, there is limited research using female rats which are known to be less sensitive to MCT-treatment than male rats [320]. There is evidence in the literature that this female-resistance to MCT-induce PAH may be afforded by the protective effects of oestradiol [320]. Moreover, my decision to investigate the role of KLF2 in male rats
is a first step in elucidating a role for KLF2 in PH, and future studies should consider experiments conducted in female rats.

OUTSIDE OF THE VESSEL WALL: OTHER CELLS EXPRESSING KLF2

Intravascular jetPEI-mediated DNA delivery may result in transgene expression in cells other than lung ECs which may have implications for the interpretation of these data [321]. We are not concerned about overexpression of Klf2 in VSMCs, since Klf2 is not normally expressed in SMCs and our results from early experiments forcing Klf2 expression in SMCs had no effect on cell proliferation and/or survival (Appendix L). I have no direct evidence to exclude the possibility that Klf2 gene transfer may have occurred in the systemic vasculature; even if this did occur it would be highly unlikely to affect the development of PH. However, my data do provide some evidence that macrophages were transfected (Appendix Q). Some leukocytes do express KLF2, and thus it is possible that its overexpression may have altered macrophage phenotype. Whether this a potential mechanism for KLF2 blunting PH development involved was not addressed by the current study, and might be an interesting avenue for future investigations.

Alveolar macrophages are known to accumulate in PH and alterations in their activity may be beneficial by reducing inflammatory cell-mediated injury in at least the inflammatory MCT model of PAH. KLF2 is not only known as a central regulator of EC gene expression but is also known to reduce proinflammatory monocyte/macrophage gene expression and result in blunting macrophage lipid accumulation [299]. Others have shown that Klf2 overexpression reduced macrophage activation and inhibited their production of pro-inflammatory cytokines such as CD40L, MCP-1, IL-1β, and TNF [322]. We therefore hy-
pothesize that alveolar macrophages transfected with Klf2, would have reduced inflammatory activation and thus lessen their inflammatory-mediated injury during PH development. On the other hand, if transfected with shKlf2, we would expect the macrophages would become activated, infiltrating and injuring the pulmonary vasculature. A simple test to quantify the DNA transfection to the non-target alveolar macrophages is to perform bronchiolar lavage twenty-four hours following intravascular jetPEI gene delivery and then isolate RNA for gene detection.

Other leukocytes such as T-cells, and mast cells (MCs), if transfected, may have a role in KLF2-mediated protection against PH development. From studies by Tamosiuniene et al. showing that T-cell activity functions to control EC injury and limit vascular injury in PAH [323], one might predict that KLF2’s gene regulation in T-cells would maintain T-cell quiescence [206], exacerbating PH. Since this was not the case, it supports the hypothesis that it is unlikely that T-cells were transfected. Hoffman et al. recently demonstrated a negative role for MCs in a rat model of PH in left heart disease (LHD) [324]. In these experiments, vascular remodeling and PH was reduced by treatment with MC stabiliser, ketotifen, or in MC-deficient rats [324]. KLF2 was recently found to be expressed in MCs and could affect MC activity [325], offering another possible mechanism that could be explored in future studies.
This study elucidates a novel role for KLF2 providing protection against the development of PH by inhibiting vascular remodeling, preventing EC apoptosis and/or by preserving endothelial-dependent dilatory function. A major finding is that KLF2 overexpression prevents both MCT-induced and CH-induced PH. The other discovery is that KLF2 is involved in preventing vascular remodeling in the MCT model, but not the CH model. We provided the first mechanistic evidence that KLF2 is required to maintain normal endothelium-dependent dilatory function in the lung vasculature and suggest that this is a mechanism for maintenance of normal pulmonary hemodynamics since RVSP significantly increased after Klf2 inhibition in normal rats. Future studies are needed to further dissect the underlying mechanisms of protection afforded by Klf2 gene transfer, such as the relative roles of KLF2’s multiple endothelial transcriptional targets such as: eNOS, ANGPT-2, S1P1, and HO-1. As well, the role of KLF2 in the regulation of inflammatory cell activity in PH models, as alluded to above, would be a fertile avenue for more investigation.

Other results from the present study warrant further investigation, including the role of the recovery of Klf2 expression in the reversibility of CH-induced PH when rats are returned to a normoxic environment. Furthermore, future studies should include assessment of KLF2’s role in a model of PH exhibiting intimal and proliferative lesions, such as the Sujen-CH model. These will be expanded upon below.

**KLF2’S ROLE IN REVERSIBILITY OF THE CH MODEL**

In the present study we postulated that the recovery of KLF2 levels following its early reduction is what related to the reversibility of the CH model of PH; which is in stark contrast to the irreversible MCT model in which the decrease in KLF2 was sustained. In
other words, the recovery of KLF2 expression may prevent progression of hemodynamic and arterial remodeling despite continued hypoxia exposure, and allow the disease to be reversible once the hypoxic stimulus is removed. Future experiments could use the 21 day CH model of PH, where rats are known to have established PH, and compare RVSP, RV hypertrophy and vascular remodeling in animals with and without KLF2 deficiency prior to and two weeks after being returned to normal air. We hypothesize that animals receiving Klf2 inhibition will have maintained the PH phenotype whereas comparative groups, transfected controls and sham controls, will have recovered.

**KLF2’S ROLE IN THE SUGEN 5416 MODEL OF PAH**

A shortcoming in both our MCT and CH model of PH is that neither model presents the hallmark intimal and complex plexiform lesions that are commonly seen in human disease. These highly proliferative lesions are known to express angiogenesis-associated markers such as VEGFR2 and unregulated vascular cell growth can ultimately obstruct the pulmonary arteries and arterioles contributing to loss of functional pulmonary vasculature [150]. Others have shown that KLF2 has a role in ordered angiogenesis by stabilizing newly formed blood vessels [196]. In the rat Sugen-CH model of PAH, a single injection of SU5416, a VEGF receptor blocker, is administered followed by 3 week exposure to CH. This results in a severe and progressive PH phenotype, despite the return to normoxia, which is characterized by proliferative lesions that develop after 8 weeks [64]. This phenotype is dependent of EC apoptosis in response to the SU compound, and can be abrogated by the co-administration of a non-specific caspase inhibitor. Even though KLF2 is also known to down-regulate the VEGF receptor it does so in a controlled context while at the same time promoting endothelial quiescence (non-activation) and survival [36, 235, 297]. We
therefore hypothesize that KLF2 would inhibit the development of PAH in this model and that KLF2 would arrest the progression of established disease, contributing to disease reversal. Alternatively, the pneumonectized rats in the MCT model also provides an angio-proliferative model of PAH in which KLF2 should be tested.

**KLF2’s Role in Human PAH and Beyond**

Future translational work should investigate *Klf2* gene modulation as a promising predictor and therapy for PH in pediatric congenital heart disease patients after corrective surgery or carriers of *Bmpr2* mutations. Levels of *Klf2* expression obtained from lung biopsies in these populations may be an important indicator of pulmonary microvascular health and could be used as an identifier of individuals initiating PH development. If the results from these studies correlate low *Klf2* expression with PH development years later, this could fundamentally alter disease treatment allowing us to provide preventative care. Specifically, targeting *Klf2* to induce its expression in these patients with low *Klf2* levels early-on during disease initiation should be then tested as a preventative therapy in clinical trials. *Klf2* gene transfer, used in my study, is only one possible means to augment *Klf2* levels *in vivo*. Opportunity, very recent reports suggest that an antagomir designed to inhibit miR92a could be the “drug” to establish *Klf2* levels which maintain EC quiescence [326]. Furthermore, in the clinic, increasing *Klf2* levels in other lung vascular diseases where it is possible to prevent progression, particularly those involving inflammatory pathways that mediate lung injury, such as acute lung injury, sepsis, and ventilator-induced lung injury should be investigated. (There is already evidence in the literature to support KLF2 as protective in endotoxin-mediated sepsis [327]).
CONCLUSION

In conclusion, we report that $Klf2$ mRNA expression is decreased early-on in models of pulmonary hypertension. In the MCT rat model of PAH, jetPEI-mediated gene transfer of $Klf2$ to the pulmonary endothelium inhibited the development of MCT-induced PAH. Exogenous $Klf2$ reduced both RVSP and RV hypertrophy, likely by disallowing the pathological remodeling of the pulmonary arterioles, and by reducing apoptosis in pulmonary ECs. Additionally, in established severe MCT-induced PAH, $Klf2$ overexpression was not sufficient to reverse the disease. The reversible CH model of PH was associated with a transient decrease in $Klf2$ expression and lung $Klf2$ gene transfer, at the time-point where $Klf2$ was reduced, inhibited the development of PH. The mechanism was not through altered vascular remodeling but rather we provide evidence that it may be through maintaining endothelial dilatory function. Supporting a direct role for KLF2 in PH, selective pulmonary $Klf2$ knockdown increased RVSP in healthy normoxic rats, likely through reduced endothelium-dependent dilation. In addition, preventing the recovery of $Klf2$ levels in the CH model using gene silencing significantly worsened both RVSP and RV hypertrophy, which was associated with underlying increased vascular remodeling, compared to sham-transfected controls.

Taken together, these results suggest that KLF2 may be highly protective against the development of PH by reducing EC apoptosis, vascular remodeling, and preventing EC dysfunction. Thus, we have established the first evidence for KLF2 as a novel therapeutic target for PH.
"Using in vivo-jetPEI, we estimate that approximately 5% of cells are transfected in the lung. This is sufficient in some models to observe an effect (see publications)."

"If you would like to observe in vivo-jetPEI-FluoR distribution on the whole animal, I would recommend using a CDD camera, and observe immediately i.e as 30 sec after injection."

"The reagent penetrates the tissues rapidly and the fluorescence may be difficult to see at the whole animal level. It needs to be checked at the cellular level, on slides." . . . "To detect in vivo-jetPEI-FluoR labelled cells on slides, I would look 2 to 4 hours after injection. At 24h after injection, the complexes are disassembled and the delivery reagent is excreted."

Best regards,
Géraldine

Géraldine Guérin-Peyrou M.Sc.
Scientific and Technical Support Specialist

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Appendix B - Hematology Data in MCT-induced PAH

Appendix B. A selection of hematology results in the MCT model of PAH. * P>0.05 versus saline-injected control. #P>0.05 versus sham-transfected control +MCT. Linear segments depict the data mean.
Appendix C. A selection of hematology results in the CH model of PH. * P>0.05 versus saline-injected control. Linear segments depict the data mean.
Appendix D - Results from Preliminary Dosing Experiments Before Selection of 150 μg of plasmid for in vivo KLF2 Gene Therapy Studies

Number of nucleated red blood cells (RBCs) per 100 WBCs:

<table>
<thead>
<tr>
<th>VEHICLE</th>
<th>PEI-P-CMV-KLF2 (150 MG OF PLASMID)</th>
<th>PEI-P-CMV-KLF2 (200 MG OF PLASMID)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>2.00</td>
<td>7.00</td>
</tr>
<tr>
<td>0.00</td>
<td>0.00</td>
<td>4.00</td>
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</table>
Appendix D. Previous page, above left, quantification of KLF2 mRNA, twenty-four hours post intravenous administration of jetPEI complexed with either 150 µg or 200 µg of KLF2 plasmids. Both of these doses were manufacturer-recommended and practical for the jetPEI system. Early results suggest that delivery of both quantities of KLF2 plasmids result in increasing lung KLF2 mRNA expression (n=2). Above right, early data suggests that 150 µg KLF2 plasmid delivery is more efficient at increasing eNOS mRNA levels than the higher 200 µg dose. Shown in the chart, below, hematology data was collected in Fisher 344 rats twenty-four hours after jetPEI-mediated tail-vein administration of 150 µg or 200 µg of plasmids for KLF2, or saline as a control. Baseline hematology data for Charles River Fisher 344 rats corresponded to values in all groups (data not shown) with the exception of an increased quantity of nucleated RBCs detected following transfection of 200 µg of KLF2 plasmids. Typically, in Fisher 344 rats the baseline quantity of nucleated RBCs does not vary with age and/or sex and consistently is 0-2 nucleated RBCs per 100 WBCs (data available on company website, www.criver.com). The increase in nucleated blood cells may be a result of off-target gene delivery to the circulating RBCs when transfection is performed at higher doses of KLF2 plasmids, since there is evidence in the literature that KLF2 is involved in the maturation and stability of primitive erythroid cells, thus KLF2 overexpression could increase nucleated RBC population [202]. Taken together, early data suggests that for this study jetPEI is used best by delivering 150 µg of KLF2 plasmids to the rat lungs.
Appendix E - Sample Calculation for Comparing Multiple Data Sets

Data was fit to sigmoidal dose-response curves and calculations were made according to Ratkowsky’s (1983) established method for evaluating whether two data sets are significantly different [328].

**Goodness of fit for separate curves:**

<table>
<thead>
<tr>
<th></th>
<th>RS (1)</th>
<th>SHKLFL (2)</th>
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<tbody>
<tr>
<td>Degrees of Freedom (df)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Absolute Sum of Squares (SS)</td>
<td>25.60</td>
<td>45.16</td>
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</tbody>
</table>

**Goodness of fit for pooled curve:**

<table>
<thead>
<tr>
<th></th>
<th>POOLED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degrees of Freedom (df)</td>
<td>44</td>
</tr>
<tr>
<td>Absolute Sum of Squares (SS)</td>
<td>102.4</td>
</tr>
</tbody>
</table>

\[
\begin{align*}
\text{df}_{\text{pooled}} &= 44 \\
\text{SS}_{\text{pooled}} &= 102.4 \\
\text{df}_{\text{separate}} &= \text{df}_1 + \text{df}_2 \\
\text{df}_{\text{separate}} &= 20 + 20 \\
\text{df}_{\text{separate}} &= 40 \\
\text{SS}_{\text{separate}} &= \text{SS}_1 + \text{SS}_2 \\
\text{SS}_{\text{separate}} &= 25.60 + 45.16 \\
\text{SS}_{\text{separate}} &= 70.76 \\
F &= \frac{[\text{SS}_{\text{pooled}} - \text{SS}_{\text{separate}}]/(\text{df}_{\text{pooled}} - \text{df}_{\text{separate}})}/(\text{SS}_{\text{separate}}/\text{df}_{\text{separate}}) \\
F &= \frac{(102.4 - 70.76)/(44 - 40)}{(70.76/40)} \\
F &= \frac{(31.64/4)}{(69.83/40)} \\
F &= 7.91/1.769 \\
F &= 4.47
\end{align*}
\]

Numerator = \text{df}_{\text{pooled}} - \text{df}_{\text{separate}}
Numerator = 44 - 40
Numerator = 4

Denominator = \text{df}_{\text{separate}}
Denominator = 40

Using the calculated F-value and F tables, P=0.004.
Appendix F. After twenty-one days of chronic hypoxia (CH) rat lungs were excised and prepared for histological evaluation. All vessels found in two medial pulmonary cross-sections in each rat were imaged on a Nikon Eclipse E800 microscope at 40X magnification and later Image J software was used to measure pulmonary arterial medial area. Medial area of pulmonary arteries and arterioles with an external vessel diameter less than 30µm, shown on left, and results from vessels of 50 to 100µm in size shown on right. Means are not significantly different between groups (N.S., P>0.10). Data are mean ± SEM.
Appendix G. Right ventricular systolic pressure (RVSP) and right ventricular weight ratio, RV/(LV+S), was assessed 21 days post-MCT administration. Either KLF2 knockdown (pRS-shKLF2) or control transfection (pRS-null) was achieved at day 7 post-MCT injection. Compared to sham-transfected controls, exogenous KLF2 knockdown neither significantly altered RVSP (64.8 ± 8.3 mmHg versus 62.6 ± 3.2, respectively; n=5) nor RV hypertrophy (0.31 ± 0.03 versus 0.32 ± 0.01, respectively; n=5-6). N.S. P>0.10. Data are mean ± SEM.
After twenty-one days of CH medial area of pulmonary arteries and arteri-oles with an external vessel diameter less than 30µm, shown below, and results from vessels of 50 to 100µm in size shown above. In large vessels, KLF2 knockdown trended towards increased medial area compared to transfected sham controls (P=0.14, n=5-6).

*P<0.05 versus normoxic shams. Data are mean ± SEM.
Appendix I - Increase in eNOS protein Following human KLF2 gene Transfer in HUVECs

Appendix I. Twenty-four hours following transduction using Ad-GFP-KLF2 at 10 MOI, eNOS and KLF2 protein expression was assessed by Western blot in HUVEC (n=3). eNOS antibody species is mouse (BD Biosciences) and the KLF2 antibody species is goat (Abcam).
Appendix J. Half a million COS-1 cells, grown in DMEM medium containing 10% FBS, were seeded into 100 mm dish. Twenty-four hours later 10 µg DNA (plasmids containing either GFP, or human KLF2, KLF4, or KLF6) were diluted in 300 µl serum-free DMEM medium. Then 60µl of Superfect reagent (Qiagen) was added and mixed by pipetting and incubated for 10 to 15 minutes at room temperature. COS-1 cells were washed with PBS, and 3mL DMEM (10% FBS) was added into each 100mm dish. The DNA/Superfect mixture was added drop-wise and treated cells were returned to incubator for 4 hours. Protein was isolated from cells and 50µg was loaded for western blot; while the positive control was obtained from endothelial cells exposed to steady-shear stress tests completed in Dr. Lowille Langille’s laboratory. Shear tests were conducted in endothelial cells 24 to 48 hours post-confluence in fibronectin-coated 10 mm cell culture dishes. Physiological steady shear was maintained for 18 hours pre-protein collection. Plasmids for KLF4 and KLF6 were a generous donation from Dr. Mukesh Jain’s laboratory.
Appendix K - Counterstain for Fluorescent Microangiography

1. Place lung sections in wells (using 24 well plate)
2. Blocking: (0.25% Triton X-100 + 2% BSA + 3-5% Goat serum) in PBS for 1 hour @ RT
3. 1° Antibody: (α-smooth muscle Cyanine-3 conjugated, 1:200 dilution)
4. Wash with PBS 3 x 5’
5. TO-PRO-3 Iodide in PBS (1:2000 dilution) for 15’ @ RT
6. Wash with PBS 4 x 5’
Appendix L - In vitro pilot study: SMC Proliferation following KLF2 gene transfer

![Graph showing cell count over time for different groups (control, GFP, KLF2)]
Appendix M - Early test study: KLF2 mRNA expression 24 hours following in vitro jetPEI-mediated pCMV-KLF2 transfer in rat aortic endothelial cells

RAECs - 24hrs following transfection

Fold change of KLF2 gene expression /Cyclophilin A

n=2

RAECs - 24hrs following transfection

Fold change of eNOS gene expression /Cyclophilin A

n=2
Appendix N - Pilot study: KLF2 mRNA expression in human circulation-derived endothelial progenitor cells (EPCs)

![Graph showing the fold change in KLF2 mRNA expression in human circulation-derived EPCs across healthy controls and patients with PAH after 7 days in culture.](image-url)
**Appendix O - KLF2 mRNA expression in human PAH: from the National Institute of Health GeoSets Database GSE15197**

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**Appendix O.** There was no significant difference in KLF2 mRNA expression levels between controls (healthy part of Cancer lungs, n=13) and PAH lung tissue, n=18.
Appendix P. The results are from data described as from pooled RNA from laser-microdissected arteries from lungs of 3 IPAH patients: male and female, age 28-64, IPAH Heath & Edwards grade 3-4, and arteries were <750 µm diameter. Results were that KLF2 mRNA expression is not significantly different in IPAH arteries versus control (P=0.81; n=3).
Appendix Q. Twenty-four hours following delivery of jetPEI-pGFP complexes, containing a total of 150µg pGFP, lungs were harvested for immunostaining for green fluorescent protein (GFP). Since lung tissue is highly green auto-fluorescing, GFP presence was assessed using immunohistochemistry. In lung samples from animals receiving the GFP plasmid, shown above right, immunostaining for GFP protein was positive (brown staining; using only a biotin-conjugated primary antibody); control lung sections, above left, stained negative. Arrows point out macrophages.
Appendix R - Evidence of GFP Detection in Pulmonary Arterioles

Appendix R. Twenty-four hours following delivery of jetPEI-pGFP complexes, containing a total of 150µg pGFP, lungs were harvested for immunostaining for green fluorescent protein (GFP). In lung samples from animals receiving the GFP plasmid, shown above right, immunostaining for GFP protein was positive (brown staining; using only a biotin-conjugated primary antibody); control lung sections, above left, stained negative.
REFERENCES


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