Role of the SphK1/S1P Signaling Axis in the Pathogenesis of Pulmonary Arterial Hypertension

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THESIS

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This thesis is dedicated to my parents, Roman and Robyn Sysol, for their unwavering love and support. They instilled in me the value of hard work and have always encouraged me to pursue my dreams. I am forever grateful for the sacrifices they have made to allow me to pursue and achieve a higher education.

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Bear Ithaca always in your thoughts.
Arriving there is the goal of your journey;
but take care not to travel too hastily.
Better to linger for years on your way;
better to reach the island's shores in old age,
enriched by all you've obtained along the way.
– Cavafy, *Ithaca*

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LIST OF ABBREVIATIONS

3'-UTR	3' untranslated region
BMPR2	Bone morphogenetic protein receptor type II
BRDU	5-bromo-2'-deoxyuridine
ChIP	Chromatin immunoprecipitation
EGR1	Early growth response protein 1
ERK	Extracellular-signal-regulated kinase
ET-1	Endothelin 1
GLUT1	Glucose transporter 1
GPCR	G-protein-coupled receptor
GSK-3	Glycogen synthase kinase 3
HIF1α	Hypoxia-inducible factor 1 alpha
hPASMC	Human pulmonary artery smooth muscle cells
HPH	Hypoxia-mediated pulmonary hypertension
MEK	MAPK/ERK Kinase; Mitogen-activated protein kinase kinase
miR	MicroRNA
mPAP	mean pulmonary arterial pressure
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NO	Nitric oxide
PA	Pulmonary artery
PAEC	Pulmonary artery endothelial cell
PAH	Pulmonary arterial hypertension
PASMC	Pulmonary artery smooth muscle cell

LIST OF ABBREVIATIONS (continued)

PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PH	Pulmonary hypertension
pVHL	Von Hippel-Lindau protein
PVR	Pulmonary vascular resistance
ROS	Reactive oxygen species
RVH	Right ventricular hypertrophy
RVSP	Right ventricular systolic pressure
S1P	Sphingosine-1-phosphate
S1PR1-5	Sphingosine-1-phosphate receptor 1-5
SGPL1	Sphingosine-1-phosphate lyase 1
SPHK1	Sphingosine kinase 1
SPHK2	Sphingosine kinase 2
STAT3	Signal transducer and activator of transcription 3
VEGFA	Vascular endothelial growth factor A
WT	Wild type

SUMMARY

Pulmonary arterial hypertension (PAH) is a severe, progressive and fatal disease for which there is currently no curative treatment available. Pathologic changes in this disease involve remodeling of the pulmonary vasculature, including marked proliferation of smooth muscle and endothelial cells, leading to occlusion of vessels via poorly understood mechanisms. This remodeling causes increased pulmonary vascular resistance (PVR) and subsequent pulmonary artery pressure, ultimately leading to right heart failure due to pressure overload, a major cause of death. Despite active research in PAH pathobiology, therapies to hinder or reverse the pathologic vascular remodeling in this debilitating disease are lacking. The data presented in this dissertation provide novel insight into the role of the sphingosine kinase 1 (SphK1) and sphingosine-1-phosphate (S1P) pathways in the pathogenesis of PAH.

SphK1/S1P are important mediators of vital biological processes including regulation of cell survival, proliferation, and migration in a wide variety of cell types. SphK1 phosphorylates sphingosine to form S1P, which then acts as a bioactive signaling molecule via ligation to a family of five G-protein coupled receptors (S1PR1-5). Given the recognized role of SphK1/S1P in the lung vasculature and its promotion of cell proliferation, we hypothesized that this pathway may be important in the pathogenesis of pulmonary vascular remodeling in PAH. Together, our data demonstrates that SphK1/S1P signaling axis is a novel therapeutic target in PAH.

We have demonstrated that both SphK1 and S1P levels are upregulated in the lungs of patients with PAH and in preclinical rodent models of hypoxia-mediated pulmonary hypertension (HPH). Mice with genetic deficiency of SphK1, but not SphK2, are protected from HPH development, including elevations in right ventricular systolic pressure (RVSP), right ventricular hypertrophy (RVH), and pulmonary vascular remodeling. Mice heterozygous for sphingosine-1-phosphate lyase 1 (Sgpl1), a critical enzyme in mammalian sphingolipid metabolism which cleaves

S1P, are more susceptible to HPH. Pharmacological inhibition of sphingosine kinases prevents HPH development in rats. Together, these studies highlight the novel role of SphK1/S1P in PAH.

Next, we explored the role of SphK1/S1P signaling in mediating human PASMC (hPASMC) proliferation, a major process involved in pulmonary vascular remodeling. Intracellular overexpression of SphK1 and exogenous S1P both promote hPASMC proliferation, and the effects of S1P are mediated via signaling through S1PR2. In support of these findings, PASMCs isolated from S1PR2-deficient mice have reduced proliferative capacity, and genetic deficiency or pharmacologic inhibition of S1PR2 prevents the development of HPH in mice. The mechanism of S1P/S1PR2-mediated hPASMC proliferation was demonstrated to involve activation of both the ERK and STAT3 signaling cascades.

Both S1P and hypoxia induce hPASMC proliferation and contribute to the development of PAH, so we next studied the potential interactions of these signaling pathways. We demonstrated that S1P/S1PR2 induces nuclear expression of the transcription factor hypoxia-inducible factor-1 alpha (HIF1α), a critical mediator of the cellular response to hypoxia, and led to increased expression of several downstream targets of HIF1α in hPASMCs. In exploration of the mechanism of this HIF1α activation, we found that S1P/S1PR2 induces expression of early growth response protein 1 (Egr-1) via ERK signaling and that this transcription factor has a potential binding site within the proximal HIF1α promoter. We also demonstrated that S1P induces phosphorylation of GSK-3β in hPASMCs via ERK signaling, a process which is known to stabilize HIF1α and lead to its nuclear accumulation in cells.

We have demonstrated the importance of SphK1 and its production of S1P in PAH, but little is known about how SphK1 expression is regulated in hPASMCs. Here we demonstrate that stimulation of hPASMCs with platelet-derived growth factor (PDGF), a known factor contributing

to the development of PAH, leads to induction of SphK1 expression. This process is mediated by activation of nuclear Egr-1 expression and direct binding of Egr-1 to the SphK1 promoter. PDGF-induced Egr-1 and downstream SphK1 expression was also shown to be important for mediating hPASMC proliferation via ERK signaling.

We also investigated the potential role of microRNAs (miRs) in modulating the expression of SphK1 given their recently identified importance as a potential therapeutic target in PAH. Our data demonstrate that miR-1 directly targets the 3'-UTR of SphK1 and mediates down-regulation of its expression in hPASMCs. Expression of miR-1 is reduced in PASMCs from PAH patients and is decreased by hypoxia in normal hPASMCs, leading to increased expression of SphK1. MiR-1 overexpression was shown to inhibit key phenotypical aspects of hPASMCs, including cell migration and proliferation. Lastly, systemic miR-1 delivery protected mice from the development of HPH, indicating its potential as a novel target in PAH therapy.

In summary, this dissertation identifies the relevance of the SphK1/S1P signaling pathway to the pathobiology of pulmonary vascular remodeling and PAH. Several distinct signaling mechanisms of S1P involved in the induction of PASMC proliferation were characterized. Key factors mediating the genetic regulation of this pathway in the context of PAH development were also demonstrated. The evidence presented here highlights the SphK1/S1P signaling axis as a potential target in the development of novel therapies in PAH.

I. INTRODUCTION

A. Background

Pulmonary arterial hypertension (PAH) is a progressive and fatal disease for which the pathogenic mechanisms are poorly understood and no curative treatments are available (1). PAH is characterized by increases in pulmonary vascular resistance (PVR) primarily due to uncontrolled pulmonary vascular remodeling, sustained vasoconstriction, or thrombosis *in situ* (2,3). Increased PVR leads to progressive elevations in pulmonary artery pressure, resulting in right ventricle hypertrophy and heart failure, the major cause of death in PAH. Pulmonary vascular remodeling is associated with marked medial hypertrophy due to unrestrained pulmonary artery smooth muscle cell (PASMC) proliferation and apoptosis resistance (4). These changes narrow the luminal space of vessels and obstruct blood flow, contributing to increased PVR. Despite active research in PAH, most current therapies are limited to targeting pulmonary vasoconstriction and patients continue to have a poor long-term prognosis. There is a critical need to understand the mechanisms contributing to pulmonary vascular remodeling in order to develop new therapeutics in PAH.

Originally named after the mythological Sphinx due to their enigmatic properties, sphingolipids are now highly recognized for their contributions to pulmonary disease (5). Our group and others have demonstrated that the bioactive lipid sphingosine-1-phosphate (S1P) is involved the pathogenesis of pulmonary fibrosis and bronchopulmonary dysplasia, and in regulation of the vascular endothelial barrier during acute lung injury (6-12). As a potent signaling molecule, S1P mediates a vast array of important biological functions including cell proliferation, differentiation, motility and resistance to apoptosis (13). These effects are mediated primarily through binding to a family of five G-protein coupled receptors (S1PR1-5). The synthesis of S1P is regulated by sphingosine kinase (SphK) isoforms 1 and 2, with SphK1 predominant in the lungs and blood (14). SphK1, a highly conserved, oncogenic enzyme, has itself been implicated in the promotion of cell

proliferation, apoptosis resistance and angiogenesis (15,16), and preclinical studies have shown the efficacy of SphK1 inhibition in decreasing tumor size (17,18). Whether SphK1/S1P are involved in the pathogenesis of PAH and how these factors may influence the pulmonary vascular remodeling phenotype is currently not well understood.

The goal of this dissertation was to characterize the molecular mechanisms underlying pathogenic pulmonary vascular remodeling in PAH to promote the discovery of novel therapies.

B. Statement of Hypothesis

We hypothesize that the SphK1/S1P signaling axis is involved in the pathogenesis of PAH by regulating the degree of pulmonary vascular remodeling. Specifically, we propose that elevations in SphK1/S1P induce and maintain a proliferative phenotype of PASMCs mediated through S1PR-mediate signaling. The resulting elevations of pulmonary vascular resistance would therefore contribute to pulmonary and cardiac dysfunction. We also hypothesize that targeting this pathway would have therapeutic benefit in preventing and/or reversing the development of PAH.

C. Significance of the Study

The data presented in this thesis identifies and characterizes a novel role of the SphK1/S1P pathway in mediating pulmonary vascular remodeling in PAH, a disease process for which few treatment options are available clinically. Using human PAH samples, cell culture models, preclinical rodent models, and *in silico* tools, these experiments highlight the SphK1/S1P molecular pathway as a novel therapeutic target in PAH to improve patient outcomes. The project synthesizes data on the genetic regulation of SphK1 expression with several downstream signaling mechanisms of S1P to provide robust insight into the role this pathway in the context of PAH.

II. OVERVIEW OF RELATED LITERATURE

A. Pulmonary Circulation and Pulmonary Hypertension

1. Structure and Function of the Pulmonary Circulation

The pulmonary circulation is the part of the vasculature which plays the important role of carrying deoxygenated blood from the heart to the lungs and returning oxygenated blood back to the heart for delivery to the systemic circulation. Venous blood is carried by pulmonary arteries (PAs) to the capillaries of the lung, where CO_2 diffuses from the blood cell into the alveoli and O_2 diffuses out of the alveoli into the blood. Arterial, oxygenated blood leaves the capillaries to the left heart via the pulmonary veins. Though the pulmonary circulation is faced with the entire cardiac output, low pressure and pulmonary vascular resistance (PVR) is normally maintained due to abundance of small PAs and capillaries with high cross-sectional area. More capillaries are recruited during exercise to maintain low PA pressure (19). PVR is calculated based on Ohm's Law as [(mean pulmonary arterial pressure – mean left atrial pressure)/cardiac output] (20). The mean left atrial pressure can be approximated based on measurement of pulmonary capillary wedge pressure, where one of the PA branches is occluded and the pressure is measured downstream of the occlusion.

Several factors regulate vascular resistance as described by an adapted form of the Hagen–Poiseuille equation in fluid dynamics. This equation defines resistance as being directly proportional to the length of the blood vessel and viscosity of the blood and indirectly proportional to the radius of the blood vessel to the fourth power. Therefore, small reductions in the radius of blood vessels can lead to dramatic increases in PVR. Elevations in PVR with subsequent increases in PA pressure are observed in the development of PAH.

Structurally, the pulmonary trunk branches into two pulmonary arteries and approximately 15 higher-order branches to the pre-capillary level (21). The PAs are comprised of three layers: the

inner intima comprised of pulmonary artery endothelial cells (PAECs), the middle medial layer comprised on pulmonary artery smooth muscle cells (PASMCs), and the outer adventitial layer comprised mostly of fibroblasts (**Figure 1**). All of these cell types have been implicated in the process of pulmonary vascular remodeling in of PAH.

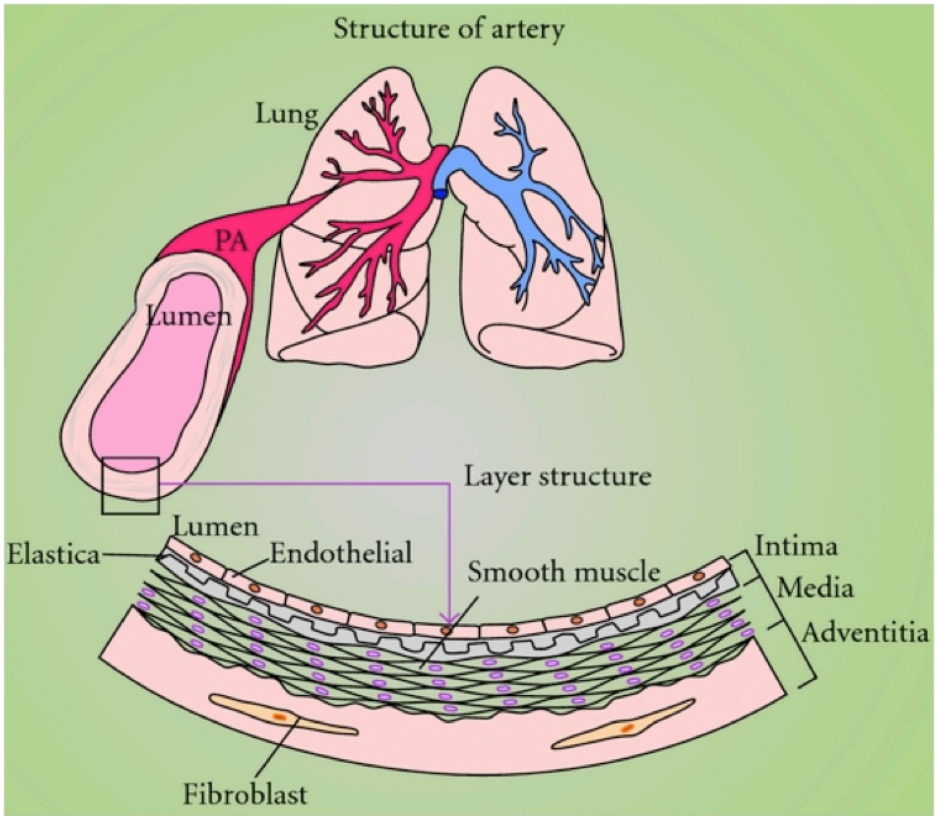

Figure 1. Structure of the pulmonary vasculature. The pulmonary artery (PA) wall is comprised of three layers: the inner intima, middle media, and outer adventitia. Each layer is comprised of distinct cell types as indicated. This figure was reprinted under the Creative Commons Attribution License from: Fernandez R.A., Sundivakkam P., Smith K.A., Zeifman A.S., Drennan A.R., Yuan J.X. "Pathogenic role of store-operated and receptor-operated ca(2+) channels in pulmonary arterial hypertension." *J Signal Transduct*. 2012; 2012:951497. Copyright 2012. doi: 10.1155/2012/951497.

2. Clinical Classification and Diagnosis of Pulmonary Hypertension

Pulmonary hypertension (PH) is a severe condition of multiple etiologies characterized by elevations in blood pressure within the pulmonary circulation. PH is hemodynamically defined as a PA pressure greater than 25 mmHg at rest, with right heart catheterization used as the gold standard for diagnosis. Augmented right ventricular afterload and strain can result from sustained elevations in pulmonary blood pressure, ultimately progressing to right ventricular failure and death. PH is an increasingly recognized comorbidity to numerous common disease processes and is associated with poor prognosis, and therefore accurate diagnosis of this condition is imperative. The World Health Organization (WHO) currently recognizes five separate groups of PH, demarcated based on shared disease histology and pathophysiology, clinical presentation and therapeutic strategies which can be divided into five distinct groups based on etiology, common disease manifestations, clinical presentation and therapeutic strategies (**Table I**) (22). The development of detailed clinical classification schemes, diagnostic criterion and novel therapeutics has lead to improved survival in PH over the past several decades, yet current therapies are unable to prevent disease progression and outcomes remain poor.

Changes in mean PA pressure (mPAP) in PH may occur after significant alterations and damage within the pulmonary vasculature have occurred, so even small pressure elevations require extensive workup. The diagnosis of PAH can be challenging due in part to the non-specificity of symptoms during early stages of pathogenesis. Physical exam and other noninvasive tests can help to delineate the subset of patients that require more invasive diagnostic procedures. Recent improvements in clinical diagnostic tools have led to the ability to distinguish subtler changes in structure and function of the heart and lungs in PH, and to better estimate mPAP without cardiac catheterization.

TABLE I

CURRENT CLASSIFICATION OF PULMONARY HYPERTENSION

1. Pulmonary arterial hypertension (PAH)
- 1.1 Idiopathic PAH
- 1.2 Heritable PAH
  - 1.2.1 BMPR2
  - 1.2.2 ALK-1, ENG, SMAD9, CAV1, KCNK3
  - 1.2.3 Unknown
- 1.3 Drug and toxin induced
- 1.4 Associated with:
  - 1.4.1 Connective tissue disease
  - 1.4.2 HIV infection
  - 1.4.3 Portal hypertension
  - 1.4.4 Congenital heart diseases
  - 1.4.5 Schistosomiasis
- 1' Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis
- 1" Persistent pulmonary hypertension of the newborn (PPHN)

2. Pulmonary hypertension due to left heart disease
- 2.1 Left ventricular systolic dysfunction
- 2.2 Left ventricular diastolic dysfunction
- 2.3 Valvular disease
- 2.4 Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies

3. Pulmonary hypertension due to lung diseases and/or hypoxia
- 3.1 Chronic obstructive pulmonary disease
- 3.2 Interstitial lung disease
- 3.3 Other pulmonary diseases with mixed restrictive and obstructive pattern
- 3.4 Sleep-disordered breathing
- 3.5 Alveolar hypoventilation disorders
- 3.6 Chronic exposure to high altitude
- 3.7 Developmental lung diseases

4. Chronic thromboembolic pulmonary hypertension (CTEPH)

5. Pulmonary hypertension with unclear multifactorial mechanisms
- 5.1 Hematologic disorders: chronic hemolytic anemia, myeloproliferative disorders, splenectomy
- 5.2 Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis
- 5.3 Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
- 5.4 Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure, segmental PH

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Patients with PH typically present with symptoms indicating poor oxygen transport and impaired cardiac output, including unexplained dyspnea with exertion, fatigue, chest pain, syncope, hemoptysis, and Raynaud's phenomenon (associated with connective tissue disease). A high level of clinical suspicion is required for PH diagnosis due to the non-specificity of these symptoms, and a detailed family history is important to identify potential hereditary PH cases. Clinical signs on physical exam may include jugular venous distension, hepatomegaly, presence of hepatojugular reflex, mottled extremities, cyanosis, diminished peripheral pulses, peripheral edema, and ascites. Cardiac auscultation can identify several abnormal sounds associated with PAH, including RV S3 and S4 sounds, accentuated pulmonic valve component (P2) of the 2nd heart sound, systolic murmur indicating tricuspid regurgitation, and diastolic murmur (indicating pulmonary regurgitation), and a parasternal lift may be detectable.

Numerous invasive and non-invasive procedures are required for accurate diagnosis of pulmonary hypertension, such as electrocardiography, pulmonary function testing, chest radiography, echocardiography, serologic testing and right heart catheterization. Despite improvements in clinical diagnostics and understanding of the underlying pathogenic mechanisms of pulmonary hypertension, current therapies are limited to supportive care and targeting pulmonary vasoconstriction.

B. Pulmonary Arterial Hypertension

1. Definitions and Epidemiology

Pulmonary arterial hypertension (PAH; Group 1 PH) is a group of diseases with the shared features of progressively increased PVR and mPAP due to obstructive changes within the pulmonary vasculature. The similarities between different types of PAH may reflect common underlying pathogenic mechanisms, which can ultimately lead to right ventricular failure and

premature death. The causes of PAH include idiopathic, heritable, drug and toxin induced, and associated disorders such as connective tissue disorders, HIV infection, portal hypertension, congenital heart diseases, and schistosomiasis. PAH can also result from pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis (Group 1') and persistent pulmonary hypertension of the newborn (Group 1"). The identification of gene mutations in bone morphogenetic protein receptor 2 (BMPR2) in most cases of familial PAH, as well mutations in ALK-1, ENG, SMAD9, CAV1, and KCNK3, has led to many important discoveries in the pathobiology of this disease (23,24).

The median life expectancy in PAH was less than 3 years in the 1980s before targeted therapies were available, with diagnosis occurring more often in young females (25,26). Outcomes currently remain poor even with the best available medications (27). The epidemiology of PAH is constantly evolving, and the variability in disease etiology and limited number of studies available makes it challenging to accurately determine outcomes and survival (28). In a recent prospective study of 482 patients diagnosed with PAH in the United Kingdom and Ireland, the estimated incidence of PAH was 1.1 cases per million per year with a prevalence of 6.6 cases per million in 2009 (29). This study also found a change in the demographics of PAH, with younger patients having more severe and hemodynamic impairment but better survival compared to older patients with more comorbidities. Despite recent improvements in the classification and diagnosis of PAH, more studies are needed to better understand the epidemiology of this fatal disease.

2. **Causes and Pathogenic Mechanisms**

The fundamental mechanism underlying elevations in PVR in PAH include sustained vasoconstriction, uncontrolled pulmonary vascular remodeling, and thrombosis *in situ* (2,3). The course of PAH is believed to be multifactorial and heterogeneous, and a wide variety of cell types

within the PA vessel walls, including PAECs, PASMCs, fibroblasts, inflammatory cells, and platelets, are implicated in the disease process (30). Initial vasoconstriction of the pulmonary vasculature leads to muscularization of peripheral arteries and medial hypertrophy of muscular arteries (**Figure 2**), with genetic risk factors increasing susceptibility to these changes. PAEC damage and dysfunction due to environmental triggers is also thought to be an early insult in PAH, and the repair process can lead to vasoocclusive neointimal formation and plexiform lesions that increase PVR (**Figure 2**) (31,32).

Pulmonary vasoconstriction is an early pathogenic process in PAH and can be induced by hypoxia, leading to narrowing of the luminal area of the PA branches. Hypoxia is known to inhibit voltage-gated potassium channels in PASMCs, which leads to opening of voltage-gated calcium channels due to membrane depolarization (33). The resulting rises in cytosolic calcium levels can induce PASMC contraction and proliferation, a process specific to the pulmonary vasculature (34). Down-regulation of potassium channels have been demonstrated in PASMCs and lungs of PAH patients (35,36). Several appetite suppressants implicated in the development of PAH, including fenfluramine, directly inhibit potassium channels and cause pulmonary vasoconstriction (37). Importantly, sustained vessel constriction causes dysfunction of PAECs, leading to a chronic reduction in the production of vasodilators prostacyclin and nitric oxide (NO) and increased production of the vasoconstrictors endothelin 1 (ET-1) and thromboxane A2 (30). These changes can also induce vascular remodeling, and therapies to target these pathways have been used (38).

Pulmonary vascular remodeling is associated with marked medial hypertrophy due to unrestrained PASMC proliferation and apoptosis resistance and neointimal formation due to PAEC dysfunction and proliferation (4,32). These changes can lead to obstructive lesions which narrow the luminal space of vessels and impede blood flow, contributing to increased PVR (39). Current investigations are underway to elucidate the mechanisms of abnormal cell proliferation contributing

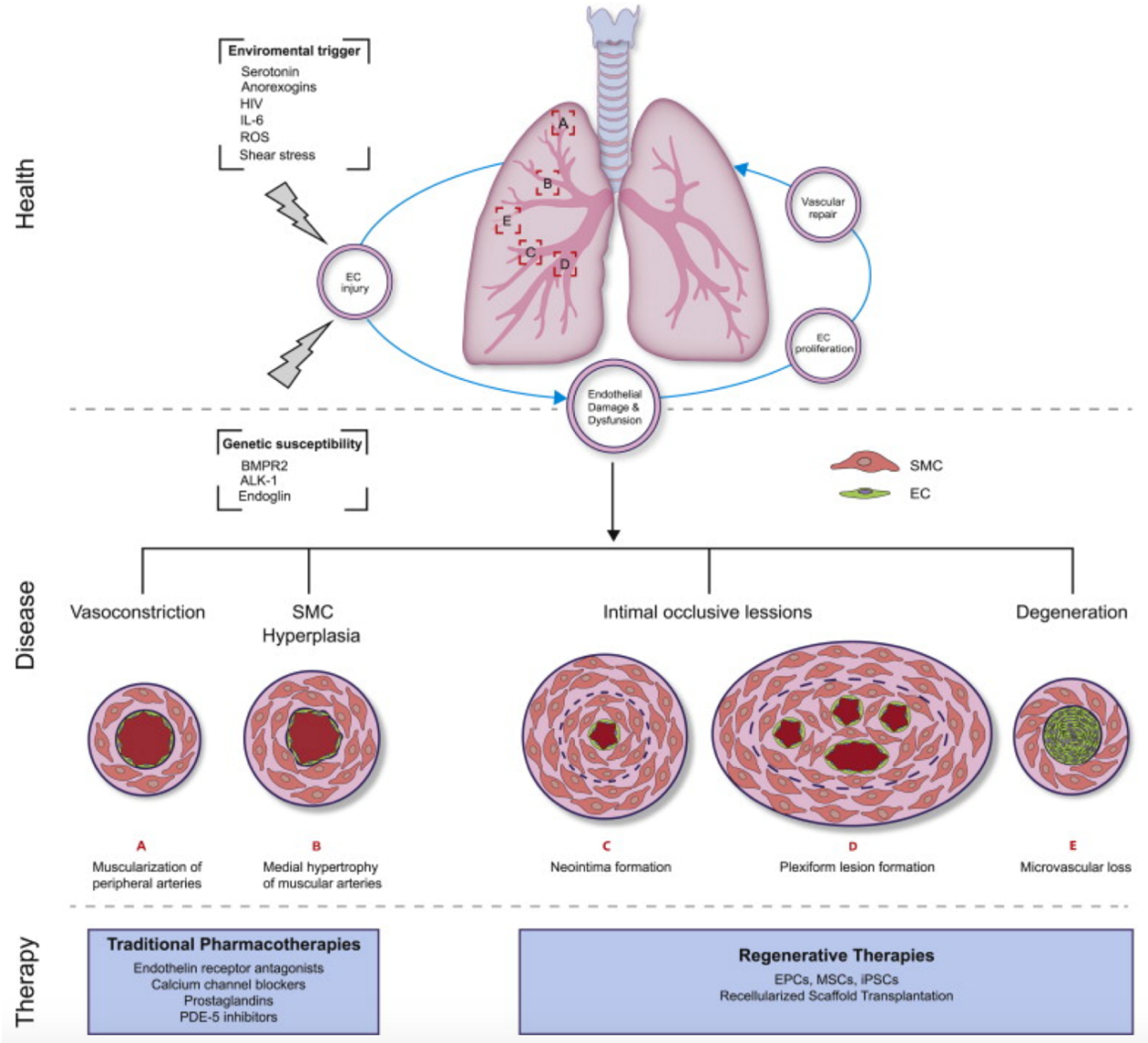

Figure 2. Pathophysiological mechanisms of PAH. Exposure to environmental insults can contribute to PAEC damage and injury. In healthy individuals, physiological repair processes restore normal lung function via proliferation of nearby ECs and/or the recruitment of circulating endothelial progenitor cells (EPCs). In PAH, pulmonary vascular cell damage contributes to the degeneration of microvasculature and/or arteriolar remodeling. In patients with hereditary PAH underlying genetic mutations are associated with increased susceptibility to PAEC damage and injury. Traditional pharmacotherapies aimed at restoring imbalances in vasoactive factors are presented alongside emerging therapies aimed at regenerating the microvasculature. iPSCs, induced pluripotent stem cells; MSCs, mesenchymal stromal/stem cells; PDE-5, phosphodiesterase type 5; ROS, reactive oxygen species; SMC, smooth muscle cell. Reprinted from *Can J Cardiol*, Volume 30(11), Foster W.S., Suen C.M., Stewart D.J. "Regenerative Cell and Tissue-based Therapies for Pulmonary Arterial Hypertension." Pages 1350-60, Copyright 2014, with permission from Elsevier. doi: 10.1016/j.cjca.2014.08.022.

to the formation of pathogenic lesions. Other mechanisms of remodeling in PAH include increased adventitial matrix production and impaired proteolysis of extracellular matrix (30). Evidence suggests that platelets may also play an important role in PAH pathogenesis given their ability to occlude vessels via thrombotic lesion formation and production of vasoconstrictive mediators, such as NO (40). Platelets from idiopathic PAH patients have been shown to have reduced levels of endothelial nitric oxide synthase (eNOS) (41), which may contribute to vasoconstriction.

3. **Current Therapeutic Strategies**

Current evidence-based therapy in PAH incudes the use of pharmacological agents in several drug classes to target pulmonary vasoconstriction and proliferation, some of which are FDA-approved. Given the complex etiology of PAH and heterogeneity in the patient population, decisions on the most appropriate therapies require accurate diagnosis and assessment of disease severity. The most commonly used drugs in PAH include prostanoids, endothelin receptor antagonists, phosphodiesterase type 5 inhibitors, soluble guanylate cyclase stimulators, and calcium channel blockers (**Figure 3**) (32,38). Though studies have examined the best combination of therapies for certain groups of PAH patients, more studies are needed to better determine the effectiveness of therapies in other subsets of the PAH population. Future promising therapies in PAH are aimed at regenerating the damaged microvasculature using stem/progenitor cell approaches and bioengineered lung scaffolds (42). Despite these advances, there remains a critical need to develop drugs that specifically target the mechanisms of cell proliferation in PAH to combat pulmonary vascular remodeling.

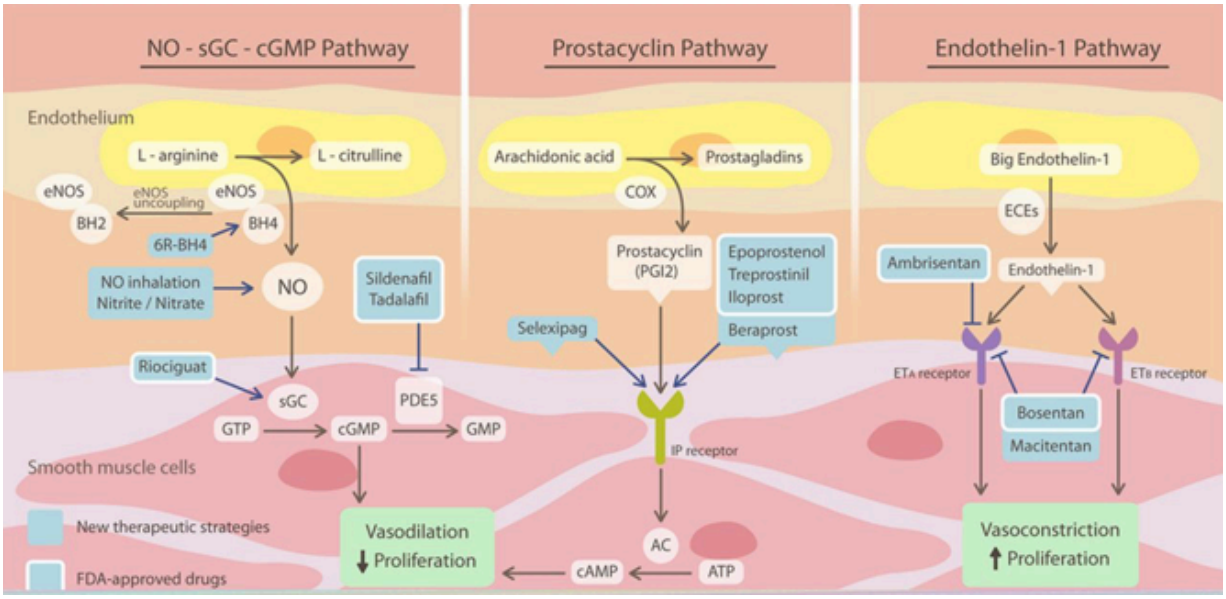

Figure 3. Classic vasodilator/vasoconstrictor systems and their translational therapies for PAH. Nitric oxide (NO) activates vasodilation and antiproliferation of smooth muscle cells via a cGMP-dependent mechanism. Inhalation of NO, administration of nitrite or nitrate, soluble guanylyl cyclase (sGC) stimulator (riociguat), phosphodiesterase 5 (PDE5) inhibitors (sildenafil and tadalafil), and tetrahydrobiopterin (BH_4) analogue (6R-BH_4) have been shown effective in the treatment of PAH. Prostacyclin activates vasodilation and inhibits proliferation of smooth muscle cells via a cAMP-dependent mechanism. Prostacyclin and its derivatives (epoprostenol, treprostinil, iloprost, and beraprost) and I-prostanoid (IP) receptor agonist (selexipag) provide therapeutic benefit in PAH. Endothelin-1 (ET-1) stimulates vasoconstriction and proliferation via activation of both ET_A and ET_B receptors on smooth muscle cells. ET_A blocker (ambrisentan) and dual ET-1 blockers (bosentan and macitentan) prove useful in the treatment of PAH. AC indicates adenylyl cyclase; BH_2, dihydrobiopterin; COX, cyclooxygenase; ECEs, endothelin-converting enzymes; eNOS, endothelial NO synthase; and FDA, Food and Drug Administration. Reprinted from Lai Y.C., Potoka K.C., Champion H.C., Mora A.L., Gladwin M.T. "Pulmonary Arterial Hypertension: The Clinical Syndrome." *Circ Res.* 115(1):115-30. Copyright 2014, with permission from Wolters Kluwer Health, Inc. doi: 10.1161/CIRCRESAHA.115.301146.

III. MATERIALS AND METHODS

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A. Reagents and Materials

S1P was purchased from Avanti Polar Lipids (Alabaster, AL). The SphK inhibitors SKI2 and PF-543 was purchased from Cayman Chemical Company (Ann Arbor, MI). PD98059 and U0126 were purchased from Cell Signaling Technology (Danvers, MA). JTE-013 was purchased from Tocris Bioscience (Bristol, United Kingdom). Recombinant human PDGF-BB was purchased from Sigma-Aldrich (St. Louis, MO). WP1066 was purchased from Santa Cruz Biotechnology (Dallas, TX). A murine SphK1 cDNA was digested and cloned into the pEGFP-C1 vector from Clontech

(Mountain View, CA) for transfection studies. The dominant-negative mutant (G82D) SphK1 plasmid was made by site-directed mutagenesis with the QuikChange mutagenesis kit from Agilent Technologies (Santa Clara, CA). Vector inserts were confirmed by DNA sequencing. For immunofluorescence studies, Cy3 labeled mouse anti-α-SMA antibody was purchased from Sigma-Aldrich (St. Louis, MO), rabbit anti-PCNA antibody was from Santa Cruz Biotechnology (Dallas, TX), and donkey anti-rabbit IgG Alexa Fluor 488 conjugate secondary antibody was purchased from Thermo Fisher Scientific (Waltham, MA). The primary antibodies for SphK1, Egr-1, Lamin B1, Erk1/2, phospho-Erk1/2 (Thr202/Tyr204), STAT3, phosphor-STAT3 (Tyr705), HIF1α, and phosph-GSK-3β (Ser9), and the HRP-conjugated β-Actin antibody and secondary anti-rabbit and anti-mouse IgG HRP-linked antibodies were purchased from Cell Signaling Technology (Danvers, MA). The primary antibody for SphK2 was purchased from Abcam (Cambridge, MA). The primary antibody for S1PR2/EDG-5 was purchased from Santa Cruz Biotechnology (Dallas, TX). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

B. *In Silico* Transcription Factor and MicroRNA Binding Site Analysis

For *in silico* transcription factor binding prediction analysis, putative Egr-1 binding sites were identified within the proximal ~2kb promoter of SphK1 and HIF1α using Genomatix software (similarity threshold >0.95). The genomic sequences were obtained and the identified binding sites were confirmed using the publically available UCSC Genome Browser (Human Feb. 2009, GRCh37/hg19 Assembly) and the public ENCODE ChIP-seq database (46,47).

For *in silico* microRNA binding prediction analysis, the publically available MicroRNA.org target prediction resource was used, which includes miRanda sites and miRSVR scoring algorithms (48). The target sites predicted using miRanda are scored for likelihood of mRNA down-regulation using a regression model trained on sequence and contextual features of the predicted

miRNA:mRNA duplexes. The genomic sequence of the SphK1 3'-UTR was used for analysis and confirmed with the UCSC Genome Browser.

C. *In Vitro* Experiments

1. Human Pulmonary Artery Smooth Muscle Cells and Lung Tissues

Human PASMCs were isolated from donors not suitable for lung transplantation and patients with idiopathic PAH, provided by the Pulmonary Hypertension Breakthrough Initiative and isolated as previously described (49). In addition to these cell lines, a primary hPASMC cell line from Lonza (Allendale, NJ) was used for cell signaling, transfection, migration, and proliferation assays. Cells were cultured at 37°C in Medium-199 supplemented with 10% fetal bovine serum and penicillin-streptomycin antibiotics and studied at passage 4 to 8. Hypoxic conditions (3% O_2) were generated using a controlled CO_2 incubator (Thermo Fisher Scientific). For treatment studies, sub-confluent PASMCs plated in multi-well plates were subjected to serum deprivation (0.2% FBS) overnight and stimulated described before collection and/or analysis. In some studies, pretreatment with chemical inhibitors (U0126, PD98059, PF-543, WP1066) for 45 min to 1 hr was used before stimulation. Further details of treatment timing are provided in the respective figure legends. The NE-PER kit from Thermo Fisher Scientific (Waltham, MA) was used for nuclear and cytoplasmic fraction extractions. Approval for the use of human lung tissues and cells was granted by the UIC Institutional Review Board. De-identified human explanted peripheral lung tissues used in this study were from four control subjects (two unsuitable organ donors and two chronic obstructive pulmonary disease patients without PH) and patients with idiopathic PAH (diagnosed on the basis of National Institutes of Health PAH Registry).

2. **Transfection and Luciferase Assays**

For SphK1 plasmid transfection studies, hPASMCs were transfected with SphK1 WT and G82D plasmids (5 μg DNA/well in 6-well plate) using Xfect Transfection reagent from Clontech (Mountain View, CA) per manufacturer's instructions. Cells were plated the day prior to transfection in complete media and grown to 75-85% confluence before transfection.

For S1PR2 siRNA-mediated silencing studies, non-targeting control siRNA and S1PR2 siRNA were purchased from GE Dharmacon (Lafayette, CO) and transfected into primary hPASMCs using DharmaFECT 2 transfection reagent from GE Dharmacon at a final concentration of 50 nM, without serum or antibiotics, per manufacturer's protocol. Complete media was replaced after 24 hrs to reduce cytotoxicity. For Egr-1 siRNA-mediated silencing studies, ON-TARGETplus siRNA specific for Egr-1 and non-targeting control were purchased from GE Dharmacon. Transfection of siRNAs (50 nM) was completed using Lipofectamine RNAiMAX reagent per manufacturer's instructions (Thermo Fisher Scientific). Cells were grown to 75-85% confluence before transfection. Silencing efficiency was determined by Western blotting of samples 48-72 hrs post-transfection.

For promoter studies using the *Gaussia* Luciferase (GLuc) and Secreted Alkaline Phosphatase (SEAP) system, a human SphK1 promoter reporter clone was purchased from GeneCopoeia (Rockville, MD). Activities of GLuc/SEAP were analyzed with the Secrete-Pair Dual Luminescence Assay Kit (GeneCopoeia) per manufacturer's guidelines, using a GloMax luminometer (Promega). For promoter deletion studies, indicated fragments of the SphK1 promoter were amplified by PCR, purified, and ligated into pGL4.10[*luc2*] promoterless luciferase reporter vectors purchased from Promega (Madison, WI). Vector inserts were confirmed by DNA sequencing. The *hRluc Renilla* luciferase reporter vector pGL4.74[hRluc/TK (Promega) was used as a transfection normalization control in these studies. Luciferase activity was measured using the Dual

Luciferase Reporter Assay System (Promega). FuGENE HD transfection reagent (Promega) was used for all promoter vector transfections in hPASMCs per manufacturer's guidelines.

MiScript miRNA-1 mimics or antagomirs were used for all miR transfection studies (Qiagen) per manufacturer's guidelines, with AllStars Negative Control siRNA used to account for non-sequence effects. The reporter construct containing the firefly luciferase gene fused to the the full-length human SphK1 3'-UTR within the pMirTarget vector was purchased from OriGene Technologies. This vector was co-transfected with a control vector expressing a *hRluc Renilla* luciferase reporter gene and a HSV-TK promoter for normalization of transfection efficiency (pGL4.74[hRluc/TK], Promega). hPASMCs at passage 4-8 were grown to 75-85% confluence before transfection with miR mimics (50-100 nM) or antagomirs (100 nM) with controls of the same concentrations using Lipofectamine (Thermo Fisher Scientific) per manufacturer's instructions. Cells were harvested for downstream analysis 24-72 hrs post-transfection. For luciferase reporter assays, cells were transfected concurrently with reporter vectors or controls. Cells were harvested at 24 hrs and luciferase activity was measured with the Dual Luciferase Reporter Assay System (Promega) per manufacture's protocol, using a GloMax luminometer (Promega). Transfection efficiency was determined by normalizing *Firefly* luciferase light units to *Renilla* luciferase light units.

3. **Cell Proliferation Assays**

Cell proliferation was determined using either a 5-bromo-2'-deoxyuridine (BrdU) incorporation assay or cell counting. A BrdU assay kit (Calbiochem) was used in a 96-well format according to manufacturer's instructions, using starting cell densities of 4,000 cells/well and incubation of cells in the indicated conditions for 24-48 hrs. Proliferation of cells results in incorporation of BrdU into the newly synthesized DNA, and this process is quantified by the intensity of absorbance of the final reaction. For cell counting, cells were trypsinized and

resuspended in basal culture media after experimental procedures; densities were counted with a TC10 automated cell counter (Bio-Rad).

4. Cell Migration Assays

Cell migration was determined using a quantitative transwell assay (50). Cells were seeded into a transwell insert with 8-µm pores (Sigma-Aldrich) in 2 ml of basal medium at a density of 50,000 cells/insert. Cells were then incubated in normoxic or hypoxic (3% O_2) conditions, fixed and stained using Diff-Quik on the transwell insert, and imaged with a standard light microscope. Five random fields were imaged per sample to obtain a total cell count. Unmigrated cells were then scraped from the top of the filter and the migrated bottom layer of cells were imaged and quantified. Migration was calculated as the percent of migrated cells on the filter. For miR transfection studies, cells were transfected 24 hr prior to transferring to the transwell insert.

5. Immunoblot Analysis for Tissues and Cells

Frozen, saline-perfused lung tissues were homogenized using RIPA buffer (Sigma-Aldrich) supplemented with protease and phosphatase inhibitor cocktails (Calbiochem). Following the indicated treatments, cells were washed twice with cold PBS and protein was extracted using the same modified RIPA buffer solution. Protein lysates (10–25 µg) were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) in laemmli sample buffer with 2-mercaptoethanol used as a reducing agent, and boiled for 5 minutes. Samples were then separated using SDS PAGE on Mini-Protean TGX precast gels (Bio-Rad), transferred to nitrocellulose membranes, and blocked in 5% nonfat dry milk. Membranes were then probed with primary and HRP-linked secondary antibodies as indicated, and bands were visualized by ECL (Pierce) on X-ray film per manufacturer's instructions. Densitometry of bands was performed to quantify protein

levels using ImageJ software (NIH), and expression levels were normalized to the expression of the indicated control protein.

6. RNA Extraction and Quantitative Real-Time PCR Analysis

For mRNA expression studies, total RNA was isolated from hPASMCs and lung tissue samples using the RNeasy Mini kit from Qiagen (Valencia, CA) and quantified with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). Two micrograms of RNA were reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Expression of all mRNAs were determined using recommended gene-specific TaqMan primer assays with GAPDH expression used as an internal control (Thermo Fisher Scientific). For miRNA expression studies, total RNA containing miRNA was isolated from hPASMC and lung tissue samples using a miRNeasy kit (Qiagen) and reverse transcribed with the miScript II RT kit (Qiagen) following RNA quantification. MiRNA-specific miScript primer assays (Qiagen) were used for real-time PCR with RNU6-2 used as an internal control. Quantitative real-time PCR analysis was performed using a CFX384 system (Bio-Rad), and relative changes in mRNA and miRNA expression were calculated after normalization to their respective internal controls using the comparative Ct method.

7. Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) studies were performed using the SimpleChIP Plus Magnetic Bead ChIP kit purchased from Cell Signaling Technology (Danvers, MA) per manufacturer's instructions. Cross-linking was completed after cell stimulation, followed by nuclei preparation and chromatin digestion. DNA gel electrophoresis was used to confirm adequate digestion. ChIP was then performed using the EGR1, positive control Histone H3, and negative

control normal rabbit IgG antibodies. Elution of chromatin from antibody/beads and reversal of crosslinks was performed. DNA was purified and analyzed by both standard PCR and quantitative real-time PCR. Primers used to amplify the EGR1-B binding site were forward: 5'-GCCTGTCGCCTGCTCTAC-3' and reverse: 5'-CCAGCTTCCCTCTTTCTTCC-3'.

D. *In Vivo* Experiments

1. Animal Models of Hypoxia-Mediated Pulmonary Hypertension

All animal protocols were reviewed and approved by the University of Illinois at Chicago Animal Care and Use Committee. In the mouse model of HPH, 8-wk old male C57BL/6 mice (Charles River), SphK1-deficient (*SphK1*$^{-/-}$) mice on the C57BL/6 background, SphK2-deficient [*SphK2*$^{-/-}$] mice on the C57BL/6 background, S1PR2-deficient (*S1PR2*$^{-/-}$) mice on the C57BL/6 background S1P lyase heterozygous [*Sgpl1*$^{+/-}$] mice on the 129/Sv background, and their WT siblings were exposed to normoxia or hypoxia (10% O_2 in a ventilated chamber) for 1d-6wks (n=3-5/group). Sgpl1 heterozygous mice were exposed to hypoxia for 6 wks due to resistance to HPH development under 4 wk conditions. Before assessment of HPH development, animals were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) given by intraperitoneal injection. HPH development was assessed by measuring right ventricular systolic pressure (RVSP) via a pressure transducer catheter (Millar) as a surrogate of pulmonary artery pressure, right ventricular hypertrophy as a weight ratio of the right ventricle divided by the sum of left ventricle and septum (RV/LV+S), and pulmonary artery wall thickness quantified and imaged using lung histological sections stained with hematoxylin and eosin (Aperio ImageScope). Vessel thickness measurements were done using approximately 20 muscular arteries with diameters 50–100 μm or less than 50 μm per lung section. Vessel remodeling was calculated as ([external vessel area–internal vessel area]/external vessel area), as previously described (43). Lung and heart tissues were snap-frozen in

liquid nitrogen or fixed in 4% paraformaldehyde for further analysis. Fixed tissues were changed to 70% ethanol after 24 hrs for extended storage.

For mouse HPH studies using the inhibitor JTE-013, both prevention and reversal protocols were performed. For prevention studies, JTE-013 (8 mg/kg, intraperitoneally, once every other day) was started on the first day of a 3.5-week hypoxia exposure. For reversal studies, JTE-013 was given similarly starting after 2 weeks of hypoxia and followed by 2 additional weeks of hypoxia. HPH assessment and tissue isolations were performed as described above.

For HPH prevention studies using microRNA mimics, animal-grade *mir*Vana miRNA mimics or negative miRNA control #1 from Thermo Fisher Scientific were prepared with Invivofectamine 3.0 reagent from Thermo Fisher Scientific per manufacturer's instructions and injected to mice retro-orbitally (7.8 mg/kg body weight) one day prior to exposure to normoxia or hypoxia (10% O_2) for four weeks. Injections were then repeated once per week during the experimental period. One day after the final injection, HPH development was assessed by measuring RVSP, RVH, and pulmonary arterial remodeling as described above. Lung, heart, and heparinized blood were collected for further analysis. Plasma was isolated by centrifugation before freezing.

For rat HPH studies, male Sprague Dawley rats (190–200 g) purchased from Charles River were exposed to hypoxia (10% O_2) for 3.5 wks. For prevention studies, rats were treated with vehicle or SKI2 (10 mg/kg body weight, intraperitoneally) every two days for 3.5 wks, followed by HPH assessment and tissue isolations as described above.

2. **Lung Tissue Immunofluorescence Microscopy**

Fixed lung tissues were paraffin-embedded and sections were used for immunostaining. Sections were de-paraffinized with xylene and rehydrated. Antigen retrieval was performed using Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA solution, 0.05% Tween-20, pH 9.0) before

blocking in PBS with 10% normal goat serum, 0.1% BSA, 0.3% TX-100. Antibodies for SphK1 and PCNA were used at 1:100 dilutions, and the anti-α-SMA antibody was used at 1:300. The secondary donkey anti-rabbit IgG Alexa Fluor 488 antibody for SphK1 and PCNA staining was used at 1:500. An anti-fade mounting media with DAPI (Life Science, Inc.) for nuclear staining was used to fix the coverslips to microscope slides. The slides were examined and imaged using a Nikon Eclipse E800 fluorescence microscope, and the images were processed by Photoshop 7.0.

3. S1P Measurements

C18-S1P levels from lung tissues (humans, mice, and rats) and PAs isolated from mice and rats were measured using a method described previously (51). Lipids were extracted from lung tissues and PAs by a modified Bligh and Dyer procedure with the use of 0.1 N HCl for phase separation. C17-S1P (40 pmol), used as an internal standard, was added during the initial step of lipid extraction. S1P content was determined by liquid chromatography/tandem mass spectrometry with electrospray ionization using API 5500 QTRAP mass spectrometer equipped with turbo-V ion source and normalized to total phospholipid content in the sample. Results were reported as fmol/nmol lipid.

4. Mouse PASMC Isolation

PASMCs from WT and *S1PR2*$^{-/-}$ mice (male, 8 wk) were isolated and cultured as previously described (52). Mice were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) given by intraperitoneal injection. Surgical exposure of the trachea and catheterization was performed, and mice were exsanguinated via the carotid artery. A solution of 5 ml Medium-199, 25 mg agarose, and 25mg iron oxide (heated to dissolve agarose, then cooled to body temperature) was injected into the right ventricle to perfuse the lungs. A solution of 7 ml Medium-199 and 25 mg agarose was then

injected into the trachea. Lungs were then bathed in cold PBS, removed, and finely minced. Lung tissues were washed three times with Medium-199 over a magnetic column to retain blood vessels filled with iron and agarose. Vessels were then digested in collagenase A (80 U/ml) for 1 hr, then disrupted by passing through a 21-guage needles several times. Vessel pieces were washed again twice using a magnetic column, then cultured in Medium-199 supplemented with 20% FBS and antibiotics. Cell purity as assessed by immunostaining for α-smooth muscle actin as demonstrated in the results section.

E. Statistical Analysis and Data Presentation

Statistical analyses of experimental data were performed using GraphPad Prism software (La Jolla, CA). Results are expressed as mean ± SEM from at least three experiments, and statistical significance between groups was calculated with Student's t-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. controls or as indicated; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. controls or as indicated). Figures were made using GraphPad Prism software and Microsoft PowerPoint.

IV. SPHK1 AND S1P CONTRIBUTE TO THE DEVELOPMENT OF PULMONARY HYPERTENSION

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1. Chen J, Tang H, Sysol JR, Moreno-Vinasco L, Shioura KM, Chen T, Gorshkova I, Wang L, Huang LS, Usatyuk PV, Sammani S, Zhou G, Raj JU, Garcia JG, Berdyshev E, Yuan JX, Natarajan V, and Machado RF. 2014. The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension. *Am J Respir Crit Care Med.* 190(9):1032-43. doi: 10.1164/rccm.201401-0121OC. (43) Reprinted with permission of the American Thoracic Society. Copyright © 2016 American Thoracic Society.

A. Rationale

PAH is a progressive and fatal disease with no curative treatments available (1). Most of the present therapies in PAH are limited to targeting pulmonary vasoconstriction, and patients continue to have poor long-term prognoses. There is therefore an urgent need to understand the pathogenic mechanisms contributing to and regulating pulmonary vascular remodeling in PAH so that new drug targets can be identified. A major hallmark of pulmonary vascular remodeling is marked medial hypertrophy due to unrestrained PASMC proliferation and apoptosis resistance (4). There is currently a gap in our knowledge of how and why these changes occur in patients with PAH. Using a combination of *in vitro* and *in vivo* experiments, the studies in this Chapter aimed to identify novel cellular signaling pathways that may contribute to pulmonary vascular remodeling.

Sphingolipids, including S1P, contribute to the pathogenesis of many pulmonary diseases, such as pulmonary fibrosis, bronchopulmonary dysplasia, and acute lung injury (6-12). S1P, which is generated by SphK1 and SphK2, can ligate and signal through S1PRs to mediate important cellular processes, including proliferation, differentiation, motility, and resistance to apoptosis (13). SphK1 is

predominant in the lungs and blood (14) and itself promotes cell proliferation, apoptosis resistance, and angiogenesis, including in many cancers (15-18). Given its role in the pulmonary vasculature and in promoting cell proliferation, we hypothesized that the SphK1/S1P signaling axis may be upregulated in PAH and contribute to pulmonary vascular remodeling.

B. SphK1 and S1P Are Significantly Increased in PAH Patients and in Experimental Rodent Models of PH

In order to determine the role of SphK1 and S1P in PAH, we first measured their levels in lung tissues and PASMCs isolated from PAH patients. Protein levels of SphK1, but not SphK2, were significantly elevated in the lungs (**Figures 4A**, **4B**, **4D**, and **4E**) and PASMCs (**Figures 5A–5C**) of patients with PAH when compared with control subjects. Similar changes were also observed in SphK1 and SphK2 mRNA expression levels in PAH patient PASMCs (**Figure 5D-E**). In addition, levels of S1P were significantly higher in the lungs of patients with PAH when compared with controls (**Figure 4C**).

Next, we tested whether elevations in SphK1 and S1P were also present in experimental rodent models of hypoxia-mediated PH (HPH). The protein expression of SphK1, but not SphK2, was significantly elevated in the lungs of both mice (**Figures 6A-C**) and rats (**Figures 6E-G**) exposed to hypoxia (10% O_2) for 4 weeks. Additionally, endogenous C18-S1P levels were significantly increased in pulmonary arteries isolated from the hypoxia-exposed mice (**Figure 6D**) and rats (**Figure 6H**).

The consistent observations in tissues between human PAH and rodent models of PH suggest that the SphK1 and S1P signaling axis may play a role in the pathogenesis of this condition. Together with the pro-proliferative effects of this pathway characterized in other cell types and

diseases, these studies prompted us to further explore the influence of SphK1/S1P in PAH development, including pulmonary vascular remodeling.

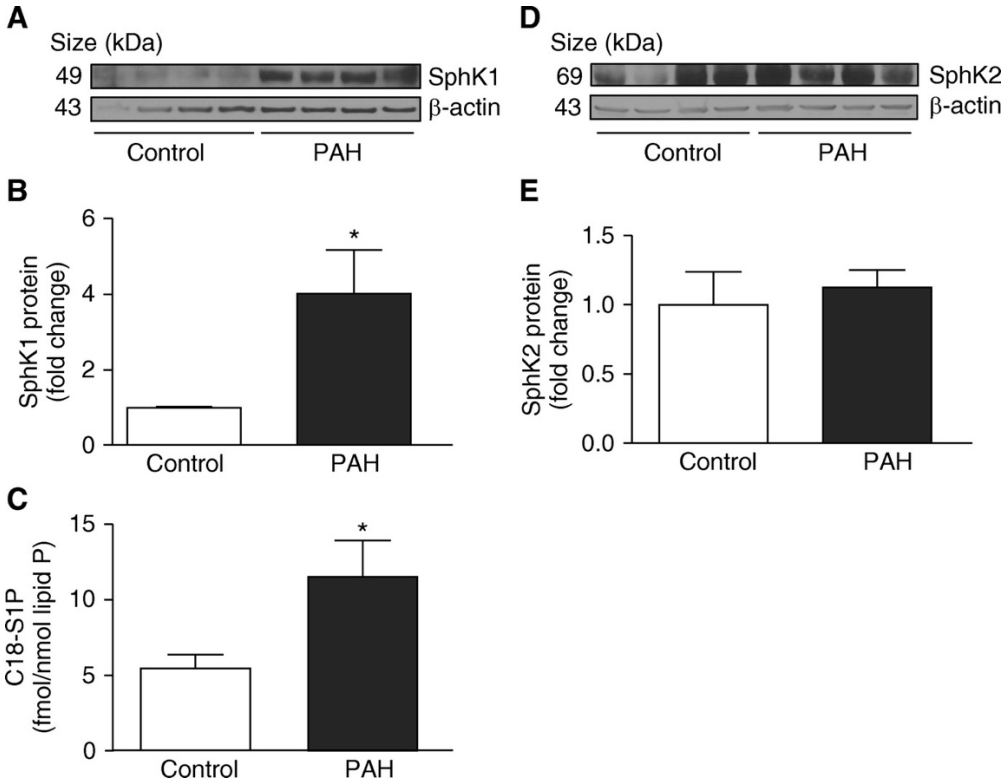

Figure 4. SphK1 and S1P are increased in the lungs of patients with PAH. (A and B) Representative Western blotting images and β-actin–normalized quantification of protein demonstrate that SphK1 expression is significantly increased in lungs from patients with PAH when compared with control subjects. (C) C18-S1P levels are increased in the lungs of patients with PAH. (D and E) Representative Western blotting images and β-actin–normalized quantification of protein demonstrate that SphK2 expression is not increased in lungs from patients with PAH when compared with control subjects. Results are expressed as mean ± SEM; n = 4 per group. *$p < 0.05$ versus control. This figure was reprinted with permission from Chen J., Tang H., Sysol J.R., et al. 2014. "The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension." *Am J Respir Crit Care Med.* 190(9):1032-43. doi: 10.1164/rccm.201401-0121OC. Copyright © 2016 American Thoracic Society.

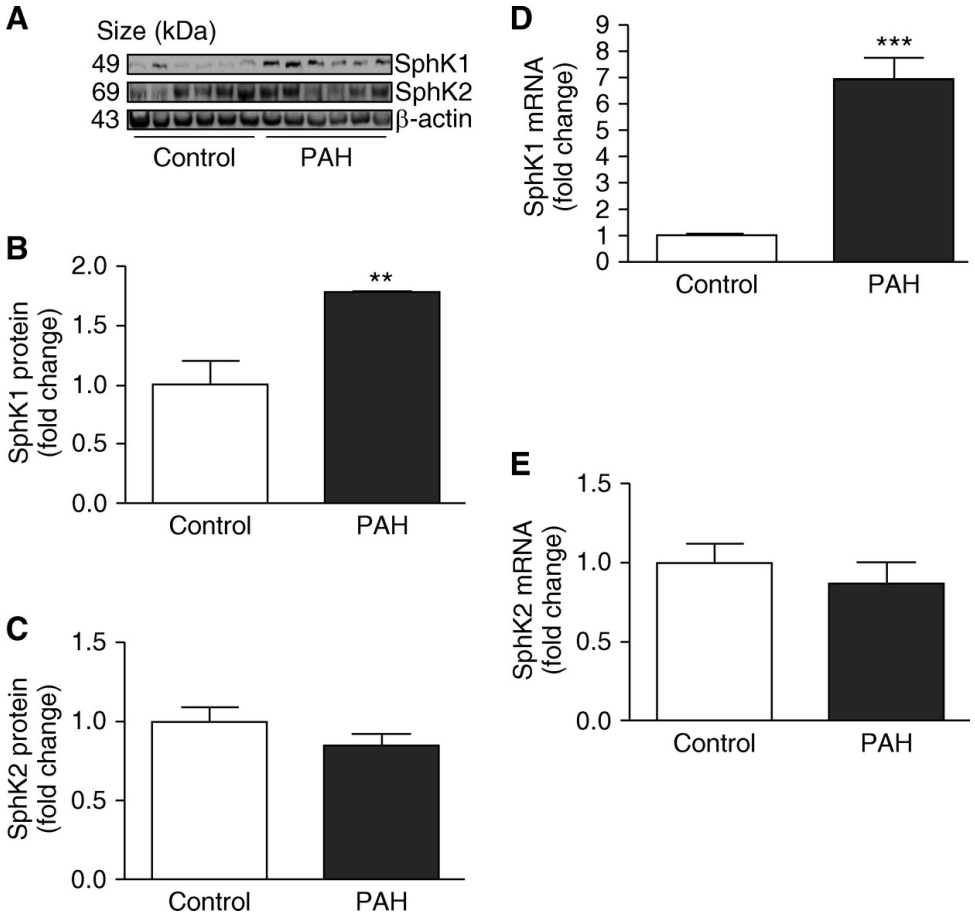

Figure 5. Expression levels of SphK1 are increased in PASMCs from patients with PAH. (A–C) Representative Western blotting image and β-actin–normalized quantification of protein demonstrate that SphK1 expression is significantly increased in PASMCs from patients with PAH when compared with control subjects, whereas no changes in SphK2 expression are demonstrated. (D and E) A similar pattern was demonstrated for SphK1 and SphK2 mRNA levels in PASMCs isolated from control subjects and patients with PAH (n = 6 per group). $^{**}p < 0.01$; $^{***}p < 0.001$ versus control. This figure was reprinted with permission from Chen J., Tang H., Sysol J.R., et al. 2014. "The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension." Am J Respir Crit Care Med. 190(9):1032-43. doi: 10.1164/rccm.201401-0121OC. Copyright © 2016 American Thoracic Society.

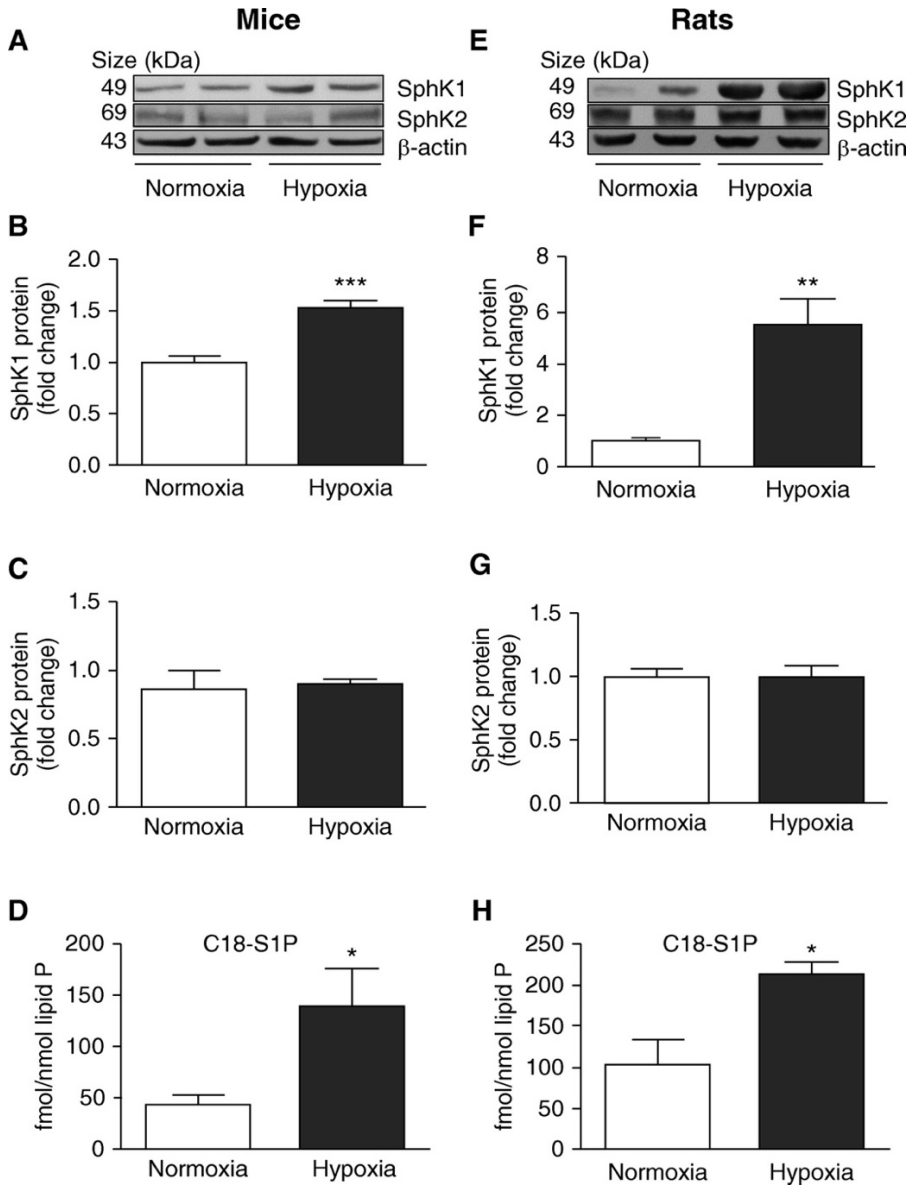

Figure 6. SphK1 and S1P levels are increased in lungs and PAs of rodent models of HPH. (A–C) Representative Western blotting images and β-actin–normalized quantification of protein demonstrate that SphK1, but not SphK2, expression is significantly increased in mouse lungs after 4-week hypoxia exposure. (D) C18-S1P levels are significantly increased in the pulmonary arteries of mice exposed to 4-week hypoxia. (E–G) Representative Western blotting images and β-actin–normalized quantification of protein demonstrate that SphK1, but not SphK2, expression is significantly increased in rat lungs after 4-week hypoxia exposure. (H) C18-S1P levels are significantly increased in the pulmonary arteries of rats exposed to 4-week hypoxia (n = 5 per group). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus normoxia. This figure was reprinted with permission from Chen J., Tang H., Sysol J.R., et al. 2014. "The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension." *Am J Respir Crit Care Med.* 190(9):1032-43. doi: 10.1164/rccm.201401-0121OC. Copyright © 2016 American Thoracic Society.

C. SphK1-Deficient, but not SphK2-Deficient, Mice are Protected from HPH

To examine the effect of SphK1 on the development of pulmonary hypertension, SphK1-deficient (*SphK1*$^{-/-}$) mice and their WT siblings were exposed to hypoxia for 4 weeks to induce development of HPH. Compared with WT controls, *SphK1*$^{-/-}$ mice exhibited decreased RVSP, RVH, and pulmonary vascular remodeling in response to hypoxia (**Figure 7**). In contrast, SphK2 knockout (*SphK2*$^{-/-}$ mice) had no significant effect on the development of HPH (**Figure 8**). Together with our previous findings of elevated SphK1 and S1P levels in PH, these studies provide compelling evidence to suggest that targeting the SphK1 enzyme may be have therapeutic potential.

D. Mice Heterozygous for Sgpl1 Are More Susceptible to HPH

Sgpl1 in a critical enzyme in mammalian sphingolipid metabolism which cleaves phosphorylated sphingoid bases, such as S1P, into fatty aldehydes and phosphoethanolamine. Deletion of both alleles of the gene encoding Sgpl1 in mice results in severe growth retardation and early mortality due to vascular development defects (53). To test whether Sgpl1 deficiency, resulting in accumulation of S1P, could contribute to the development of HPH, we examined *Sgpl1*$^{+/-}$ mice in this model. Consistent with previous results in normoxia (54), *Sgpl1*$^{+/-}$ mice exhibited increased C18-S1P levels in lung tissues under both normoxia or 4–6 weeks of hypoxia exposure compared with WT littermates (**Figure 9**). Following 6 weeks of hypoxia exposure, *Sgpl1*$^{+/-}$ mice exhibited significantly increased RVSP, RVH, and pulmonary vascular remodeling compared with WT littermates (**Figure 10**). No differences in these parameters were observed at 4 weeks of hypoxia exposure, possibly due to genetic background differences of the mice compared to other studies. Together, these data demonstrate that deficiency of Sgpl1 contributes to the development of HPH, likely due to elevations in S1P concentration.

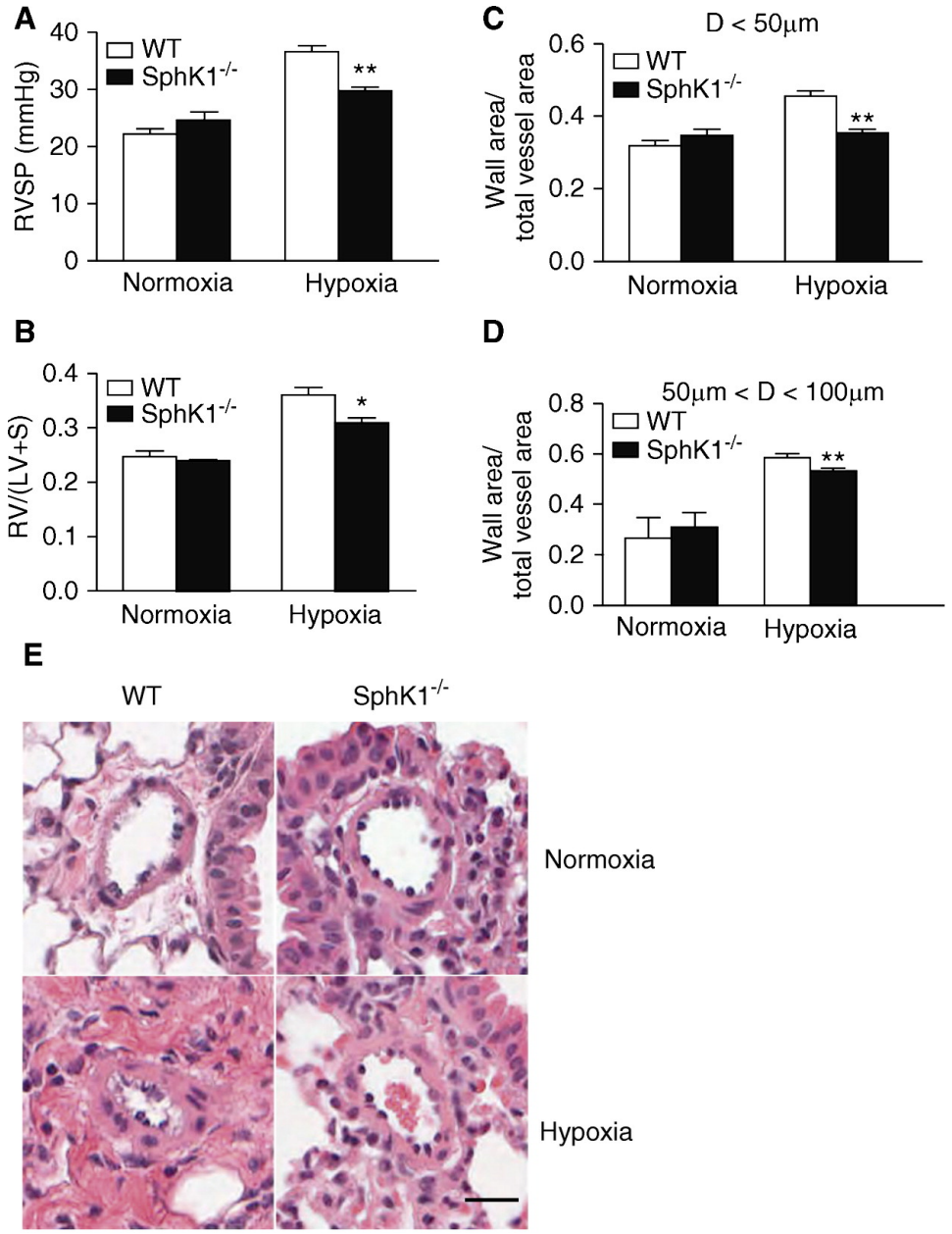

Figure 7. SphK1-deficient mice are protected against HPH development. *SphK1*$^{-/-}$ mice exposed to hypoxia (4 wks) demonstrated attenuated increases in (A) RVSP, (B) RVH, and vessel thickness of pulmonary arteries < 50 μm (C) and 50–100 μm in diameter (D) compared to WT littermates. (E) Representative pulmonary artery images from lung sections of WT and *SphK1*$^{-/-}$ mice exposed to normoxia or hypoxia. Bar size: 20 μm. Results are expressed as mean ± SEM; n = 10 per group. *$p < 0.05$; **$p < 0.01$ versus hypoxia WT group. This figure was reprinted/modified with permission from Chen J., Tang H., Sysol J.R., et al. 2014. "The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension." *Am J Respir Crit Care Med.* 190(9):1032-43. doi: 10.1164/rccm.201401-0121OC. Copyright © 2016 American Thoracic Society.

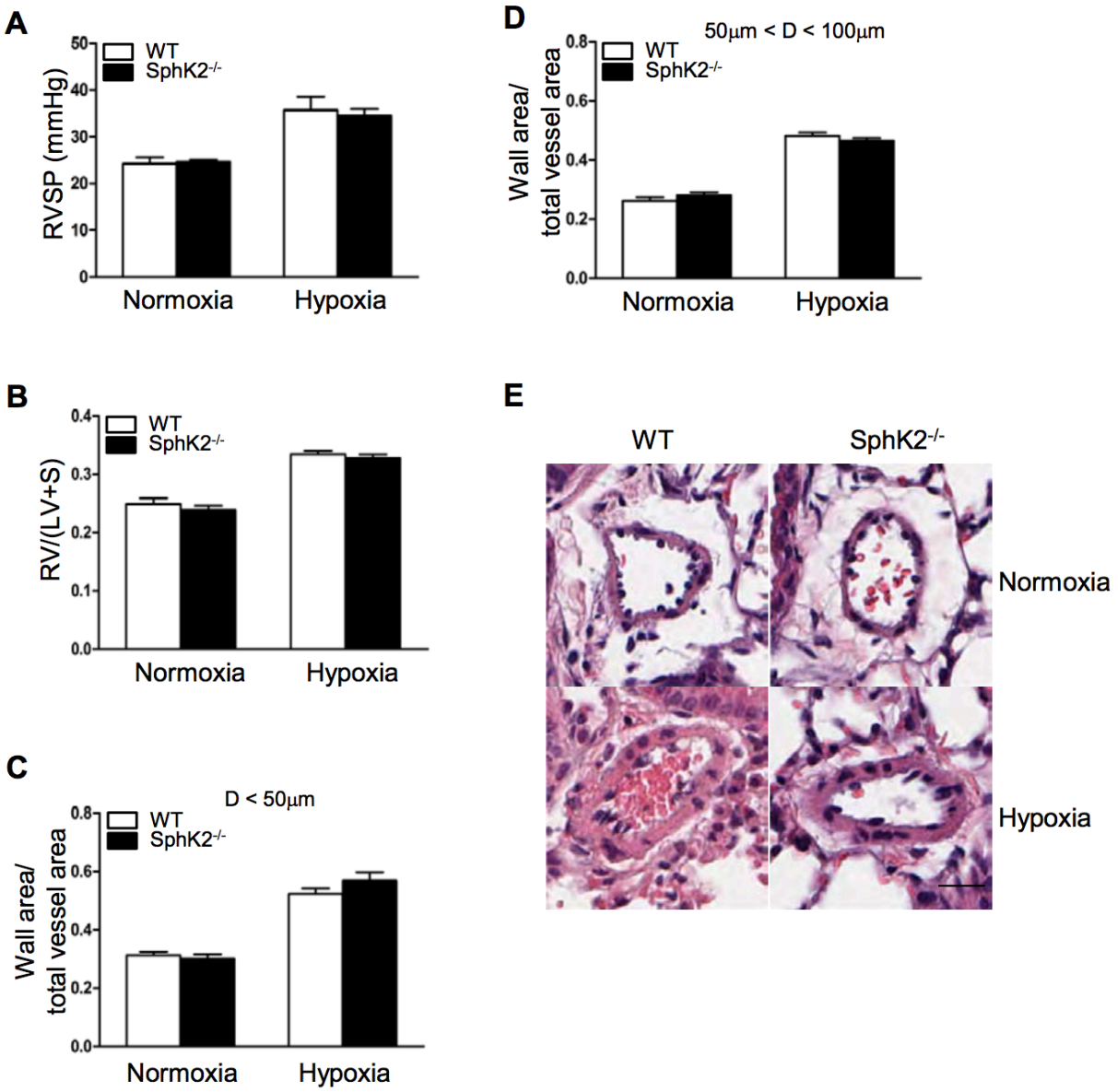

Figure 8. SphK2-deficient mice are not protected against HPH development. No significant differences were observed in *SphK2*$^{-/-}$ mice exposed to hypoxia (4 wks) in changes of (A) RVSP, (B) RVH, and vessel thickness of pulmonary arteries < 50 μm (C) and 50–100 μm in diameter (D) compared to WT littermates. (E) Representative pulmonary artery images from lung sections of WT and *SphK2*$^{-/-}$ mice exposed to normoxia or hypoxia. Bar size: 20 μm. Results are expressed as mean ± SEM; n = 10 per group. This figure was reprinted with permission from Chen J., Tang H., Sysol J.R., et al. 2014. "The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension." *Am J Respir Crit Care Med.* 190(9):1032-43. doi: 10.1164/rccm.201401-0121OC. Copyright © 2016 American Thoracic Society.

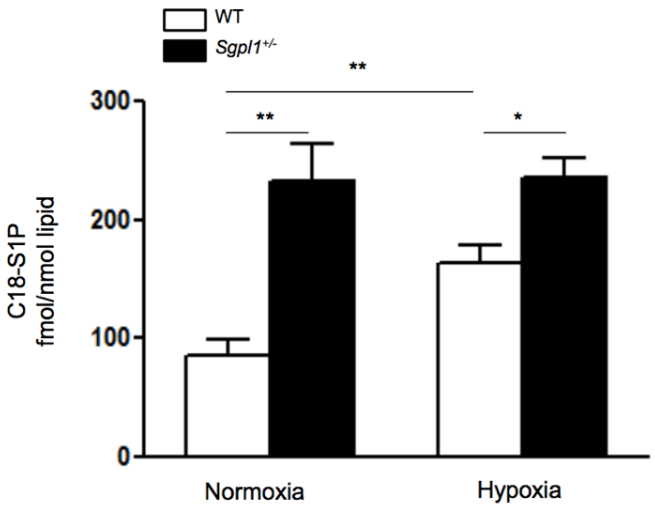

Figure 9. S1P is elevated in lung tissues of heterozygous Sgpl1 mice in normoxia and hypoxia. *Sgpl1*$^{+/-}$ mice exhibited significantly increased C18-S1P levels in lung tissues under normoxia or after 4-6-week hypoxia exposure, compared to WT littermates. N = 5 per group, *$p < 0.05$; **$p < 0.01$. This figure was reprinted with permission from Chen J., Tang H., Sysol J.R., et al. 2014. "The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension." *Am J Respir Crit Care Med.* 190(9):1032-43. doi: 10.1164/rccm.201401-0121OC. Copyright © 2016 American Thoracic Society.

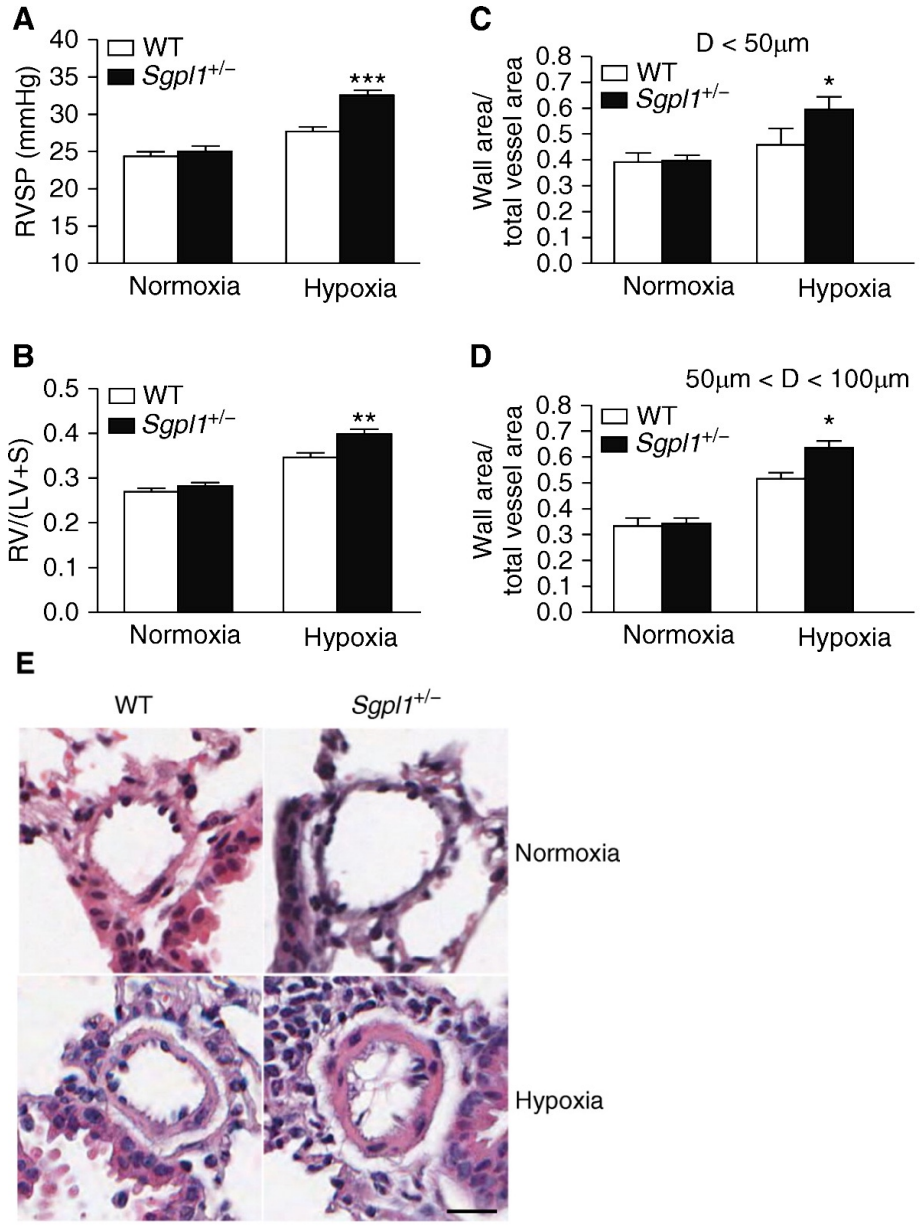

Figure 10. Mice heterozygous for Sgpl1 mice are more susceptible to HPH development. *Sgpl1*$^{+/-}$ mice exposed to hypoxia (6 wks) developed more severe elevations in (A) RVSP, (B) RVH, and pulmonary vessel thickness in arteries < 50 μm (C) and 50–100 μm in diameter (D) compared to WT littermates. (E) Representative pulmonary artery images from lung sections of WT and *Sgpl1*$^{+/-}$ mice exposed to normoxia or hypoxia. Bar size: 20 μm. Results are expressed as mean ± SEM; n = 10 per group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus hypoxia WT group. This figure was reprinted/modified with permission from Chen J., Tang H., Sysol J.R., et al. 2014. "The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension." *Am J Respir Crit Care Med.* 190(9):1032-43. doi: 10.1164/rccm.201401-0121OC. Copyright © 2016 American Thoracic Society.

E. SphK Inhibition Prevents HPH Development in Rats

To further examine the potential role of SphKs in PH, we evaluated the effect of SKI2, a selective non-lipid inhibitor of both SphK1 and SphK2, in rats exposed to hypoxia. When compared with vehicle-treated rats, treatment with SKI2 (10 mg/kg body weight, intraperitoneally, every two days for 3.5 wks) prevented the development of HPH, as assessed by RVSP, RVH, and pulmonary vascular remodeling (**Figure 11**). Together with the results from the HPH studies in *SphK1*$^{-/-}$, *SphK2*$^{-/-}$, and *Sgpl1*$^{+/-}$ mice, these data strongly support our hypothesis that S1P plays a critical role in the development of PH and that targeting its synthesis may be therapeutically beneficial. These studies led us to further explore the mechanism of how SphK1 and S1P may promote the pathogenesis of PAH, with a focus on pulmonary vascular remodeling.

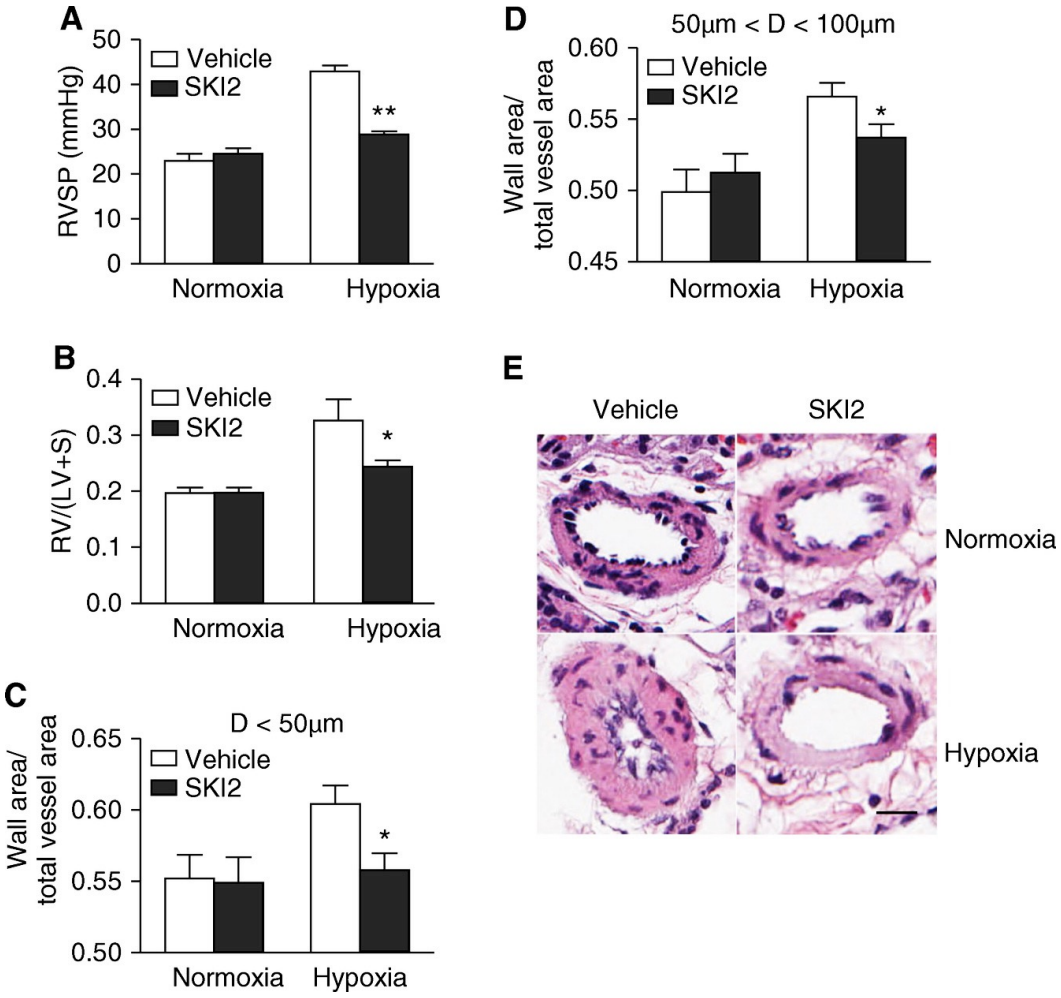

Figure 11. SphK inhibition via SKI2 prevents HPH development in rats. Rats exposed to hypoxia and treated with SKI2 (10 mg/kg body weight, intraperitoneally, every two days for 3.5 wks) demonstrated attenuated increases in (A) RVSP, (B) RVH, and pulmonary vessel thickness in arteries < 50 μm (C) and 50–100 μm in diameter (D) compared to vehicle-treated controls. (E) Representative pulmonary artery images from lung sections of rats exposed to normoxia or hypoxia and treated with either SKI2 or vehicle control. Bar size: 20 μm. Results are expressed as mean ± SEM; n = 6 per group. *$p < 0.05$; **$p < 0.01$ versus hypoxia control group. This figure was reprinted with permission from Chen J., Tang H., Sysol J.R., et al. 2014. "The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension." *Am J Respir Crit Care Med.* 190(9):1032-43. doi: 10.1164/rccm.201401-0121OC. Copyright © 2016 American Thoracic Society.

V. SPHK1 AND S1P PROMOTE PULMONARY ARTERY SMOOTH MUSCLE CELL PROLIFERATION VIA S1PR2 SIGNALING

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1. Chen J, Tang H, Sysol JR, Moreno-Vinasco L, Shioura KM, Chen T, Gorshkova I, Wang L, Huang LS, Usatyuk PV, Sammani S, Zhou G, Raj JU, Garcia JG, Berdyshev E, Yuan JX, Natarajan V, and Machado RF. 2014. The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension. *Am J Respir Crit Care Med.* 190(9):1032-43. doi: 10.1164/rccm.201401-0121OC. (43) Reprinted with permission of the American Thoracic Society. Copyright © 2016 American Thoracic Society.

A. Rationale

Recent studies have identified and described the role of SphK1 and S1P signaling in many conditions involving cell proliferation, including pulmonary diseases and cancer. Through its myriad actions as a bioactive signaling molecule, S1P has been shown to promote differentiation, motility, and resistance to apoptosis in cells (13,55). Neutralizing S1P with monoclonal antibodies has also shown therapeutic promise in reducing cell growth and invasion in multiple murine tumor lineages (56). SphK1, which produces S1P from sphingosine, is itself oncogenic and can promote cell proliferation (17,18). These effects may underlie our findings in Chapter IV that upregulation of SphK1/S1P is found human PAH and contributes to disease progression and pulmonary vascular remodeling in several animal models of HPH (43).

S1P can act both intracellularly and extracellularly, with its extracellular effects as a soluble signaling molecule mediated primarily through binding to domains of a family of five GPCRs known as S1PR1-5. Interestingly, S1P can also be transported out of the cell to act in a paracrine fashion at the cell surface, a process known as "inside-out" signaling (57). Binding of S1P to S1PRs triggers

activation of numerous downstream signaling pathways which vary based on the specific Gα subunit that is activated, including $G\alpha_i$, $G\alpha_{12/13}$, and $G\alpha_q$ (**Figure 12**). Pathways triggered by S1P can modulate cell metabolism, proliferation, survival, cell survival, vascular tone, and migration. Many of these processes are intimately associated with the development of pulmonary vascular remodeling in PAH. However, whether SphK1/S1P overexpression and signaling may regulate hPASMC proliferation in the context of PAH is largely unknown.

In this Chapter, we investigated the mechanistic role of SphK1 and S1P to promote proliferation of hPASMCs. These studies provide novel insight into the cellular events and signaling molecules contributing to proliferation of this cell type. Understanding these pathways in greater detail may lead to the discovery of new therapeutic targets to combat pulmonary vascular remodeling in PAH.

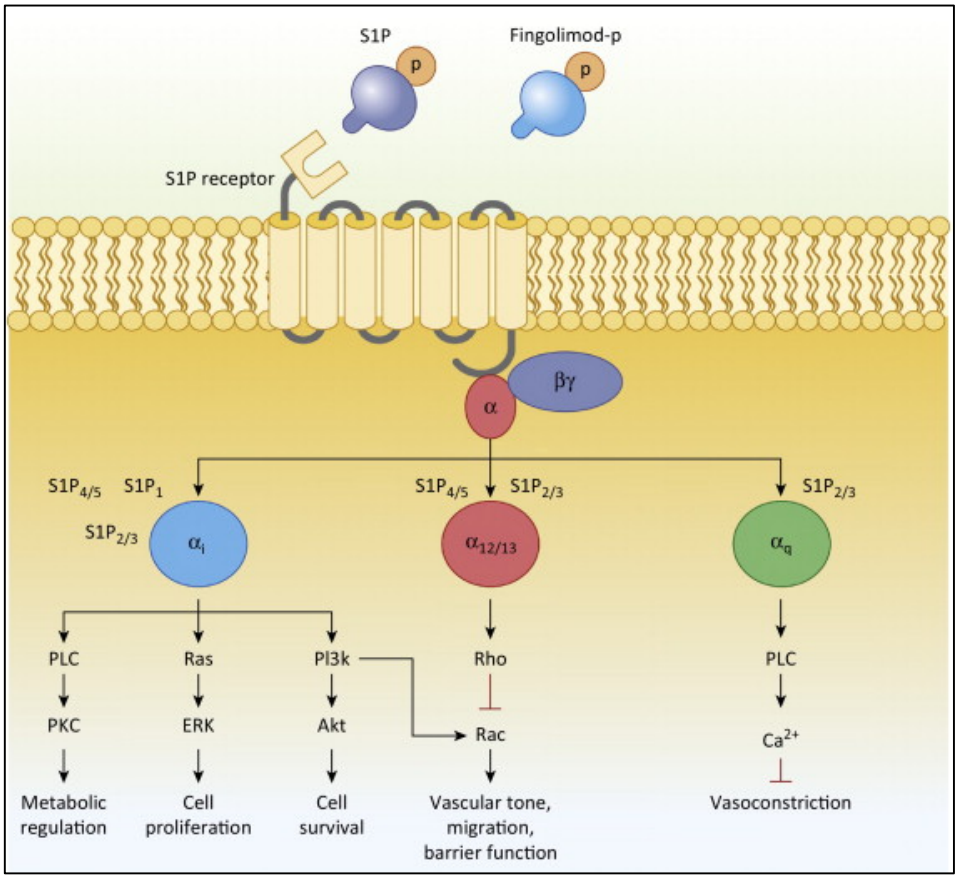

Figure 12. S1P signals through GPCRs and activates various downstream pathways. The five S1PRs activate different profiles of Gα subunits and as a result the profile of S1PR expression on a given cell type results in a pleiotropic response to S1P signaling. S1P is a soluble signaling molecule that binds the extracellular domain of the S1PR, triggering activation of the GPCR pathway and receptor internalization. Downstream signaling pathways stimulated by S1P vary depending on the Gα subunit that is activated. Pathways triggered by S1P binding modulate various signals including those involved in cell survival, vascular tone, and endothelial tight junction integrity. Reprinted from *Trends Mol Med*, Volume 21(6), Prager B., Spampinato S.F., and Ransohoff R.M. "Sphingosine 1-phosphate signaling at the blood–brain barrier." Pages 354-63, Copyright 2015, with permission from Elsevier. doi: 10.1016/j.molmed.2015.03.006.

B. SphK1 and S1P Promote hPASMC Proliferation

To begin to study the whether SphK1 and S1P may mediate hPASMC proliferation, we first characterized the role of intracellular SphK1 overexpression. In cultured hPASMCs, overexpression of SphK1 by transfection with a plasmid containing WT SphK1 significantly promoted cell proliferation as demonstrated by cell counting and BrdU incorporation assays (**Figure 13**). Overexpression of a dominant-negative form of SphK1 containing a G82D point mutation, which blocks SphK1 activity and functions (58), significantly inhibited hPASMC proliferation by these measures (**Figure 13**). To confirm these findings, the proliferation of PASMCs in *SphK1*$^{-/-}$ mice exposed to hypoxia, (10% O_2, 4 wks) was assessed by co-staining for α-smooth muscle actin, a smooth muscle cell marker, and PCNA, a proliferation marker, using immunohistochemistry in paraffinized mouse lung sections. *SphK1*$^{-/-}$ mice demonstrated attenuated proliferation of PASMCs in response to chronic hypoxia compared to WT control mice (**Figure 14**). These results are consistent with our findings in hPASMCs demonstrating the importance of SphK1 expression in proliferation of this cell type.

Next, we tested whether exogenous S1P could stimulate hPASMC proliferation *in vitro*. S1P (10-100nM, 48hrs) led to a dose-responsive increase in hPASMC proliferation compared to vehicle-only control measured by a BrdU incorporation assay, with platelet-derived growth factor (PDGF, 20ng/ml) used as a positive control (**Figure 15A**). Together with the SphK1 overexpression data, these findings confirmed our hypothesis that S1P signaling is pro-proliferative in hPASMCs.

To further explore the mechanisms of S1P-mediated proliferation, we then examined whether the effects of S1P were dependent on particular S1PR ligation. Previous studies have shown that S1PR2 and S1PR3 are the most predominant S1PRs expressed in adult hPASMCs (59). Importantly, these receptors have also been shown to mediate S1P-induced cell proliferation,

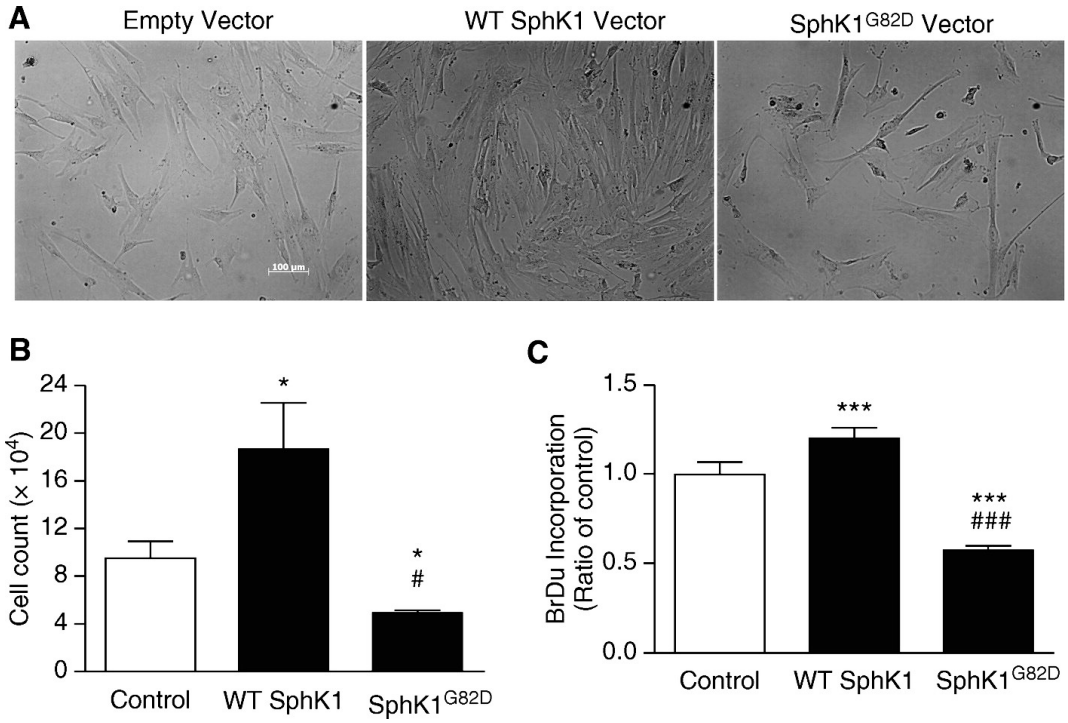

Figure 13. Overexpression of WT SphK1 promotes PASMC proliferation and overexpression of dominant-negative mutant (G82D) SphK1 inhibits PASMC proliferation. Human PASMCs were cultured at equal cell density and transfected with either empty vector (control), plasmid expressing WT SphK1, or plasmid expressing dominant-negative mutant (G82D) SphK1. (A) Representative photomicrograph of cells in culture 2 days after transfection. Cell proliferation was measured by cell counting (B) and BrdU incorporation assays (C). Bar size: 100 µm. Data are expressed as mean ± SEM; n = 4; $*p < 0.05$; $***p < 0.001$ versus control group; $\#p < 0.05$; $\#\#\#p < 0.001$ versus WT SphK1 group. This figure was reprinted with permission from Chen J., Tang H., Sysol J.R., et al. 2014. "The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension." *Am J Respir Crit Care Med.* 190(9):1032-43. doi: 10.1164/rccm.201401-0121OC. Copyright © 2016 American Thoracic Society.

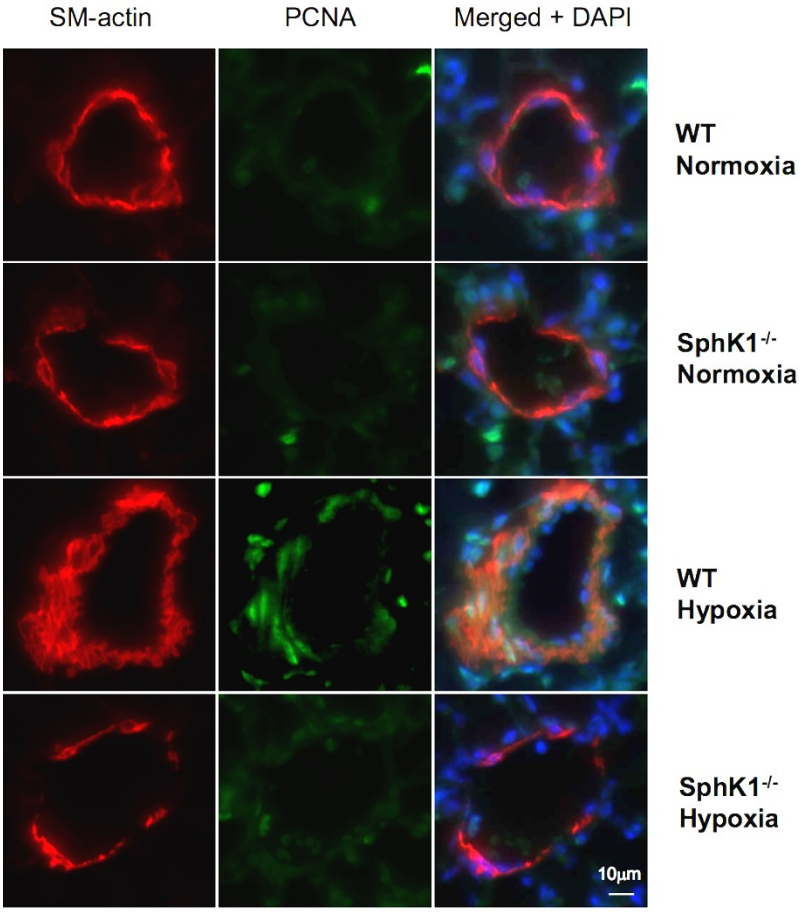

Figure 14. PASMCs from SphK1-deficient mice have attenuated PCNA expression in response to chronic hypoxia. Immunohistochemistry in paraffinized mouse lung sections from *SphK1*$^{-/-}$ and WT control mice in normoxia and hypoxia (10%O_2, 4 wks) was done to assess PASMC proliferation. PASMCs were stained with Cy3-labeled mouse anti-α-smooth muscle actin (SM-actin) antibodies (1:300) and proliferating cells were examined by rabbit anti-PCNA antibodies (1:100) followed by Alexa Fluor 488 Donkey anti-Rabbit IgG antibody (1:500) staining. Representative pulmonary artery images demonstrate that *SphK1*$^{-/-}$ mice exhibit less medial hypertrophy of PASMCs (red) in chronic hypoxia compared to WT controls, which was associated with a decreased number of proliferating PCNA-positive cells (green). Scale bar: 10 μm. This figure was reprinted with permission from Chen J., Tang H., Sysol J.R., et al. 2014. "The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension." *Am J Respir Crit Care Med.* 190(9):1032-43. doi: 10.1164/rccm.201401-0121OC. Copyright © 2016 American Thoracic Society.

survival, and related signaling events in other cell types (60). We found that the protein expression of S1PR2 was significantly elevated in hPASMCs isolated from PAH patients compared to controls (**Figure 15B-C**). However, no differences were observed in S1PR3 expression levels (data not shown). These findings led us to focus on the downstream mechanisms of S1P/S1PR2 signaling in future studies.

C. S1PR2 Mediates the Proliferative Effects of S1P in hPASMCs

Several lines of evidence support the role of S1P/S1PR2 in cell proliferation. In satellite muscle stem cells, S1P/S1PR2 signaling has been shown to promote proliferation via repression of cell cycle inhibitors (61). In addition, SphK1/S1P/S1PR2 signaling in human chronic myeloid leukemia cells exhibits oncogenic properties via enhancement of Bcr-Abl1 protein stability (62). Other studies have highlighted that S1PR2 can negatively regulate PDGF-induced motility and proliferation (63), demonstrating the complexity of this receptor in its downstream functions and crosstalk with other pathways. It is unsurprising that S1P signaling through ligation of S1PR2 may produce disparate effects in different cell types given its ability to activate the $G\alpha_i$, $G\alpha_{12/13}$, and $G\alpha_q$ families of G protein in certain contexts (64).

Given the increased expression of S1PR2 in PASMCs from PAH patients and its role of mediating proliferation in other cell types, we investigated whether inhibition of S1PR2 could attenuate the pro-proliferative effects of S1P. We found that silencing of S1PR2 expression via siRNA or S1PR2 antagonism with JTE-013 decreased basal and S1P-mediated PASMC proliferation. Treatment of hPASMCs with JTE-013 (10 μM), a potent and selective S1PR2 antagonist, reduced both basal and S1P (100 nM)-mediated hPASMC proliferation as measured by BrdU incorporation (**Figure 15D**). To confirm these findings, we used siRNA-mediated silencing of S1PR2 in similar studies, achieving approximately 80% reduction in S1PR2 protein expression

(**Figure 15E**). S1PR2-specific siRNA-mediated silencing attenuated basal and S1P (100 nM)-mediated hPASMC proliferation measured by BrdU incorporation (**Figure 15F**). Functional S1PR2 is therefore important in basal and S1P-induced hPASMC proliferation.

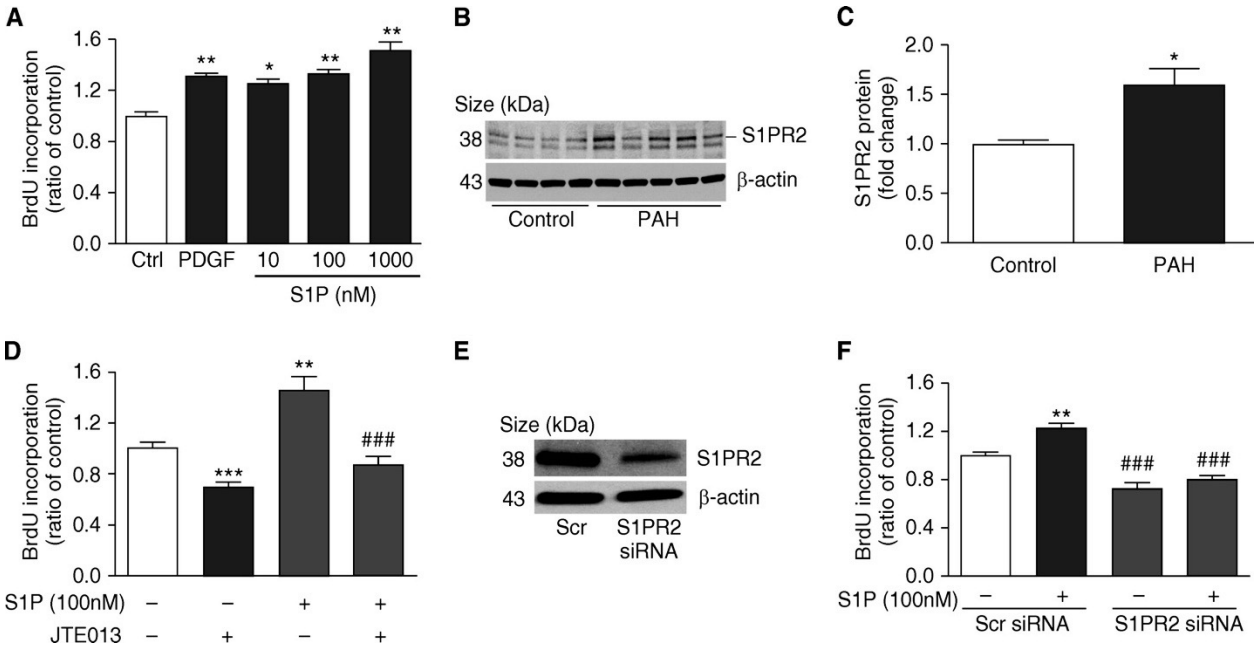

Figure 15. S1P stimulates hPASMC proliferation via S1PR2. (A) S1P (10-1000 nM, 48 hrs) stimulates hPASMC proliferation measured by BrdU incorporation with PDGF (20 ng/ml) used as a positive control. (B and C) Western blotting images and β-actin–normalized quantification of protein levels demonstrate that S1PR2 protein expression is significantly increased in PASMCs from patients with PAH compared to controls. (D) Antagonism of S1PR2 with JTE-013 (10 μM) attenuates basal and S1P (100 nM)-mediated hPASMC proliferation, measured by BrdU incorporation. (E) Representative Western blotting images demonstrate the effect of siRNA-mediated silencing of S1PR2 in hPASMCs. (F) siRNA-mediated silencing of S1PR2 attenuates basal and S1P (100 nM)-mediated hPASMC proliferation, measured by BrdU incorporation. Results are expressed as mean ± SEM; n = 6 per group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus untreated control conditions or control hPASMC cell lines; ###$p < 0.001$ versus S1P without JTE-013 condition (D) or scrambled siRNA with S1P group (F). This figure was reprinted with permission from Chen J., Tang H., Sysol J.R., et al. 2014. "The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension." *Am J Respir Crit Care Med.* 190(9):1032-43. doi: 10.1164/rccm.201401-0121OC. Copyright © 2016 American Thoracic Society.

D. PASMCs from S1PR2-deficient Mice Have Reduced Proliferative Capacity

We next investigated the proliferative phenotype of PASMCs completely deficient of S1PR2 expression. Using an established murine PASMC isolation method (52), PASMCs were obtained and cultured from *S1PR2*$^{-/-}$ mice and WT controls with ~90% cell purity as assessed by immunostaining for α-smooth muscle actin (**Figure 16A**). Compared to WT, *S1PR2*$^{-/-}$ PASMCs exhibited reduced basal and S1P-induced proliferation (**Figure 16B**). In addition, the S1PR2 antagonist JTE-013 did not inhibit S1P-induced proliferation in *S1PR2*$^{-/-}$ cells. These results corroborate our previous findings for the role of S1PR2 signaling in hPASMC proliferation.

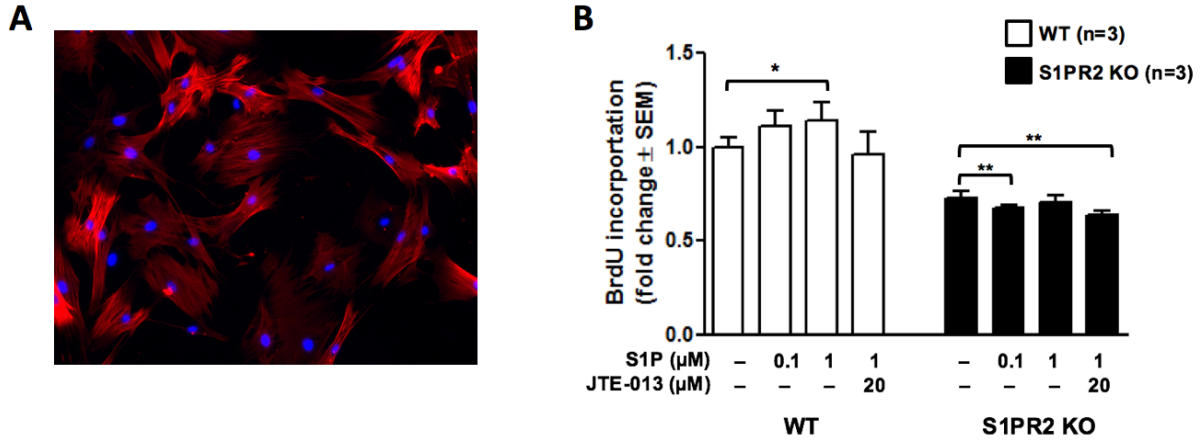

Figure 16. PASMCs isolated from S1PR2-deficient mice have reduced proliferative capacity. (A) Representative immunofluorescence image of isolated mouse PASMCs demonstrates high cell purity (red: α-smooth muscle actin; blue: DAPI). (B) PASMCs isolated and cultured from S1PR2-deficient (KO) mice have a reduced basal proliferation rate and abolished S1P (0.1-1 μM, 24 hr)-induced proliferation measured by BrdU incorporation compared to WT controls. The KO cells do not respond to the S1PR2 antagonist JTE-013 (20 μM). Results are expressed as mean ± SEM; n = 3 per group. *$p < 0.05$, **$p < 0.01$, compared to indicated condition.

E. S1PR2-Deficient Mice are Protected Against HPH Development

Given our studies establishing the importance of SphK1/S1P/S1PR2 signaling in pulmonary vascular remodeling, including reduced proliferation of PASMCs isolated from *S1PR2*$^{-/-}$ mice, we investigated the effects of S1PR2 deficiency on the development of HPH *in vivo*. We hypothesized that *S1PR2*$^{-/-}$ mice would have less severe vascular remodeling, leading to protection from HPH. After 4 wks of hypoxia exposure (10% O_2), our data demonstrate that *S1PR2*$^{-/-}$ mice have attenuated elevations in RVSP and RVH (**Figure 17**). No protection from HPH development was observed in *S1PR3*$^{-/-}$ mice (data not shown). The relatively small magnitude of the HPH protection observed in the *S1PR2*$^{-/-}$ mice may be due to compensatory expression of other S1PRs in the pulmonary vasculature, and further studies are planned to investigate this in more detail.

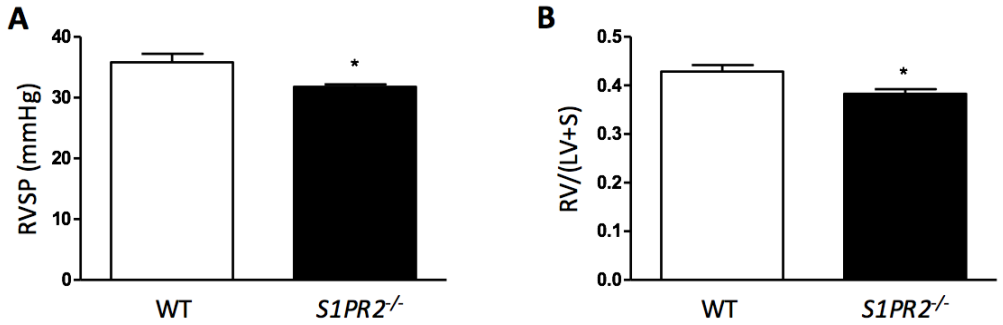

Figure 17. S1PR2-deficient mice are protected from HPH development. Following 4 wks of hypoxia exposure (10% O_2), *S1PR2*$^{-/-}$ mice have attenuated elevations in (A) RVSP and (B) RVH compared to WT littermate controls. Results are expressed as mean ± SEM; n = 5 per group. *p < 0.05 compared WT controls.

F. S1PR2 Inhibition Prevents and Reverses the Development of HPH in Mice

Since *S1PR2*$^{-/-}$ mice demonstrated protection from the development of HPH, we next sought to examine the effects of inhibiting S1PR2 on HPH development in mice. We hypothesized that pharmacological reduction of S1PR2 signaling would attenuate S1P-mediated PASMC proliferation and protect from HPH and/or reverse established HPH development. To test this, we evaluated the effect of administering JTE-013 in mice before (prevention studies) and after (reversal studies) exposure to chronic hypoxia. For prevention studies, mice were injected intraperitoneally with JTE-013 (8 mg/kg body weight) once every two days, starting on the first day of a 3.5-wk hypoxia exposure time course. For prevention studies, mice were similarly injected with JTE-013 starting at day 14 of a 4-wk hypoxia exposure time course. These studies demonstrated that JTE-013 prevents and reverses the development of HPH in mice, as assessed by RVSP, RVH, and pulmonary vascular remodeling (**Figure 18**). The beneficial effects of JTE-013 in reversing established HPH are promising since symptoms of PAH often do not manifest until significant vascular remodeling has occurred. These findings suggest that S1P/S1PR2 may mediate hPASMC proliferation throughout the disease course and act to maintain cell survival in remodeled pulmonary arteries.

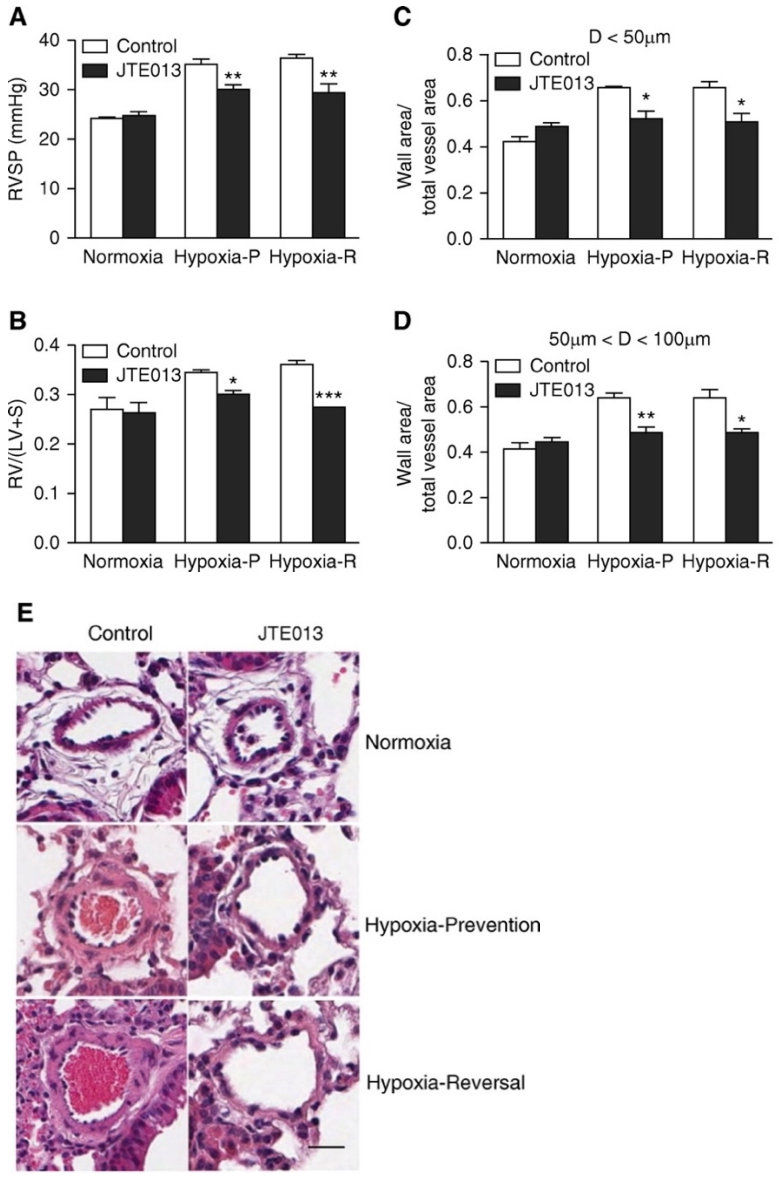

Figure 18. S1PR2 inhibition with JTE-013 prevents and reverses HPH development in mice. For prevention studies ("P"), JTE-013 (8 mg/kg, i.p., once every other day) was started on the first day of a 3.5-week hypoxia exposure. For reversal studies ("R"), JTE-013 was given similarly starting after 2 weeks of hypoxia and followed by 2 additional weeks of hypoxia. (A) Changes in RVSP, (B) RVH, and (C-D) pulmonary vascular remodeling quantification in arteries < 50 μm and 50-100 μm in diameter, respectively) in control and JTE-013-treated mice after normoxia or hypoxia exposure studies. (E) Representative PAs from lungs of control and JTE-013-treated groups after normoxia or hypoxia exposure. Bar size: 20 μm. Results are expressed as mean ± SEM; n = 6 per group. *p < 0.05; **p < 0.01; ***p < 0.001 versus respective hypoxia group. This figure was reprinted/modified with permission from Chen J., Tang H., Sysol J.R., et al. 2014. "The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension." *Am J Respir Crit Care Med.* 190(9):1032-43. doi: 10.1164/rccm.201401-0121OC. Copyright © 2016 American Thoracic Society.

G. S1P/S1PR2-mediated hPASMC Proliferation Involves ERK Activation

The MAPK/ERK signaling pathway plays a central role in regulating the growth and survival of mammalian cells including a wide variety of cancers, and several specific inhibitors of this pathway have entered clinical trials (65). ERK1/2 (referred to here as ERK) signaling regulates cell proliferation and differentiation in response to numerous signals. In the basal state ERKs are catalytically inactive, and their activity requires phosphorylation by a family of upstream MEKs.

Several lines of evidence have demonstrated that ERK activation is involved in mediating hPASMC proliferation induced by a variety of stimuli *in vitro*. For example, pro-proliferative effects of hypoxia and brain-derived neurotrophic factor (BDNF) in hPASMCs have been attenuated using U0126, a highly selective MEK inhibitor (66,67). Another recent study demonstrated that both U0126 and inhibition of PDGF receptor signaling blocked peroxynitrite-induced proliferation and ERK phosphorylation in hPASMCs (68). Importantly, both SphK1 and S1P have also been shown to activate ERK (55,60,69). These studies highlight the multifaceted mechanisms by which ERK may be activated and regulate hPASMC proliferation. We therefore hypothesized that S1P/S1PR2-induced hPASMC proliferation may involve ERK signaling.

To test whether ERK activation was required for S1P-induced proliferation, we pretreated hPASMCs with PD98059 (10 μM, 45 minutes), a selective inhibitor of MEK1 activity and the ERK cascade, and stimulated with S1P (100 nM, 24 hrs). These studies demonstrated that S1P-induced hPASMC proliferation was significantly attenuated with PD98059, measured by BrdU incorporation (**Figure 19A**). We also investigated whether S1P could activate ERK in hPASMCs and the role of S1PR2 in this process. Inhibition of S1PR2 with JTE-013 (10 μM) significantly attenuated dose-responsive S1P-mediated ERK phosphorylation (1-100 nM) (**Figure 19B**). These studies suggest that S1P/S1PR2-mediated hPASMC proliferation involves activation of ERK signaling.

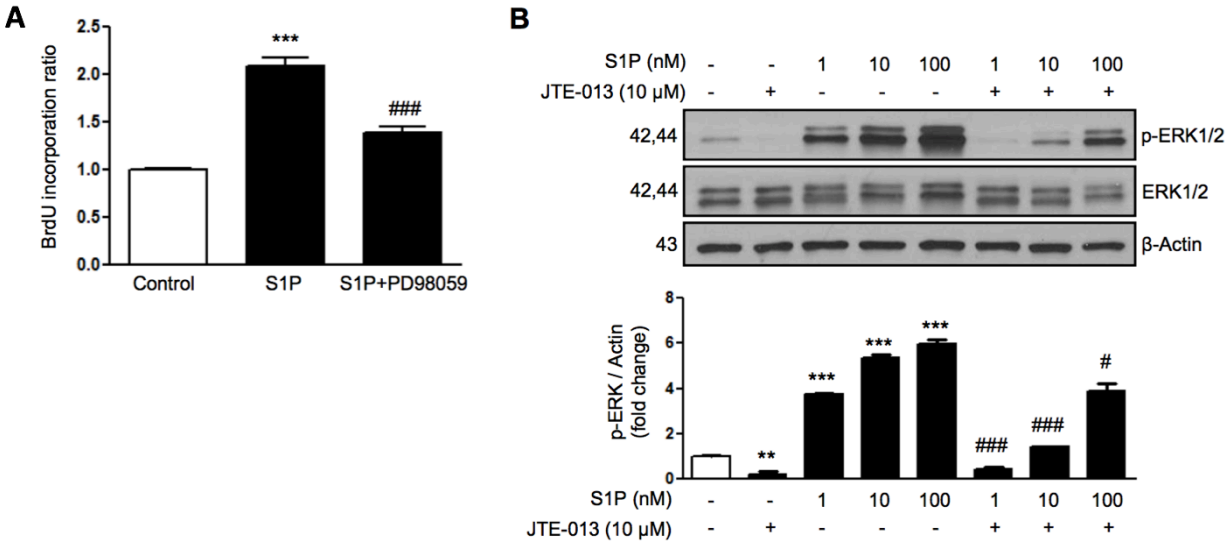

Figure 19. Role of ERK activation in S1P/S1PR2-induced hPASMC proliferation. (A) Inhibition of ERK activation (PD98059, 10 μM, 45 min pre-treatment) attenuates S1P-mediated hPASMC proliferation measured by BrdU incorporation assay (***p < 0.001 versus control; ###p < 0.001 versus S1P group). (B) S1P (1-100 nM, 15 min) promotes ERK phosphorylation while inhibition of S1PR2 by JTE-013 (10 μM, 45 min pre-treatment) attenuates basal and S1P-induced ERK phosphorylation (*p <0.05, **p < 0.01 ***p < 0.001 versus control; #p <0.05, ##p < 0.01 ###p < 0.001 versus S1P group). This figure was reprinted/modified with permission from Chen J., Tang H., Sysol J.R., et al. 2014. "The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension." *Am J Respir Crit Care Med.* 190(9):1032-43. doi: 10.1164/rccm.201401-0121OC. Copyright © 2016 American Thoracic Society.

H. S1P/S1PR2 Signaling Activates STAT3 in hPASMC

We also examined whether S1P affects other key signaling pathways involved in PAH. Several recent studies have demonstrated that activation of of the signal transducer and activator of transcription 3 (STAT3) transcription factor is involved in the pro-survival and proliferative pulmonary vascular phenotype of PAH (70-72). STAT3 belongs to a protein family which regulates diverse cellular processes including proliferation and survival, and is frequently dysregulated in cancer (70). Its activation requires phosphorylation (Y705), which leads to its nuclear translocation, dimerization, and subsequent binding to different regulatory regions. This process can occur in

response to many different growth factors and agonists, such as PDGF and endothelin-1 (ET1), which are elevated in PAH. Of particular interest to our studies, the SphK1/S1P axis has been shown to link persistent STAT3 activation with the development of colitis-associated cancer (73). S1P also promotes muscle stem cell progression through the cell cycle by repression of cell cycle inhibitors via S1PR2/STAT3-dependent signaling (61).

Based on these previous studies, we tested whether S1P/S1PR2 activates STAT3 signaling in hPASMCs. We found that S1P induced rapid and sustained expression and nuclear translocation of phosphorylated STAT3 (**Figure 20A**). Additionally, siRNA-mediated silencing of S1PR2 expression attenuated S1P-mediated STAT3 phosphorylation and diminished levels to below baseline values (**Figure 20B**). In support of these findings, we also demonstrated that S1PR2 antagonism with JTE-013 attenuated S1P-mediated STAT3 phosphorylation (**Figure 20C**). Pre-treatment with JTE-013 alone reduced basal STAT3 phosphorylation levels in these experiments, suggesting a role of S1PR2 in maintaining basal levels of STAT3 activation.

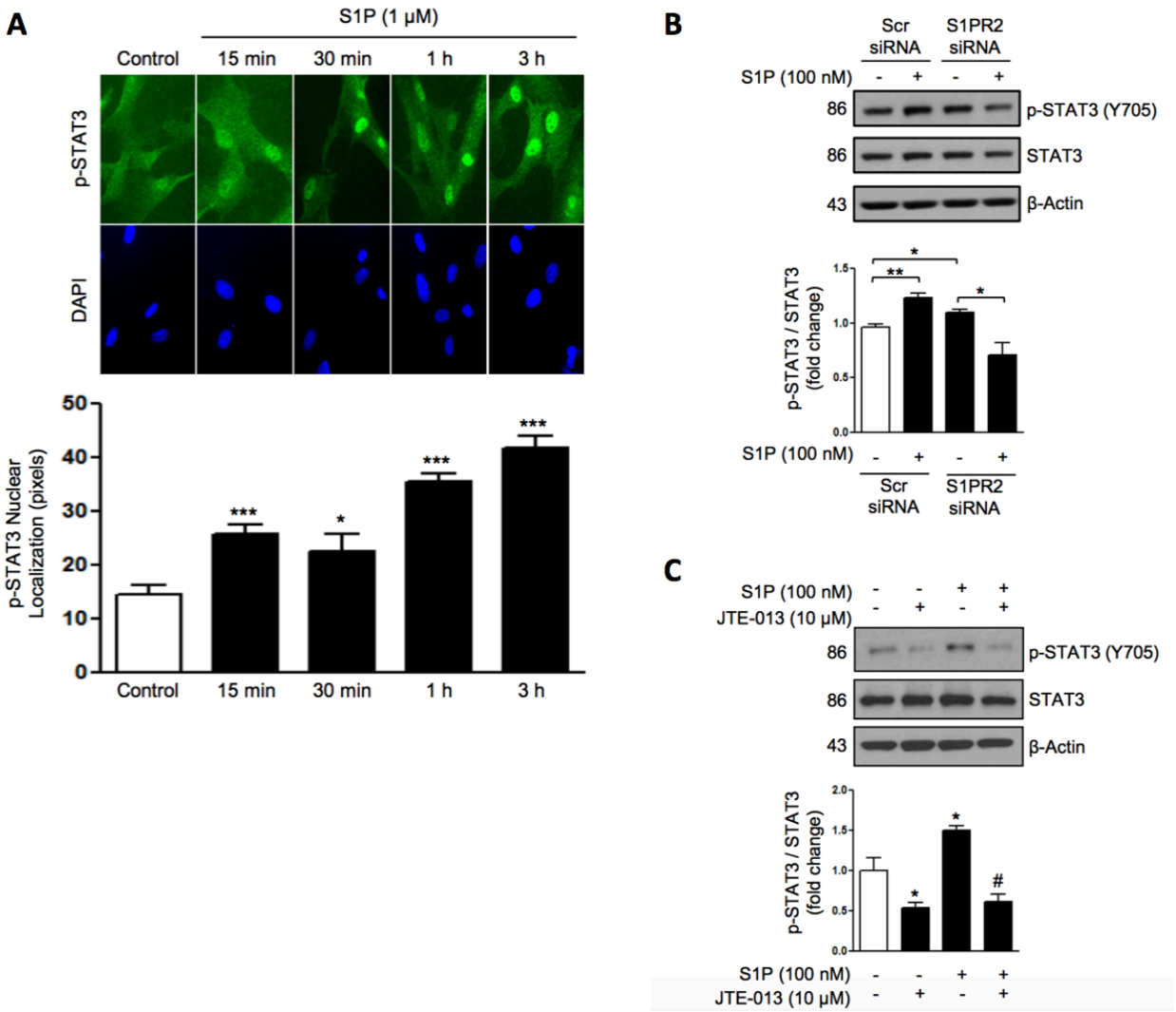

Figure 20. S1P/S1PR2 signaling activates STAT3 in hPASMC. (A) S1P (1 μM) induces a time-dependent nuclear translocation of p-STAT3 (Y705) in hPASMC (60x magnification). (B) Western blotting demonstrating S1PR2-siRNA attenuates S1P (100 nM, 30 min)-induced STAT3 phosphorylation in hPASMC. (C) Western blotting demonstrating JTE-013 (10 μM, 45 min pre-treatment) attenuates S1P (100 nM, 30 min)-induced STAT3 phosphorylation in hPASMC. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control or as indicated; #$p < 0.05$ versus S1P group. This figure was reprinted/modified with permission from Chen J., Tang H., Sysol J.R., et al. 2014. "The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension." *Am J Respir Crit Care Med.* 190(9):1032-43. doi: 10.1164/rccm.201401-0121OC. Copyright © 2016 American Thoracic Society.

I. STAT3 is Increased in PASMCs of PAH Patients and is Involved in S1P-induced hPASMC Proliferation

Since STAT3 has been previously shown to be constitutively activated in PAH (74,75), we next explored whether STAT3 was activated in PASMCs isolated from PAH patients. The ratio of phosphorylated (Y705) to unphosphorylated STAT3 was significantly increased in PAH patient PASMCs (**Figure 21A-B**), which may contribute to their increased proliferative capacity.

To examine whether STAT3 activation was involved in S1P-induced hPASMC proliferation, we tested the effect of a specific inhibitor of janus kinase 2 (JAK2), a non-receptor tyrosine kinase, that blocks downstream STAT3 activation. When pretreated with WP1066 (1 μM, 45 min), hPASMCs exhibited a reduction in S1P (1 μM, 48 hr)-induced proliferation as measured by BrdU incorporation (**Figure 21C**). Studies to further characterize the importance of S1P/S1PR2/STAT3 signaling in hPASMC proliferation are therefore warranted.

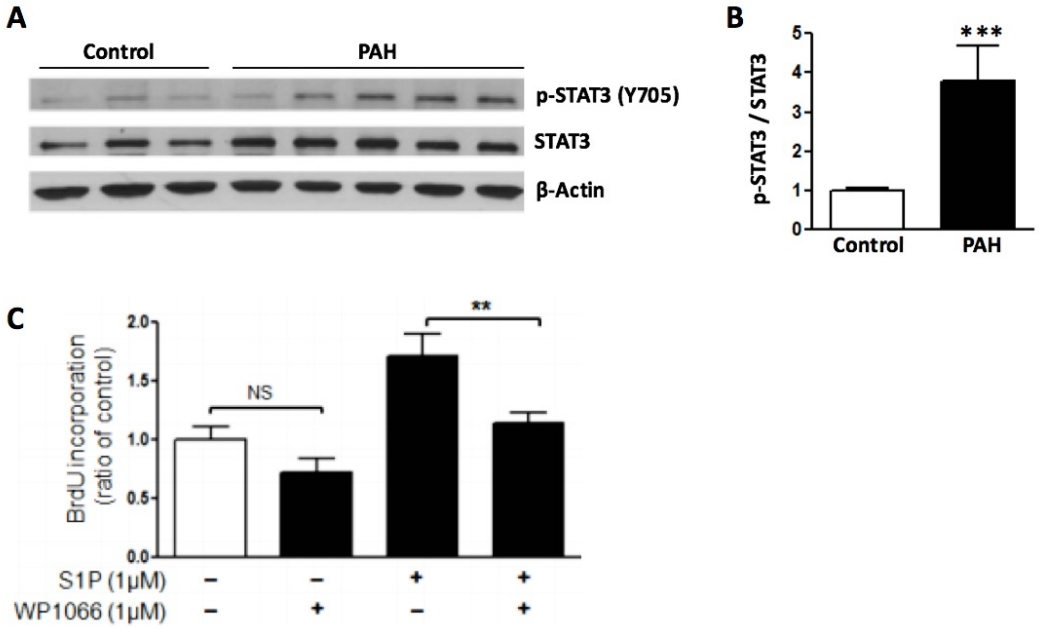

Figure 21. STAT3 phosphorylation (Y705) is upregulated in PASMCs from PAH patients and mediates S1P-induced hPASMC proliferation. (A-B) Western blot images and corresponding quantification demonstrates basal upregulation of STAT3 phosphorylation in PASMCs from PAH patients compared to control subjects. (C) WP1066 (1 μM, 45 min pre-treatment), a specific STAT3 inhibitor, prevents S1P (1 μM, 48 hr)-induced hPASMC proliferation, measured by BrdU incorporation. $^{**}p < 0.01$, $^{***}p < 0.001$, compared to controls (*B*) or S1P condition (*C*).

VI. S1P SIGNALING ACTIVATES HYPOXIA-INDUCIBLE FACTOR 1-ALPHA IN PULMONARY ARTERY SMOOTH MUSCLE CELLS

A. Rationale

As outlined in previous Chapters, hyper-proliferation of PASMCs in PAH can contribute to elevated pulmonary pressures by impairing blood flow and increasing vascular resistance. Therefore, understanding the regulation of PASMC proliferation is critical in this fatal disease. We have demonstrated the significant role of SphK1/S1P/S1PR2 signaling in the pathogenesis of PAH, including in pulmonary vascular remodeling, using human samples, *in vitro* studies, and *in vivo* disease modeling with hypoxia. In this Chapter, we aimed to explore the mechanistic interactions between S1P signaling and hypoxia in promoting hPASMC proliferation.

The role of hypoxia and its activation of the transcription factor hypoxia-inducible factor-1 alpha (HIF1α) in the pathogenesis of pulmonary vascular remodeling in PAH is well established (76-78). HIF1α is a master regulator of the cellular response to hypoxia, including the control of energy metabolism, proliferation, migration, angiogenesis, and extracellular matrix reorganization (79). Elevated levels of HIF1α in PAH pulmonary artery smooth muscle (80) and endothelial cells (81) have been reported. Interestingly, recent studies have shown that HIF1α can also be overexpressed and activated under normoxic conditions to promote cell proliferation and survival in many types of cancers via the enhanced use of glycolysis for energy, a process known as the Warburg effect (82-85). Studies have also demonstrated that normoxic HIF1α activation can be induced by S1P stimulation in cancer (86), potentially through S1PR2 signaling (87). Together with our data demonstrating S1P/S1PR2 are involved in the pathogenesis of PAH, these studies suggest that there may be a mechanistic link between S1P signaling and HIF1α in pulmonary vascular remodeling. In this Chapter, we investigated the role of S1P-mediated activation of HIF1α under normoxic

conditions in hPASMCs. We hypothesized that S1P/S1PR2 signaling would activation HIF1α activation to promote hPASMC proliferation, contributing to the development of PAH.

B. Hypoxia Stimulates HIF1α Expression and Induces hPASMC Proliferation

We first sought to confirm previous studies demonstrating the role of hypoxia and activation of HIF1α in stimulating hPASMC proliferation (76). To study this, we stimulated hPASMCs with hypoxia (3% O_2, 1-12 hrs) and measured the protein expression of HIF1α. Hypoxia caused a rapid and sustained increase in HIF1α expression from 1-6 hrs which normalized to baseline levels by 12 hrs (**Figure 22A**). Hypoxia also caused in increase in hPASMC proliferation at both 24 and 48 hrs compared to normoxic conditions (**Figure 22B**).

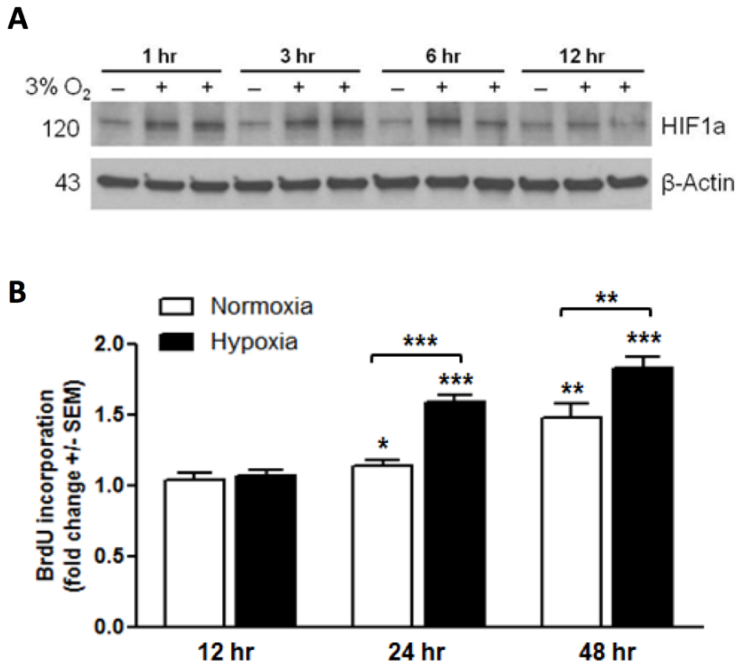

Figure 22. Hypoxia stimulates HIF1α expression and induces hPASMC proliferation. (A) Hypoxia (3% O_2, 1-12 hrs) time-dependently increases HIF1α protein expression in hPASMCs. (B) BrdU incorporation assay demonstrating hypoxia-mediated hPASMC proliferation over time (3% O_2, 12-48 hrs). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, compared to untreated control or as indicated.

C. S1P/S1PR2 Induces Nuclear HIF1α Protein Expression in hPASMCs

Given the ability of both S1P/S1PR2 and hypoxia to induce proliferation in hPASMCs in our studies, as well as recent reports that these two signaling mechanisms may be linked (86,87), we next examined whether S1P could induce HIF1α expression under normoxic conditions in hPASMCs. Cells stimulated with exogenous S1P (0.1-1 μM, 1-12 hrs) exhibited a dose-responsive increase in HIF1α protein levels at 3 hrs that returned to baseline levels by 6 hrs in whole cell lysates (**Figure 23A**). S1P (1 μM) also induced mRNA expression of HIF1A in hPASMCs at 3 hrs, indicating that transcriptional upregulation may play a mechanistic role in HIF1α activation in our studies (**Figure 23B**). Next, to determine whether this induction was present in the nucleus where HIF1α acts as a transcription factor to induce target gene expression, we stimulated hPASMCs with S1P (1 μM, 3 hrs) and collected both nuclear and cytoplasmic protein fractions. S1P caused a significant increase in nuclear HIF1α protein expression levels normalized to nuclear Lamin B1 loading control (**Figure 23C-D**). Cytoplasmic HIF1α was undetectable in these studies, likely due to the relatively low abundance in this cellular compartment compared to the nucleus. Lysates from hypoxia stimulated cells (3% O_2, 3 hrs) were used as a positive control. Interestingly, when cells were pre-treated with the S1PR2 antagonist JTE-013 (10 μM, 45 min) before S1P stimulation, the induction of nuclear HIF1α protein expression was abolished (**Figure 23C-D**), indicating that S1PR2 signaling is required for this effect.

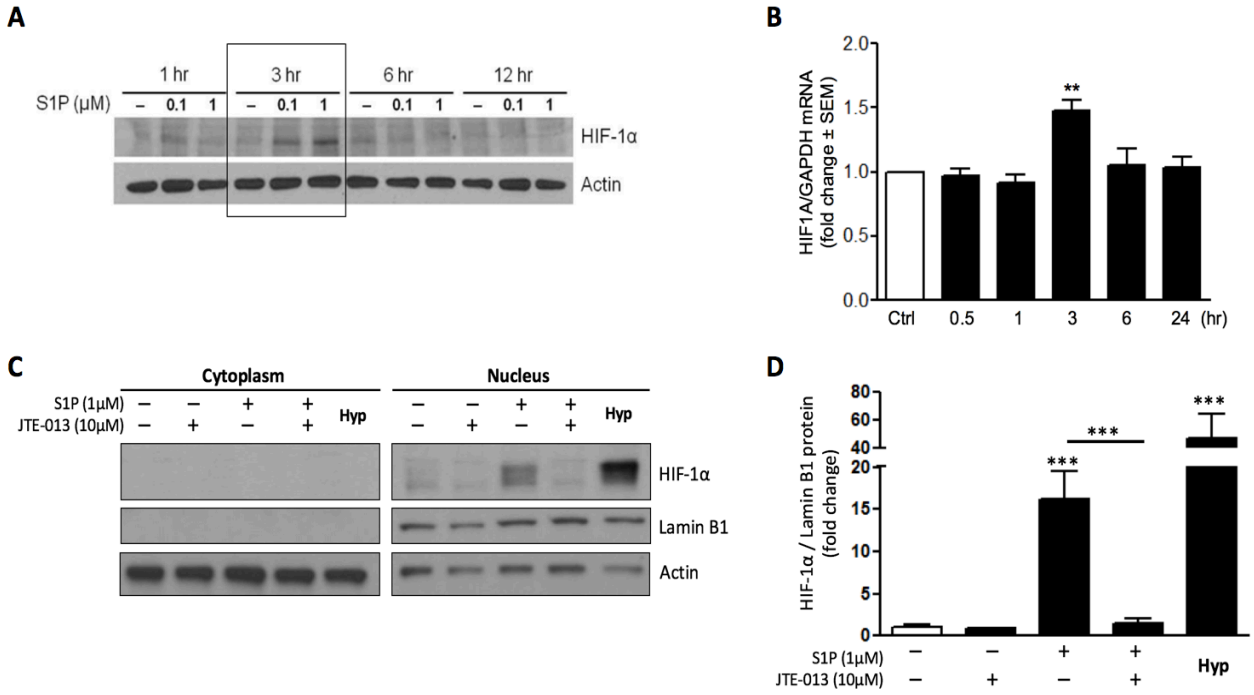

Figure 23. S1P induces HIF1α expression and nuclear translocation in hPASMCs. (A) S1P dose-responsively increases HIF1α protein at 3 hrs (100 nM: 4.7-fold, $p<0.01$; 1 μM: 6.4-fold, $p<0.01$). (B) S1P (1 μM) induces upregulation of HIF1A mRNA in hPASMCs at 3 hrs. (C) S1P induces nuclear HIF1α in hPASMC at 3 hrs, and this is inhibited with S1PR2 antagonism by JTE-013 (10 μM, 45 min pre-treatment). Hypoxia used as a positive control for induction of HIF1α protein. (D) Quantification of protein expression in panel (C) normalized to Lamin B1. ***p < 0.001, compared to untreated control or as indicated.

D. S1P Increases mRNA Expression of Downstream HIF1α Targets in hPASMCs

Our data demonstrates that S1P induces nuclear HIF1α protein expression under normoxic conditions in hPASMCs. To determine whether the HIF1α transcription factor is functionally active to induce proliferative changes in these conditions, we measured mRNA transcript levels of several classic downstream targets of HIF1α. The targets we investigated were vascular endothelial growth factor (VEGFA), which plays a major role in vascular development in hypoxia and pulmonary hypertension (88,89), and glucose transporter 1 (GLUT1, also known as solute carrier family 2 facilitated glucose transporter member 1, or SLC2A1), which is increased in both PAECs and PASMCs in pulmonary hypertension and is associated with vessel muscularization (90). We found that VEGFA and GLUT1 mRNA were significantly increased following stimulation of hPASMCs with S1P (1 μM, 0-24 hrs) at the 3-6 hr and 3 hr time points, respectively, consistent with the time-course of HIF1α protein induction in our earlier studies (**Figure 24**).

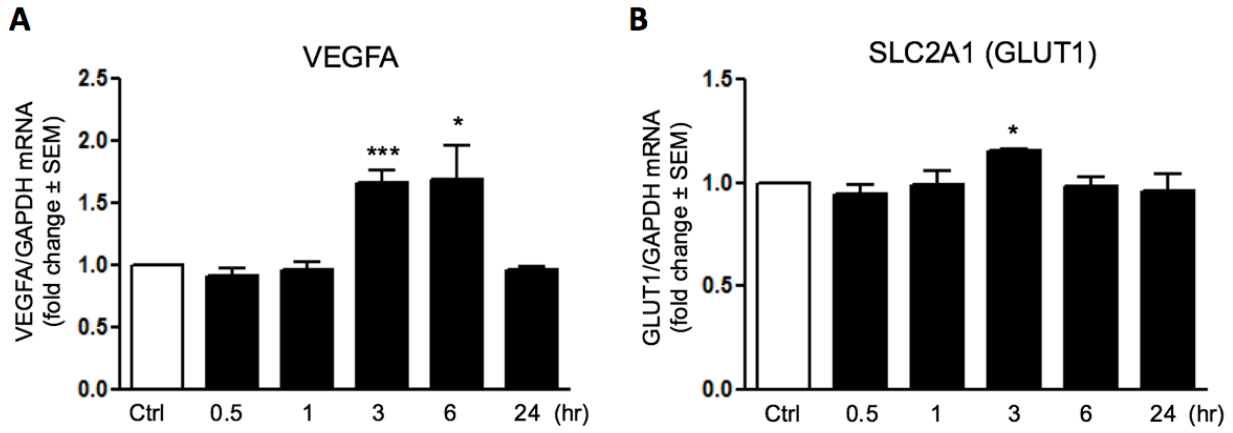

Figure 24. S1P stimulates mRNA expression of downstream HIF1α targets in hPASMCs. Stimulation of hPASMCs with exogenous S1P (1 μM, 0-24 hrs) led to a significant increase in mRNA expression of (A) VEGFA and (B) SLC2A1/GLUT1 by 3 hrs, normalized to GAPDH expression. *$p < 0.05$; ***$p < 0.001$, compared to untreated controls.

E. S1P Induces Egr-1 Expression in hPASMCs via S1PR2/ERK Signaling

Given the ability of S1P/S1PR2 signaling to induce HIF1α and expression of its downstream targets in hPASMCs, we aimed to determine the signaling mechanisms by which this may occur. Accumulation of intracellular HIF1α can occur via both oxygen-dependent and independent mechanisms. In normoxia, HIF1α is modified post-translationally by prolyl hydroxylases which promotes binding with von Hippel-Lindau protein (pVHL) and targeting for proteasomal degradation (91). Under hypoxic conditions, prolyl hydroxylation is inhibited and the interaction between pVHL and HIF1α is disrupted, leading to HIF1α accumulation. Importantly to our studies, growth factors and cytokines can also stimulate the synthesis of HIF1α in normoxia via activation of the phosphatidylinositol 3-kinase (PI3K) or ERK pathways (92-94).

The ERK signaling cascade is one of the most well-described downstream pathways mediating the pro-proliferative effects of S1P in mammalian cells (43). Interestingly, studies have demonstrated that bioactive lipids, such as S1P and lysophosphatidic acid (LPA), can induce early growth response-1 (Egr-1) protein expression via ERK signaling (95,96). Egr-1 is a tightly regulated zinc-finger transcription factor induced by several important stimuli associated with PAH, including growth factors and hypoxia (97), and studies have implicated Egr-1 in the transcriptional upregulation of HIF1α (98). Here, we sought to determine the role of S1P/ERK/Egr-1 signaling in the activation of HIF1α expression in hPASMCs.

First, we tested whether S1P could activate Egr-1 expression in hPASMCs. Cells stimulated with S1P (0.1-1 μM, 1 hr) demonstrated a significant increase in Egr-1 protein expression at either dose (**Figure 25A**). S1P-induced Egr-1 protein expression was also significantly increased at 3 hrs (data not shown). We then explored the role of ERK signaling in mediating S1P-induced Egr-1 expression. Selective inhibition of ERK kinases MEK1 and MEK2 with U0126 (10μM, 1hr pre-treatment) completely abrogated basal and S1P-induced Egr-1 expression in hPASMCs (**Figure**

25B). These studies were confirmed using PD98059 (10 μM, 45 min pre-treatment), a selective inhibitor of MEK1 activity and the ERK cascade. Both PD98059 and U0126 prevented ERK phosphorylation and Egr-1 induction by S1P in hPASMCs (**Figure 25C**).

Since S1P/S1PR2 signaling was demonstrated to be important in key aspects of PAH in Chapter V, we next tested studied whether S1PR2 was critical for S1P-induced HIF1α and Egr-1 activation. Inhibition of S1PR2 with JTE-013 (10 μM, 45 min pre-treatment) in hPASMCs abolished S1P (1 μM, 3 hr)-induced HIF1α protein expression and attenuated the induction of Egr-1 in nuclear cell extracts (**Figure 25D**). These combined studies highlight the role of S1P/S1PR2/ERK signaling in Egr-1 activation and HIF1α accumulation under normoxic conditions in hPASMCs

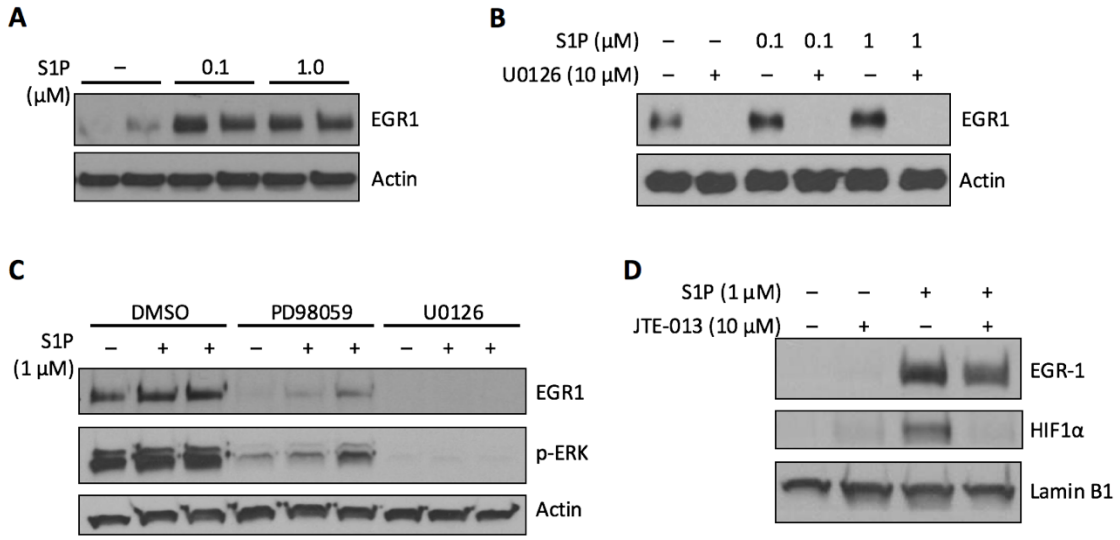

Figure 25. S1P induces Egr-1 expression in hPASMCs via S1PR2/ERK signaling. (A) S1P (0.1-1 μM, 1 hr) induces Egr-1 protein expression in hPASMCs. (B) Induction of Egr-1 expression by S1P is abrogated using U0126 to inhibit ERK. (C) Two inhibitors of ERK signaling, PD98059 and U0126, prevent ERK phosphorylation and S1P (1 μM, 1 hr)-mediated Egr-1 activation in hPASMCs. (D) Inhibition of S1PR2 with JTE-013 (10 μM, 45 min pre-treatment) attenuates S1P (1 μM, 3 hr)-induced Egr-1 and HIF1α protein expression in nuclear extracts of hPASMCs.

F. *In Silico* Analysis Identifies an Egr-1 Binding Site in the HIF1A Promoter

Our data demonstrate that S1P can induce both mRNA and protein levels of HIF1α in hPASMCs. In addition, S1P/ERK signaling in these cells activates the Egr-1 transcription factor, which has been shown to regulate HIF1α expression (98). We next examined whether any potentially functional Egr-1 binding sites were present upstream of HIF1α that may regulate its expression. *In silico* analysis using the publically available UCSC Genome Browser (Human Feb. 2009, GRCh37/hg19 Assembly) and chromatin immunoprecipitation (ChIP)-seq data from ENCODE (46) identified a single Egr-1 binding site within the HIF1A promoter region with high similarity to the canonical Egr-1 motif (**Figure 26**). To determine the functional relevance of this binding site, future experiments, including ChIP studies, can be used to determine the direct interaction of Egr-1 with the HIF1A promoter in hPASMCs following stimulation with S1P.

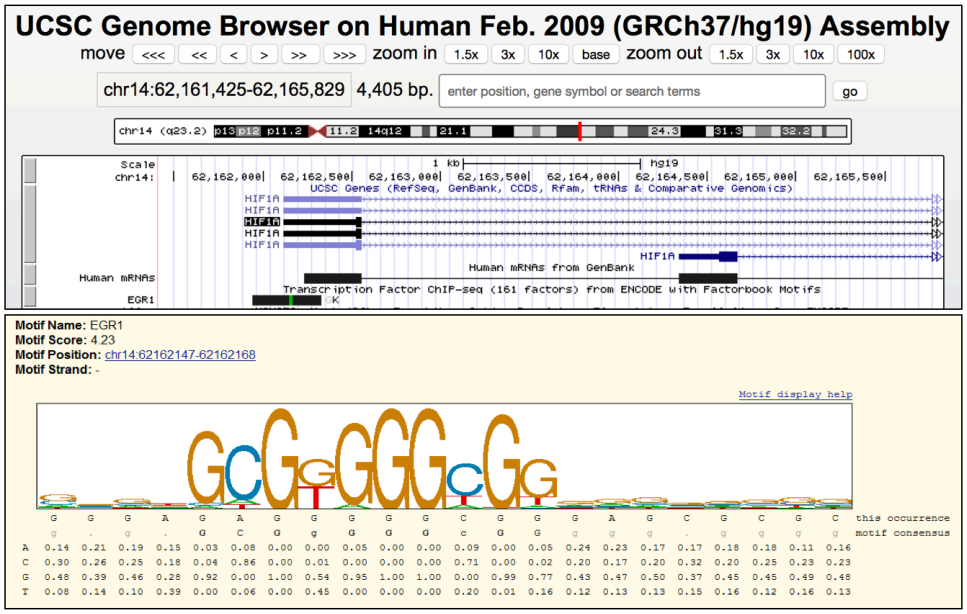

Figure 26. The HIF1A promoter contains a predicted Egr-1 binding site. *In silico* analysis using the UCSC Genome Browser (Human Feb. 2009, GRCh37/hg19) and ChIP-seq data available from ENCODE identify an Egr-1 transcription factor binding site within the HIF1A promoter region.

G. S1P Induces Phosphorylation of GSK-3β in hPASMCs via ERK Signaling

As previously described, HIF1α protein can be induced in cells under normoxic and hypoxic conditions through various mechanisms. The precise mechanisms mediating S1P-induced HIF1α in hPASMCs is unknown. While the previous Section sought to identify transcriptional activation mechanisms of HIF1α, we were also interested in the role of HIF1α accumulation due to inhibition of its proteasomal degradation.

Evidence suggests that S1P may down-regulate pVHL-mediated ubiquitination and degradation of HIF1α, leading to its accumulation in cancer cells (86). Studies also demonstrate that reactive oxygen species (ROS), which S1P can generate in some cell types (99), can induce HIF1α through inhibition of prolyl hydroxylases and indirectly through activation of Akt/GSK-3β signaling (100,101). When GSK-3β is phosphorylated (Ser9) by Akt it is inactivated, leading to stabilization and accumulation of HIF1α protein (101,102). Interestingly, SphK1 has been shown to be upstream of this process in cancer cells (103). We therefore hypothesized that stimulation of hPASMCs with S1P may lead to accumulation of HIF1α protein via the GSK-3β pathway. When hPASMCs were treated with S1P (0.1-1 μM, 1 hr), the phosphorylation of GSK-3β (Ser9) was significantly increased (**Figure 27**). These findings correlate with the ability of S1P to activate HIF1α in hPASMCs. Further studies are required to determine the exact role of GSK-3β in mediating this process.

Notably, this effect of S1P on GSK-3β phosphorylation was attenuated by inhibiting ERK signaling with U0126 (10 μM, 45 min pre-treatment) (**Figure 27**). ERK has been shown to be important for hypoxia-induced HIF1α transactivation activity in human microvascular endothelial cells (104) and crosstalk can occur between the ERK and Akt/GSK-3β signaling pathways (105) (106). ERK may indirectly activate Akt in hPASMCs, though the mechanisms of how ERK inhibition alters the phosphorylation of GSK-3β in our studies is currently unknown.

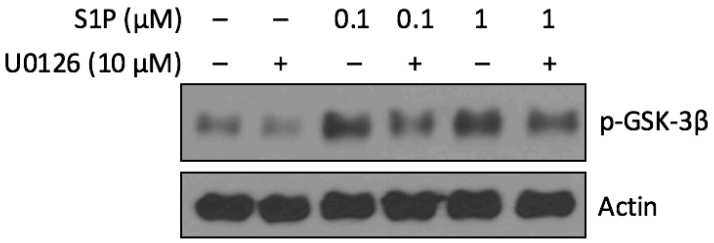

Figure 27. S1P induces phosphorylation of GSK-3β in hPASMCs. S1P (0.1-1 μM, 1 hr) induces phosphorylation of GSK-3β (Ser9) in hPASMCs. Inhibition of ERK signaling with pre-treatment of U0126 (10 μM, 45 min) attenuated the induction of GSK-3β phosphorylation (Ser9).

Together, these studies demonstrate a mechanistic link between S1P signaling and normoxic HIF1α activation in PASMCs (**Figure 28**). The potential mechanisms of this activation include both transcriptional upregulation by Egr-1 and protein stabilization via phosphorylation of GSK-3β. As S1P and HIF1α levels are both increased in PAH patients and promote hPASMC proliferation, a functional link between these pathways provides novel insight into disease pathogenesis. Future studies aim to further delineate the role of this pathway in pulmonary vascular remodeling in PAH.

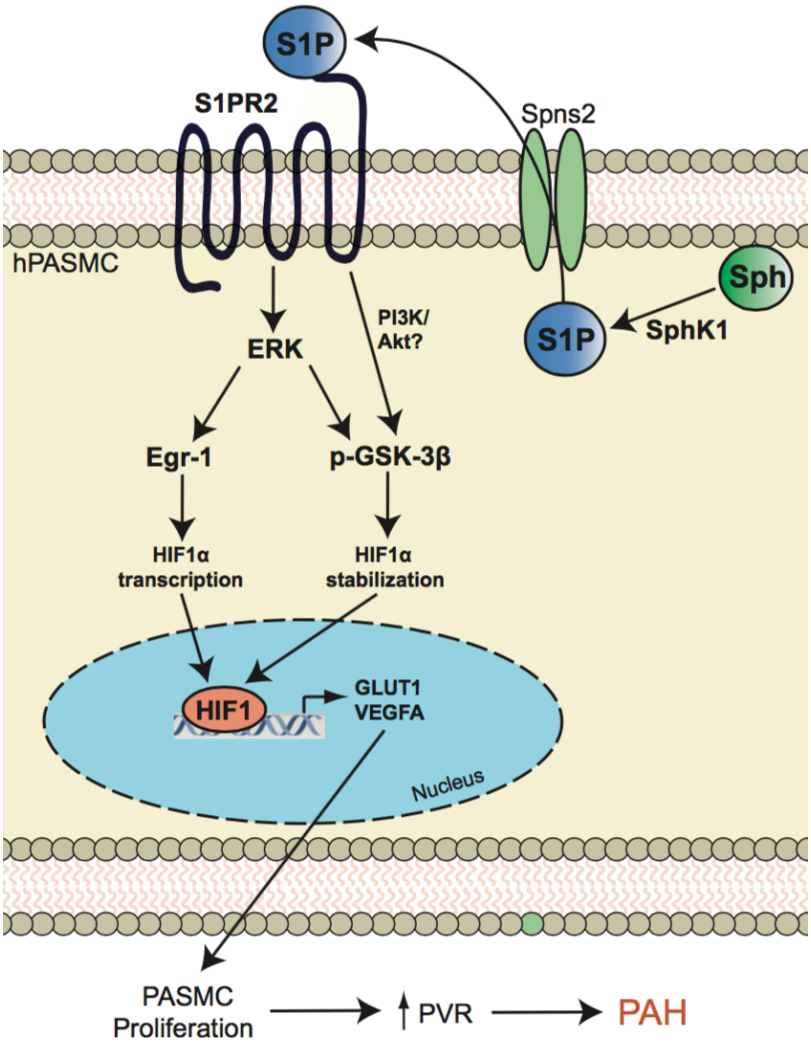

Figure 28. Hypothetical model of S1P/S1PR2-mediated HIF1α activation to promote PASMC proliferation in PAH. S1P may activate transcription and/or stabilization of HIF1α under normoxic conditions via S1PR2 signaling in PASMCs. Downstream ERK signaling may mediate induction of Egr-1 and phosphorylation of GSK-3β. HIF1α could then act as a nuclear transcription factor to activate genes mediating hPASMC proliferation and survival, leading to enhanced pulmonary vascular resistance (PVR) and the development of PAH.

VII. ROLE OF PLATELET-DERIVED GROWTH FACTOR IN INDUCING SPHK1 IN PULMONARY ARTERY SMOOTH MUSCLE CELLS

*Portions of the text and figures were reprinted/modified with permission from:

1. Sysol JR, Natarajan V, Machado RF. "PDGF Induces SphK1 Expression via Egr-1 to Promote Pulmonary Artery Smooth Muscle Cell Proliferation." *Am J Physiol Cell Physiol.* 2016 Jun 1;310(11):C983-92. (44)

A. Rationale

Understanding the mechanistic regulation of hPASMC proliferation in PAH is critical for developing novel therapeutics. With the discovery that some familial and sporadic cases of PAH arise from mutations in members of the transforming growth factor beta (TGF-beta) cell-signaling superfamily, including the BMPR2 gene (107), the role of different growth factors in disease pathogenesis have been explored. One of these growth factors, PDGF, has been demonstrated to contribute to pulmonary vascular remodeling in human PAH and experimental models and is a potential therapeutic target (108,109).

PDGF is a mitogenic and pro-migratory stimuli for hPASMCs with both autocrine and paracrine functions (108,110,111). Many cell types in the lung can synthesize PDGF, including vascular smooth muscle cells and endothelial cells, and it has been shown to be induced in alveolar hypoxia, causing vascular remodeling in lung parenchyma (112). Five ligand isoforms of PDGF are known (PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC, and PDGF-DD) along with two distinct receptor tyrosine kinase isotypes (PDGFR-α and PDGFR-β). The PDGF-BB isoform, which can bind to both PDGFR-α and PDGFR-β, is released at sites of vascular injury by endothelial cells and platelets and is a potent stimulus for hPASMC proliferation and migration (108,113). Activation of PDGFRs leads to receptor dimerization, autophosphorylation, and subsequent signal transduction

mainly via the mitogen-activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK) cascade, resulting in targeted gene transcription that promotes cell proliferation, migration, and differentiation, and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, promoting cell survival (114). Recent studies have implicated PDGF and its receptors in both lung development and disease, including lung cancer, lung fibrosis, and PAH (114-117). The circulating concentration of PDGF has been shown to be significantly elevated in patients with PAH (118), and concentrations within the pulmonary vasculature environment are likely to be much greater since small remodeled pulmonary arteries of PAH patients have increased PDGF and PDGFRs expression in the hPASMCs and PAECs (108). In addition, inhibition of PDGFR-β using the specific tyrosine kinase inhibitor, imatinib (STI-571), has been shown to reverse vascular remodeling in severe experimental PH (109). The therapeutic use of imatinib in refractory PAH has also been reported in several clinical case reports, leading to improvements in exercise capacity, hemodynamics, and functional class (119,120). The precise mechanisms by which PDGF-BB stimulates hPASMC proliferation and vascular remodeling are not fully understood.

Crosstalk between PDGF/PDGFR signaling and sphingolipid signaling has been demonstrated in numerous cell types, including airway smooth muscle cells, where PDGFRs can be trans-activated by the bioactive lipid, sphingosine-1-phosphate (S1P), to elicit pro-proliferative and pro-migratory signaling (121-123). Interestingly, PDGF has also been shown to stimulate activity of SphK1 and increase intracellular S1P in fibroblasts, while competitive inhibition of SphK1 prevents PDGF-induced cell proliferation (124,125). PDGF has also been shown to increase expression of SphK1 in coronary artery smooth muscle cells (126). We have shown that levels of S1P and SphK1 are elevated in patients with PAH and promote hPASMC proliferation (43). In addition, genetic deficiency or pharmacologic inhibition of SphK1 protects from the development of experimental PH in several rodent models (43). The mechanisms of SphK1/S1P upregulation and their role in

pulmonary vascular remodeling in PAH are still largely unknown. In this Chapter, we investigated the mechanisms by which PDGF may control SphK1 expression in hPASMCs and contribute to cell proliferation.

B. PDGF Increases SphK1 Expression in hPASMCs

Given the upregulation of both SphK1 and PDGF in PAH and their association with pulmonary vascular remodeling, we first tested whether PDGF can induce SphK1 expression in hPASMCs. Following stimulation of hPASMCs with PDGF-BB (20-100ng/ml, 0-6hr), SphK1 protein was increased by approximately 1.5-fold at 6hr as measured by Western blotting and normalized to β-actin expression (**Fig. 29A-B**). PDGF did not increase SphK2 expression in hPASMCs (data not shown). Next, quantitative real-time RT-PCR was used to measure SphK1 mRNA following stimulation of hPASMCs with PDGF-BB (20ng/ml, 0-24hr). SphK1 mRNA normalized to GAPDH expression was significantly increased at 1hr and 3hr, and expression returned to baseline levels by 24hr (**Fig 29C**). These findings demonstrate the role of PDGF-BB in SphK1 upregulation in hPASMCs.

C. The SphK1 Promoter is Activated by PDGF Signaling in hPASMCs

To investigate the mechanism(s) by which PDGF increases SphK1 expression in hPASMCs, we tested whether PDGF could induce activation of the SphK1 promoter using several approaches. First, a commercially available dual reporter construct was used to express and secrete *Gaussia* Luciferase (GLuc) under control of the ~1.3kb upstream human SphK1 promoter sequence, with Secreted Alkaline Phosphatase (SEAP) used as a control. PDGF-BB (20-100ng/ml) stimulated SphK1 promoter activity at 1.5hr and 24hr, as measured by quantification of relative luminescence

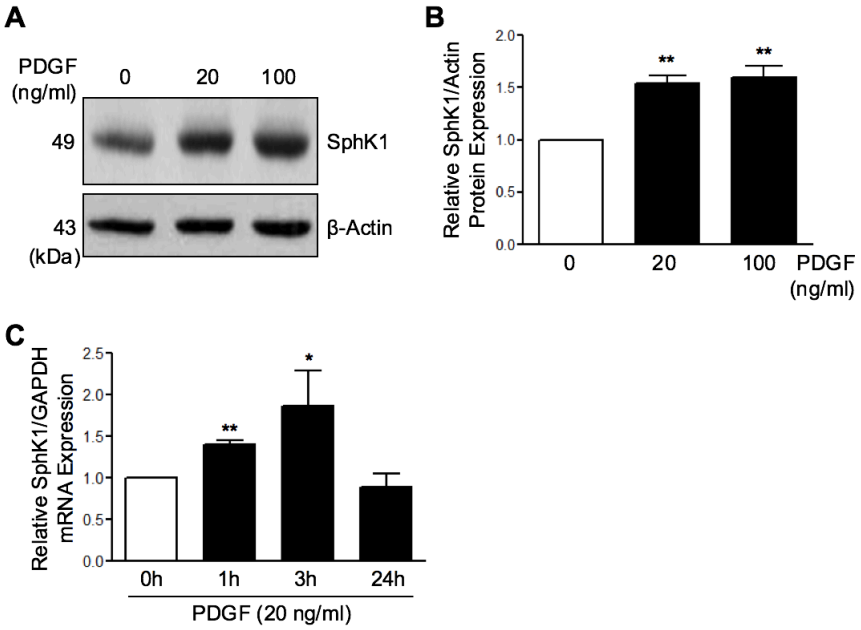

Figure 29. PDGF increases SphK1 expression in hPASMCs. (A-B) Representative Western blotting images and β-actin-normalized quantification of protein levels demonstrate increased SphK1 expression in hPASMC following stimulation with PDGF-BB (20-100ng/ml, 6hrs). (C) SphK1 mRNA expression levels are also increased in hPASMC following PDGF-BB treatment (20ng/ml, 0-24hrs). Results are shown as mean ± SEM from at least three experiments. *$p < 0.05$, **$p < 0.01$ vs. untreated control. Image source: Sysol JR, Natarajan V, Machado RF. "PDGF Induces SphK1 Expression via Egr-1 to Promote Pulmonary Artery Smooth Muscle Cell Proliferation." *Am J Physiol Cell Physiol.* 2016 Jun 1;310(11):C983-92.

of GLuc/SEAP in the culture media (**Fig. 30A**). To confirm these findings, the ~2.1kb human SphK1 promoter was isolated via PCR and cloned into the pGL4 luciferase reporter vector. Stimulation of hPASMCs transfected with this vector with PDGF-BB (20ng/ml, 1.5 hr) resulted in a 2.5-fold increase in transcriptional activity over basal levels (**Fig. 30B**). The empty control vector was not activated under basal or PDGF-BB stimulated conditions.

Next, *in silico* analysis of the SphK1 promoter was conducted to identify transcription factor binding sites that may mediate PDGF-induced SphK1 expression. Three highly predicted binding sites for Egr-1, a transcription factor by which PDGF is known to signal intracellularly (127), were identified within the SphK1 promoter (labeled EGR1-"A", "B", and "C") (**Fig. 30C**). These three

binding sites were also observed in the public ENCODE ChIP-seq database (47). To determine whether the promoter regions containing these Egr-1 binding sites were functionally important in PDGF-mediated SphK1 activation, truncated promoter fragments containing the sites were amplified by PCR and cloned into the pGL4 luciferase reporter vector (**Fig. 30C**). hPASMCs were transfected with these vector constructs and stimulated with PDGF (20ng/ml, 1.5hr). Truncations that excluded binding sites for EGR1-"B" and EGR1-"C" demonstrated a significant loss in SphK1 promoter activity, with exclusion of the EGR1-"B" site fragment showing the largest decrease in activity (**Fig. 30D**). These results suggest that Egr-1 binding to the SphK1 promoter may be important for PDGF-induced transcriptional activation.

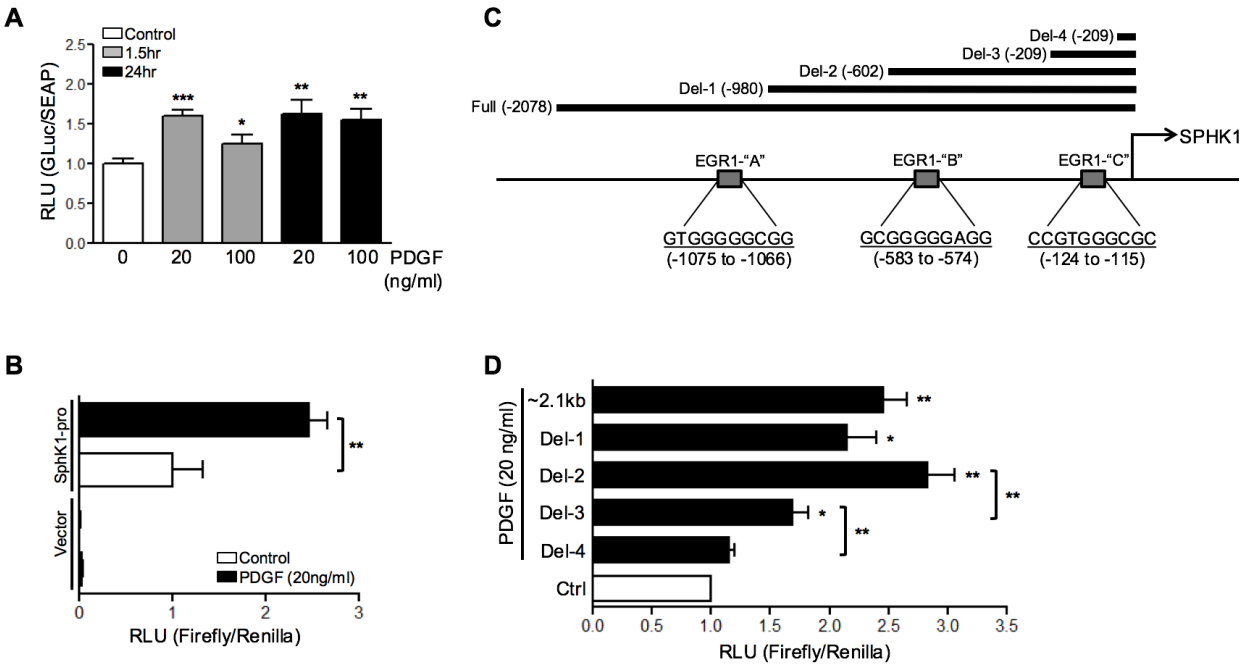

Figure 30. PDGF activates the SphK1 promoter in hPASMCs. (A) Relative luminescence of secreted *Gaussia* Luciferase (GLuc) and Secreted Alkaline Phosphatase (SEAP) in a SphK1 promoter dual-reporter system, demonstrating that PDGF (20-100ng/ml, 1.5 hr) stimulates transcriptional activity of the SphK1 promoter (1300bp) in hPASMCs. (B) Relative luminescence of a full-length human Sphk1 promoter (~2.1kb) cloned into a pGL4 luciferase reporter vector (Firefly/Renilla) demonstrating induction of SphK1 promoter transcriptional activity with PDGF (20ng/ml, 1.5 hr) in hPASMCs. (C) Representation of the SphK1 promoter containing several putative EGR1 binding sites (EGR1-A, -B, and -C) identified *in silico*, with black bars depicting SphK1 promoter deletion regions cloned into pGL4 luciferase reporter vectors. (D) Relative luminescence of Sphk1 promoter deletion constructs demonstrating induction of SphK1 promoter transcriptional activity with PDGF (20ng/ml, 1.5 hr) in hPASMCs. Results are shown as mean ± SEM from at least three experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. control unless otherwise indicated. Image source: Sysol JR, Natarajan V, Machado RF. "PDGF Induces SphK1 Expression via Egr-1 to Promote Pulmonary Artery Smooth Muscle Cell Proliferation." *Am J Physiol Cell Physiol.* 2016 Jun 1;310(11):C983-92.

D. Nuclear Expression of Egr-1 is Increased by PDGF in hPASMCs

Since Egr-1 transcription factor binding sites may play a role in PDGF-induced SphK1 activation, we tested whether PDGF-BB could increase Egr-1 expression in hPASMCs. Stimulation with PDGF-BB (20-100ng/ml, 1-3hr) resulted in a dramatic increase in Egr-1 protein expression by 1 hr that subsided by 3 hr (**Fig. 31A**). PDGF-BB also significantly increased Egr-1 mRNA levels, with maximum expression observed at 1hr (**Fig. 31B**). The basal expression of Egr-1 protein expression was also measured in three hPASMC lines derived from PAH patients, and no basal differences were observed compared to control PASMCs (data not shown). Though higher circulating PDGF concentrations within the pulmonary vascular microenvironment of PAH patients have been demonstrated (118) which may lead to elevations in Egr-1 expression, this elevation may not be retained following isolation of PASMCs and culturing *in vitro*.

Egr-1 has a bipartite nuclear localization domain allowing for nuclear expression that is pivotal for its functional role as a transcription factor (97,128), though Egr-1 expression in the cytoplasm has also been reported in some cancer cell lines (129). We next tested whether PDGF-induced Egr-1 expression was localized in the nucleus. Stimulation with PDGF-BB (20ng/ml, 0.5-3hr) resulted in abundant and transient nuclear Egr-1 protein expression that was maximal at 1 hr (**Fig. 31C**), with Lamin B1 used as a nuclear loading control. These results show up-regulation of Egr-1 by PDGF in hPASMCs.

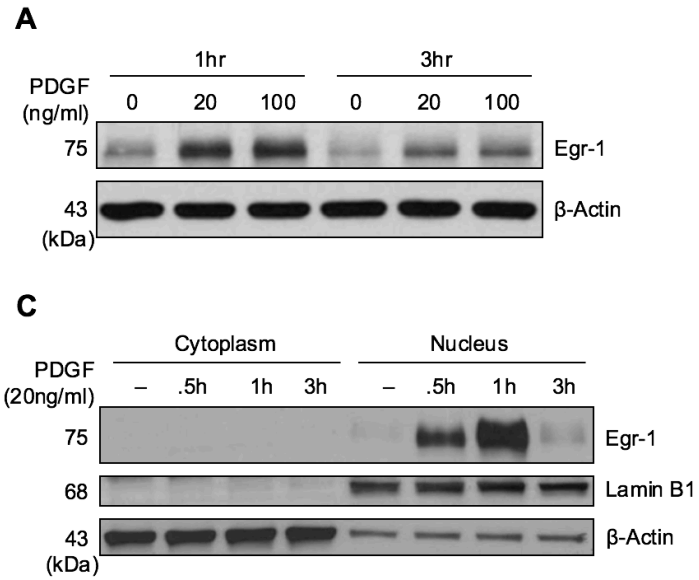

Figure 31. PDGF increases Egr-1 expression in hPASMCs. (A) Representative Western blotting images demonstrate increased Egr-1 protein expression in human pulmonary artery smooth muscle cells (hPASMC) following stimulation with PDGF-BB (20-100ng/ml, 1-3hrs). (B) EGR1 mRNA expression levels are increased in hPASMC following PDGF-BB treatment (20ng/ml, 0-8hrs) relative to GAPDH expression. (C) Western blotting with cytoplasmic and nuclear hPASMC fractions demonstrates PDGF-BB treatment (20ng/ml, 0-3hrs) induces EGR1 expression limited to the nucleus, with Lamin B1 is used as a nuclear loading control. Results are shown as mean ± SEM from at least three experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control unless otherwise indicated. Image source: Sysol JR, Natarajan V, Machado RF. "PDGF Induces SphK1 Expression via Egr-1 to Promote Pulmonary Artery Smooth Muscle Cell Proliferation." *Am J Physiol Cell Physiol.* 2016 Jun 1;310(11):C983-92.

E. PDGF Induces Egr-1 Binding to the SphK1 Promoter in hPASMCs

Regions of the SphK1 promoter containing both EGR1-"B" and EGR1-"C" transcription factor binding sites are important for PDGF-induced SphK1 expression, with loss of the EGR1-"B" resulting in the greatest reduction in expression (**Fig. 30D**). Given these results, we sought to determine whether PDGF could induce direct binding of Egr-1 to the EGR1-"B" predicted site within the SphK1 promoter. Following stimulation of hPASMCs with PDGF-BB (20-100ng/ml, 1 hr), ChIP studies coupled with quantitative real-time PCR demonstrated significantly increased

binding of Egr-1 protein to the EGR1-"B" DNA binding site within the SphK1 promoter (**Fig. 32A**). Optimal chromatin shearing was achieved for these studies (**Fig. 32B**). To confirm these findings, standard PCR was used to amplify the EGR1-"B" binding site following immunoprecipitation with Egr-1 in hPASMCs treated with PDGF-BB (20-100ng/ml, 1hr). DNA gel electrophoresis for these studies demonstrated increased Egr-1 protein binding to the EGR1-"B" site within the SphK1 promoter relative to IgG controls, with resulting quantification of signal relative to input (**Fig. 32C-D**).

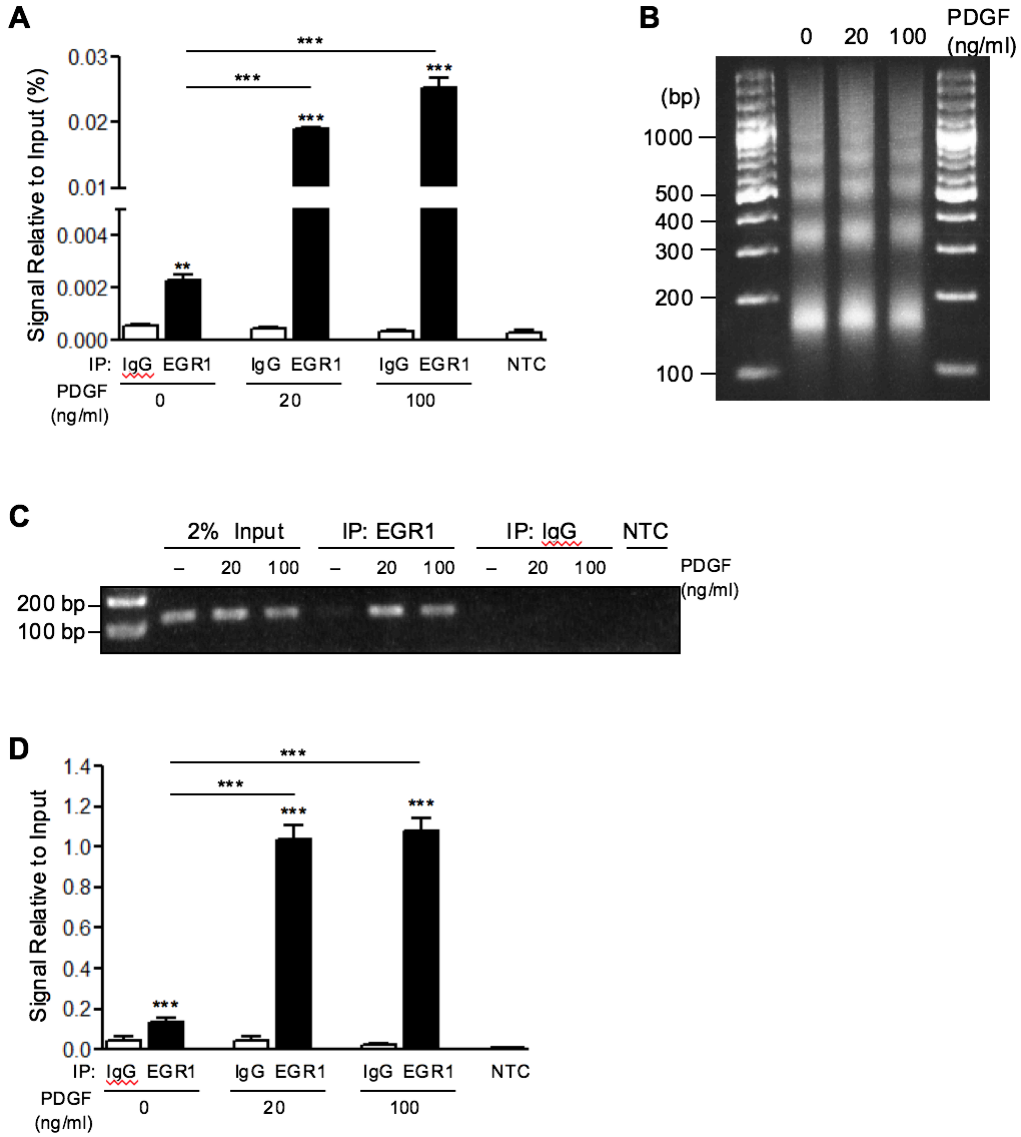

Figure 32. PDGF induces Egr-1 binding to the SphK1 promoter in hPASMCs. (A) ChIP real-time PCR analysis demonstrates Egr-1 binding to the EGR1-"B" site within the SphK1 promoter following stimulation of hPASMC with PDGF (20-100ng/ml, 1 hr), with (B) optimized chromatin digestion of ChIP samples. (C-D) Representative DNA gel electrophoresis of ChIP PCR products demonstrates enhanced Egr-1 binding to the EGR1-"B" site within the SphK1 promoter following stimulation of hPASMC with PDGF (20-100ng/ml, 1 hr), with quantification of data signal relative to input. Results are shown as mean ± SEM from at least three experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. IgG control unless otherwise indicated. Image source: Sysol JR, Natarajan V, Machado RF. "PDGF Induces SphK1 Expression via Egr-1 to Promote Pulmonary Artery Smooth Muscle Cell Proliferation." *Am J Physiol Cell Physiol.* 2016 Jun 1;310(11):C983-92.

F. PDGF Promotes SphK1 Expression and hPASMC Proliferation via EGR1.

PDGF-BB (20-100ng/ml, 48hr) increases proliferation of hPASMCs (**Fig 33A**). Since PDGF also enhances Egr-1 transcription factor expression, SphK1 expression, and binding of Egr-1 to the Sphk1 promoter, we aimed to determine whether silencing of Egr-1 could prevent PDGF-induced SphK1 expression. SiRNA-mediated silencing of Egr-1 in hPASMCs resulted in >80% reduction in basal Egr-1 protein expression after 48 hr (**Fig 33B-C**). Egr-1 silencing also prevented induction of Egr-1 expression by PDGF-BB (20ng/ml, 1hr) (**Fig. 33B-C**).

Uncontrolled proliferation of PASMCs contributes to pulmonary vascular remodeling in PAH, but the mechanisms regulating the proliferative phenotype of these cells are poorly understood. Therefore, we next tested whether silencing of Egr-1 could alter proliferation of hPASMCs. Compared to scrambled siRNA controls, silencing of Egr-1 decreased basal and PDGF-induced (20ng/ml, 48hr) proliferation of hPASMCs as measured by relative BrdU incorporation (**Fig. 33D**). In addition, silencing of Egr-1 attenuated PDGF-induced (20ng/ml, 6hr) expression of SphK1 in hPASMCs (**Fig. 33E-F**). Next, we used the highly specific SphK1 inhibitor, PF-543, to assess the importance of SphK1 in mediating PDGF-induced proliferation. Treatment of hPASMCs with PF-543 (100nM, 1hr pre-treatment) did not alter basal hPASMC proliferation, but significantly reduced PDGF-induced (20ng/ml, 48hr) proliferation (**Fig. 33G**). These results demonstrate the importance of the Egr-1/SphK1 signaling axis in the induction of hPASMC proliferation by PDGF.

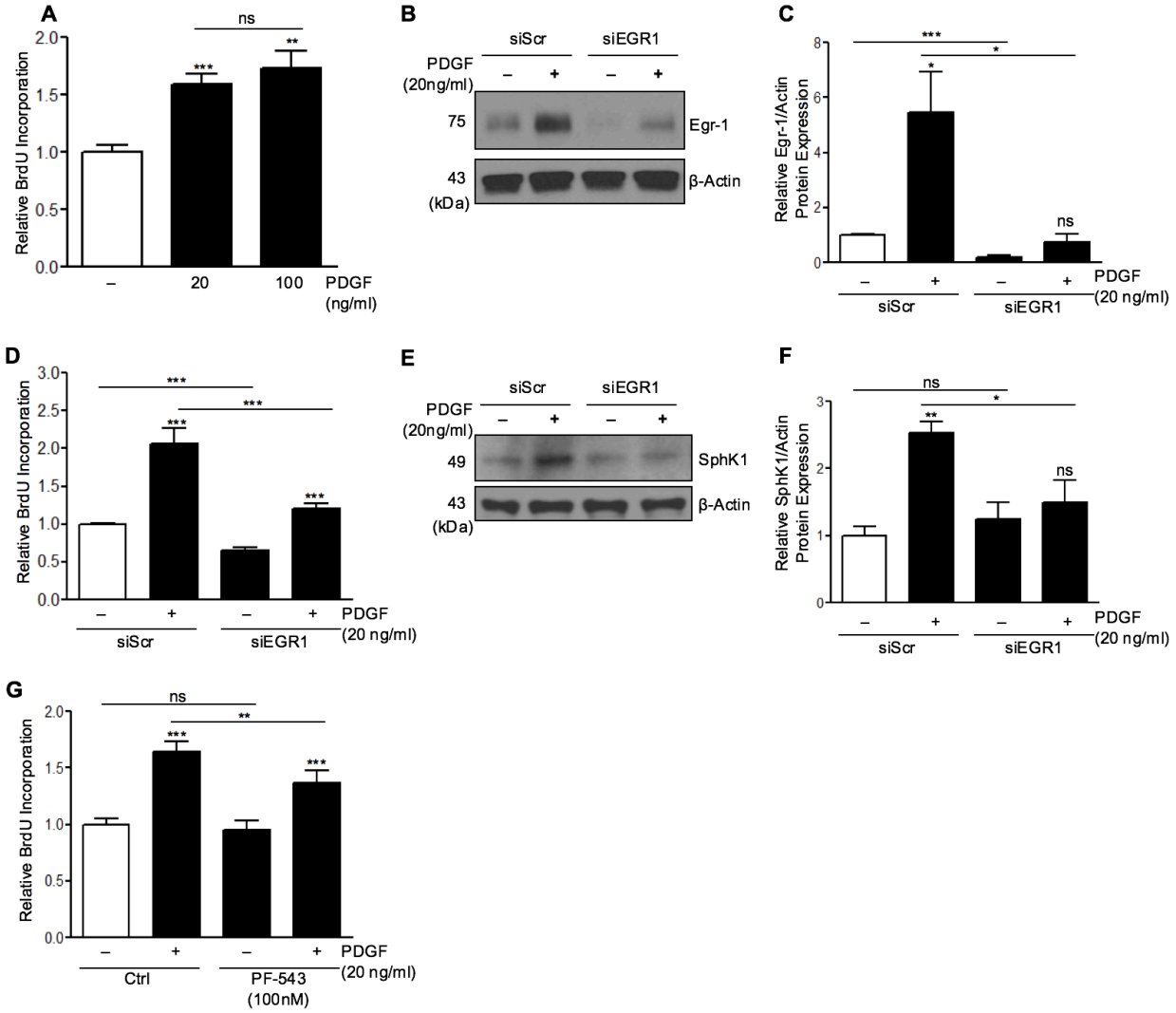

Figure 33. PDGF promotes hPASMC proliferation and SphK1 expression via Egr-1. (A) BrdU-incorporation assays demonstrate enhanced proliferation of hPASMC following stimulation with PDGF-BB (20-100ng/ml, 48hrs). (B-C) siRNA-mediated silencing of EGR1 reduces basal and PDGF-induced (20ng/ml, 1hr) Egr-1 expression, with data quantification. (D) siRNA-mediated silencing of EGR1 reduces basal hPASMC proliferation and attenuates PDGF-induced (20ng/ml, 48hr) proliferation. (E-F) siRNA-mediated silencing of EGR1 in hPASMC prevents the induction of SphK1 protein expression by PDGF-BB (20ng/ml, 6hr), with data quantification. (G) Selective SphK1 inhibition with PF-543 (100nM, 1hr pre-treatment) attenuates PDGF-induced (20ng/ml, 48hr) proliferation but not basal proliferation. Results are shown as mean ± SEM from at least three experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. untreated control unless otherwise indicated. Image source: Sysol JR, Natarajan V, Machado RF. "PDGF Induces SphK1 Expression via Egr-1 to Promote Pulmonary Artery Smooth Muscle Cell Proliferation." *Am J Physiol Cell Physiol.* 2016 Jun 1;310(11):C983-92.

G. PDGF Induces Egr-1 and SphK1 Expression via ERK in hPASMCs

Previous studies have identified the importance of the MAPK/ERK cascade in cell proliferation (43), Egr-1 activation (96), and PDGF-induced cell signaling (111). Here we explored whether MAPK/ERK signaling could be an upstream mediator of PDGF-induced EGR1 activation in hPASMCs. Treatment of hPASMCs with PDGF-BB (20ng/ml) resulted in enhanced ERK1/2 phosphorylation at 15 minutes (**Fig. 34A-B**). Inhibition of ERK phosphorylation with U0126 (10μM, 1hr pre-treatment), a highly selective inhibitor of MAPK/ERK kinases MEK1 and MEK2, resulted in reduced basal and PDGF-induced (20ng/ml, 48hr) hPASMC proliferation (**Fig. 34C**) and significant attenuation of PDGF-induced (20ng/ml, 1hr) Egr-1 protein expression in hPASMCs (**Fig. 34D-E**). U0126 (10μM) also inhibited induction of downstream SphK1 expression by PDGF (20ng/ml, 6hr) (**Fig. 34F-G**). These results demonstrate the critical importance of MAPK/ERK signaling in PDGF-induced proliferation, as well as Egr-1 and SphK1 expression in hPASMCs. The hypothesized mechanism of these events is outlined in **Figure 35**.

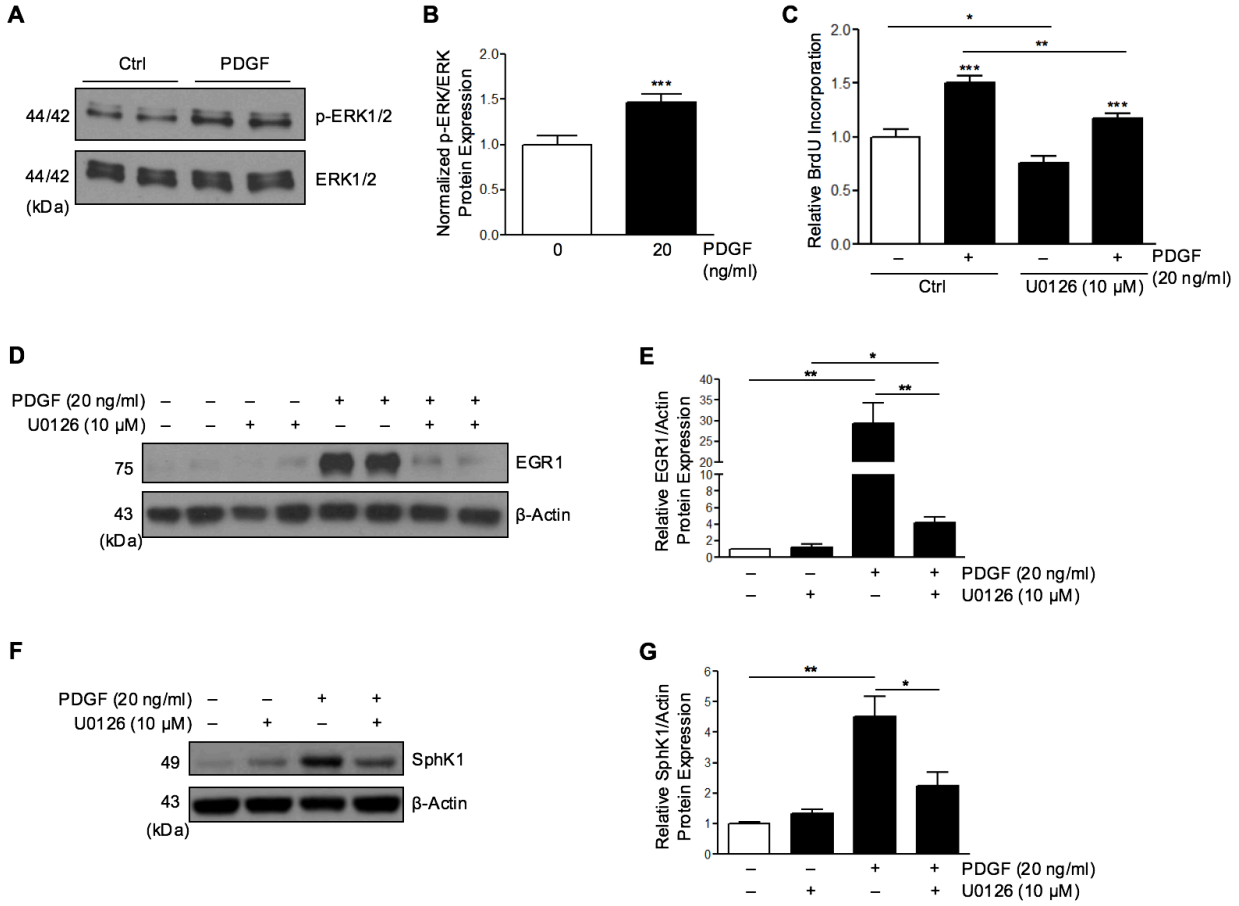

Figure 34. PDGF-induced Egr-1 and SphK1 expression is mediated by ERK in hPASMCs. (A-B) Representative Western blotting images and ERK-normalized quantification of protein levels demonstrate induction of ERK phosphorylation by PDGF (20ng/ml, 15min) in hPASMC. (C) U0126-mediated inhibition of ERK phosphorylation reduces basal hPASMC proliferation and attenuates PDGF-induced (20ng/ml, 48hr) proliferation. (D-E) Representative Western blotting images and β-actin-normalized quantification of protein levels demonstrate U0126-mediated inhibition of ERK phosphorylation attenuates Egr-1 induction by PDGF (20ng/ml, 1hr) in hPASMC and (F-G) attenuates SphK1 induction by PDGF (20ng/ml, 6hr) in hPASMC. Results are shown as mean ± SEM from at least three experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. untreated control unless otherwise indicated. Image source: Sysol JR, Natarajan V, Machado RF. "PDGF Induces SphK1 Expression via Egr-1 to Promote Pulmonary Artery Smooth Muscle Cell Proliferation." *Am J Physiol Cell Physiol.* 2016 Jun 1;310(11):C983-92.

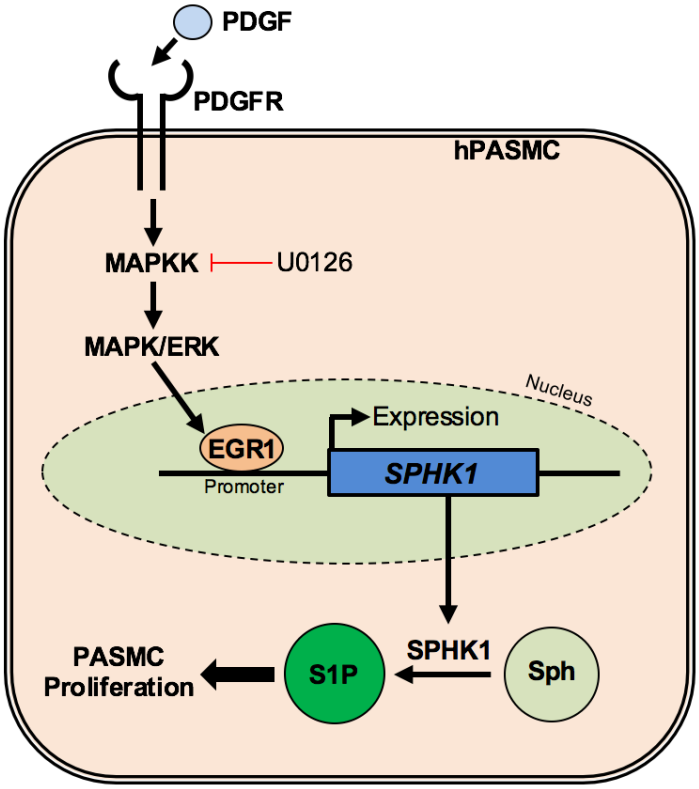

Figure 35. Potential mechanism of PDGF-induced SphK1 expression and proliferation of hPASMCs in PAH. The PDGF/PDGFR signaling pathway is upregulated in PAH. Its activation induces phosphorylation of MAPK/ERK, nuclear expression of the EGR1 transcription factor, and subsequent upregulation of SphK1 gene expression. Increased intracellular SphK1 may then lead to S1P production in hPASMCs, resulting in enhanced proliferation. Abnormal hPASMC proliferation leads to pulmonary vascular remodeling and increased pulmonary vascular resistance in PAH. Image modified from: Sysol JR, Natarajan V, Machado RF. "PDGF Induces SphK1 Expression via Egr-1 to Promote Pulmonary Artery Smooth Muscle Cell Proliferation." *Am J Physiol Cell Physiol.* 2016 Jun 1;310(11):C983-92.

VIII. REGULATION OF SPHK1 EXPRESSION BY MICRORNA-1 IN PULMONARY HYPERTENSION

*Portions of the text and figures were reprinted/modified with permission from:

1. Sysol JR, Chen J, Singla S, Zhao S, Comhair SAA, Natarajan V, and Machado RF. 2017. "MicroRNA-1 Is Decreased by Hypoxia and Contributes to the Development of Pulmonary Vascular Remodeling via Regulation of Sphingosine Kinase 1." *Am J Physiol Lung Cell Mol Physiol.* 2017 Nov 22. [Epub ahead of print] doi: 10.1152/ajplung.00057.2017. (45)

A. Rationale

We have demonstrated that the SphK1/S1P signaling axis is important in regulating the development of HPH in preclinical rodent models (43). However, the mechanisms involved in the hypoxia-mediated upregulation of SphK1 expression in PAH and its contribution to hPASMC proliferation is currently not well understood. In this Chapter, we aimed to identify novel molecular mechanisms of hypoxia-induced hPASMC proliferation, focusing on the role of SphK1 expression and its regulation by microRNAs (miRs).

MiRs are small, non-coding RNAs that play important regulatory roles in animals and plants by base-pairing with complementary sequences within mRNA molecules, resulting in cleavage, translational repression, or destabilization (130-132). The importance of miRs in modulating complex gene expression networks (133) and their contributions to human disease pathogenesis have emerged, including in the field of cardiovascular biology (134). Recent studies have identified several miRs associated with cell-specific phenotypes in PAH and experimental models of pulmonary hypertension (135,136). The regulation of miR expression by hypoxia and their involvement in modulating the phenotype of SMCs have also been described (137,138).

Arterial SMCs, unlike cardiac and skeletal myocytes, can switch to a highly proliferative and migratory state under various stimuli, including hypoxia and vascular injury (139). We hypothesized that hypoxia induces down-regulation of microRNAs targeting SphK1, contributing to enhanced hPASMC proliferation and migration. In this study, we demonstrate that miR-1 is decreased in PASMCs of PAH patients and is downregulated by hypoxia, a major contributor to PAH development, in both cultured PASMCs and in lung tissues from experimental models of PH. In addition, overexpression of miR-1 protected mice from hypoxia-induced PH and suppressed the expression of SphK1, a conserved lipid kinase that catalyzes formation of the pro-proliferative and pro-migratory lipid S1P.

B. MiR-1 Targets and Regulates the Expression of SphK1 in hPASMCs

To investigate the potential regulation of SphK1 by miRs, we performed *in silico* analysis of the human SphK1 3'-UTR to identify predicted miR response elements. This approach identified several putative binding sites, including miR-1-3p (miR-1) as the most highly predicted target of SphK1 with a high probability of down-regulation (7mer-m8 canonical site type, mirSVR score = -0.6270) (48). MiR-1 and its SphK1 3'-UTR binding site are highly evolutionarily conserved across species, suggesting its importance in gene regulation. As bioinformatic analysis identified the putative binding site of miR-1 within the 3'-UTR of *SPHK1*, we hypothesized that this miR-1 may be a negative regulator of SphK1 expression in hPASMCs. To test this hypothesis, we utilized a luciferase reporter vector containing a sequence-verified clone of the SphK1 3'-UTR designed for microRNA target validation. To determine the interaction of miR-1 with the SphK1-3'UTR, we overexpressed or inhibited miR-1 expression using human miR-1 mimics or antagomirs, respectively, in hPASMCs co-transfected with the SphK1-3'UTR reporter vector. Overexpression of miR-1 resulted in reduced luciferase activity due to SphK1-3'UTR binding, while transfection of miR-1 antagomirs had no effect (**Figure 36A-B**). In addition, both protein and mRNA expression

levels of SphK1 were significantly decreased in hPASMCs following transfection with the miR-1 mimics (**Figure 36C-D**, respectively), while miR-1 antagomirs did not alter SphK1 expression (data not shown).

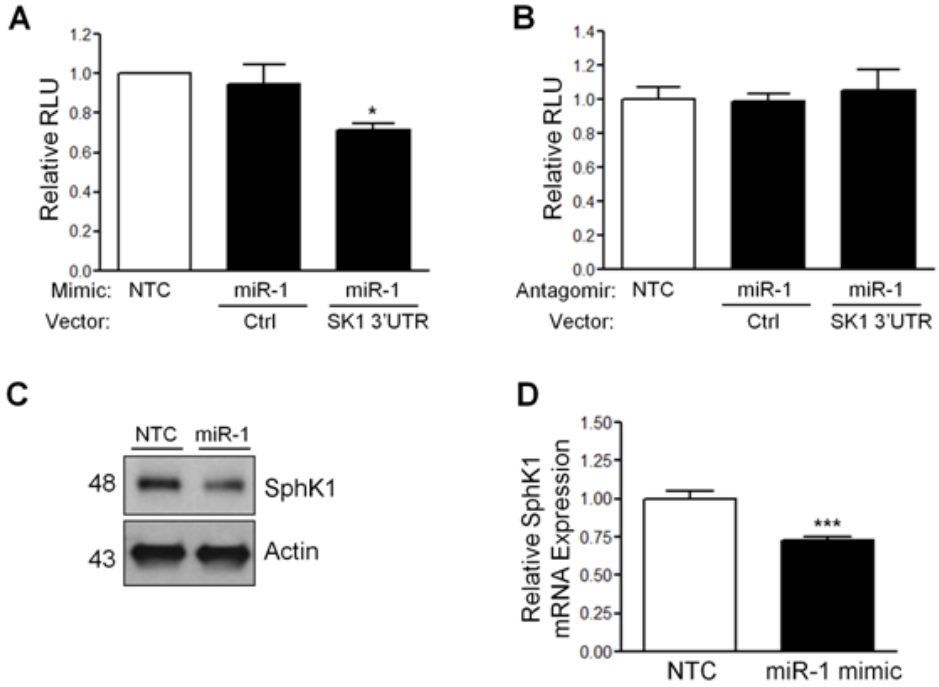

Figure 36. MiR-1 binds to the SphK1 3'-UTR and inhibits SphK1 expression in hPASMC. (A-B) Transfection of hPASMCs with miR-1 mimics (50 nM), but not antagomirs (100 nM), significantly reduces luciferase activity of a SphK1 3'-UTR reporter construct relative to control. (C) Overexpression of miR-1 mimic (50 nM) in hPASMCs significantly decreases SphK1 protein expression and (D) mRNA expression at 48hrs. *$p < 0.05$, ***$p < 0.001$ relative to control. Image source: Sysol JR, et al. 2017. "MicroRNA-1 Is Decreased by Hypoxia and Contributes to the Development of Pulmonary Vascular Remodeling via Regulation of Sphingosine Kinase 1." *Am J Physiol Lung Cell Mol Physiol.* 2017 Nov 22. [Epub ahead of print] doi: 10.1152/ajplung.00057.2017.

C. Lung Expression of MiR-1 and SphK1 are Dysregulated in Murine HPH Development

Hypoxia is associated with the pathogenesis of PAH and contributes to pulmonary vascular remodeling. We utilized a mouse model of experimental HPH to investigate the development of disease progression following 1, 7, 14, and 28 days of 10% O_2 exposure, assessed by the measurement of RVSP and RVH. Mice developed significant elevations in RVSP by Day 14, and increased RVH and heart/body weight by Day 7, with increased severity of these parameters by Day 28 (**Figure 37A-C**). Interestingly, the expression of miR-1 in whole lung was significantly reduced over time following hypoxia exposure (**Figure 37D**), and this paralleled an increase in lung SphK1 protein expression (**Figure 37E**). Significant elevation in vessel wall thickness of small pulmonary arteries, due to medial hypertrophy of PASMCs, was also observed by Day 28 (data not shown), consistent with prior reports (43). These results demonstrate the utility of the HPH mouse model in studying the role of miR-1 expression.

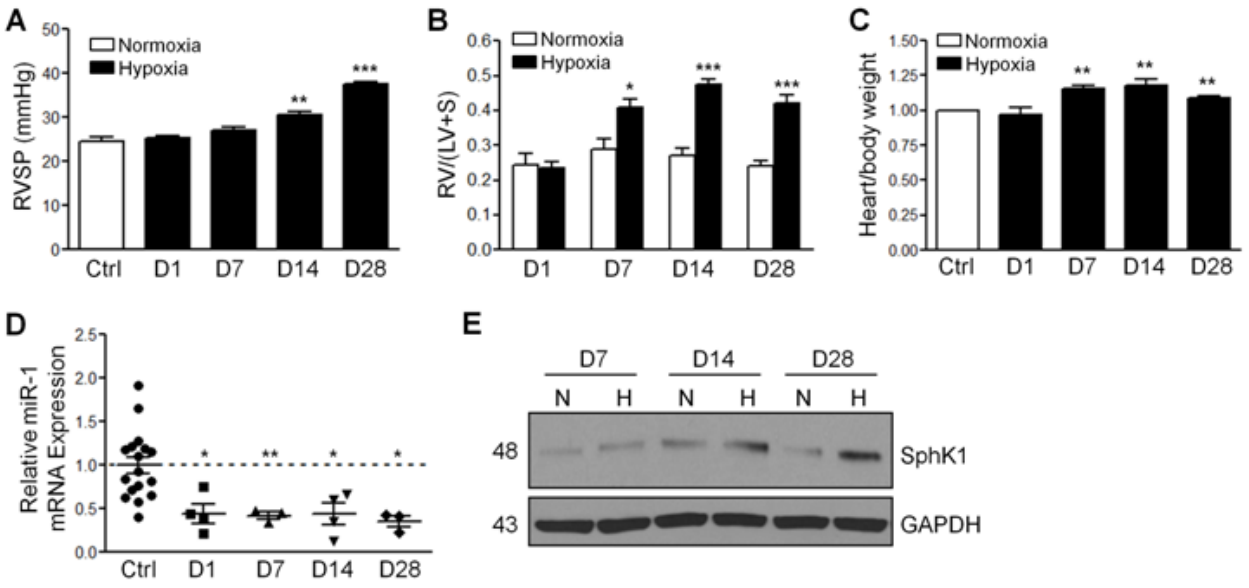

Figure 37. MiR-1 is down-regulated in mouse lungs during the progression of HPH. (A-B) HPH development in mice is demonstrated by elevations in right ventricular systolic pressure (RVSP) and right ventricular hypertrophy (RVH, measured by RV/[LV+S]) over time (Day [D] 1-28 of hypoxia). (C) Mouse heart/body weight change over the course of HPH development. (D) MiR-1 expression normalized to RNU6-2 expression is reduced and (E) SphK1 protein expression is increased in mouse lungs during HPH development. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ relative to control. Image source: Sysol JR, et al. 2017. "MicroRNA-1 Is Decreased by Hypoxia and Contributes to the Development of Pulmonary Vascular Remodeling via Regulation of Sphingosine Kinase 1." *Am J Physiol Lung Cell Mol Physiol.* 2017 Nov 22. [Epub ahead of print] doi: 10.1152/ajplung.00057.2017.

D. MiR-1 Expression is Reduced in PASMCs from PAH Patients and is Decreased by Hypoxia in hPASMCs

Since miR-1 was reduced in lungs from HPH mice, we investigated whether PASMCs isolated from patients with PAH had altered levels of miR-1. Expression of miR-1 was significantly reduced in PASMCs from PAH patients versus controls (**Figure 38A)**. We then tested whether hypoxia could alter miR-1 expression in hPASMCs. Hypoxia exposure (3% O_2) increased the proliferative phenotype of hPASMCs over time (**Figure 38B**) and significantly reduced the expression of miR-1 normalized to RNU6-2 expression (**Figure 38C**). These data are consistent

with our hypothesis that hypoxia may downregulate miR-1, leading to increased SphK1 expression and enhanced cell proliferation. Since overexpression of miR-1 mimics in hPASMCs can reduce Sphk1 protein expression under normoxic conditions (**Figure 36C**), we tested whether it could also prevent the induction of SphK1 expression by hypoxia. As shown in **Figure 38D-E**, miR-1 overexpression attenuates SphK1 expression under hypoxic conditions in hPASMC over time, suggesting a significant role of miR-1 in directly targeting the 3'-UTR of SphK1. The transient transfection of miR-1 mimics sustained expression at 72 hr, with the most robust expression at 24hrs (data not shown).

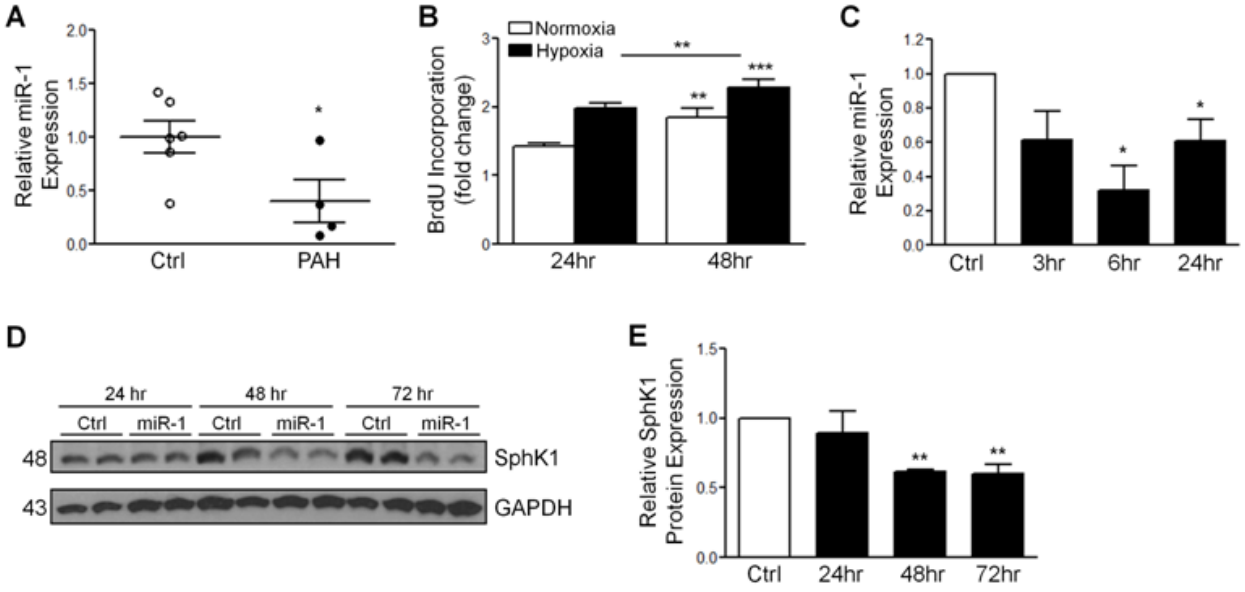

Figure 38. MiR-1 is decreased in hPASMCs from PAH patients, is downregulated by hypoxia, and prevents hypoxia-induced SphK1 expression. (A) MiR-1 expression normalized to RNU6-2 expression is reduced in hPASMCs isolated from PAH patients versus controls. (B) Stimulation of hPASMCs with hypoxia induces proliferation (24-48hrs, 3% O_2). (C) Hypoxia (3% O_2) induces down-regulation of miR-1 expression over time in hPASMCs. D-E) MiR-1 overexpression in hPASMCs attenuates hypoxia-induced SphK1 protein expression (24-72hr, 50nM mimics). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ relative to control. Image source: Sysol JR, et al. 2017. "MicroRNA-1 Is Decreased by Hypoxia and Contributes to the Development of Pulmonary Vascular Remodeling via Regulation of Sphingosine Kinase 1." *Am J Physiol Lung Cell Mol Physiol.* 2017 Nov 22. [Epub ahead of print] doi: 10.1152/ajplung.00057.2017.

E. MiR-1 Regulates the Proliferation of hPASMCs

Because miR-1 is significantly decreased in the hypoxia-induced proliferative state of hPASMCs, we investigated whether altering miR-1 levels with the transfection of miR-1 mimics could alter the proliferative phenotype in these cells. Overexpression of miR-1 mimics at increasing concentrations reduced the proliferative capacity of hPASMC in both normoxic and hypoxic conditions, respectively (**Figure 39A-B**), and these changes were not caused by enhanced cytotoxicity measured by LDH release (data not shown). Differences in proliferation were also observed by light microscopy, with a substantial reduction in the number of hPASMCs at both normoxia and hypoxia following miR-1 overexpression (**Figure 39C-D**).

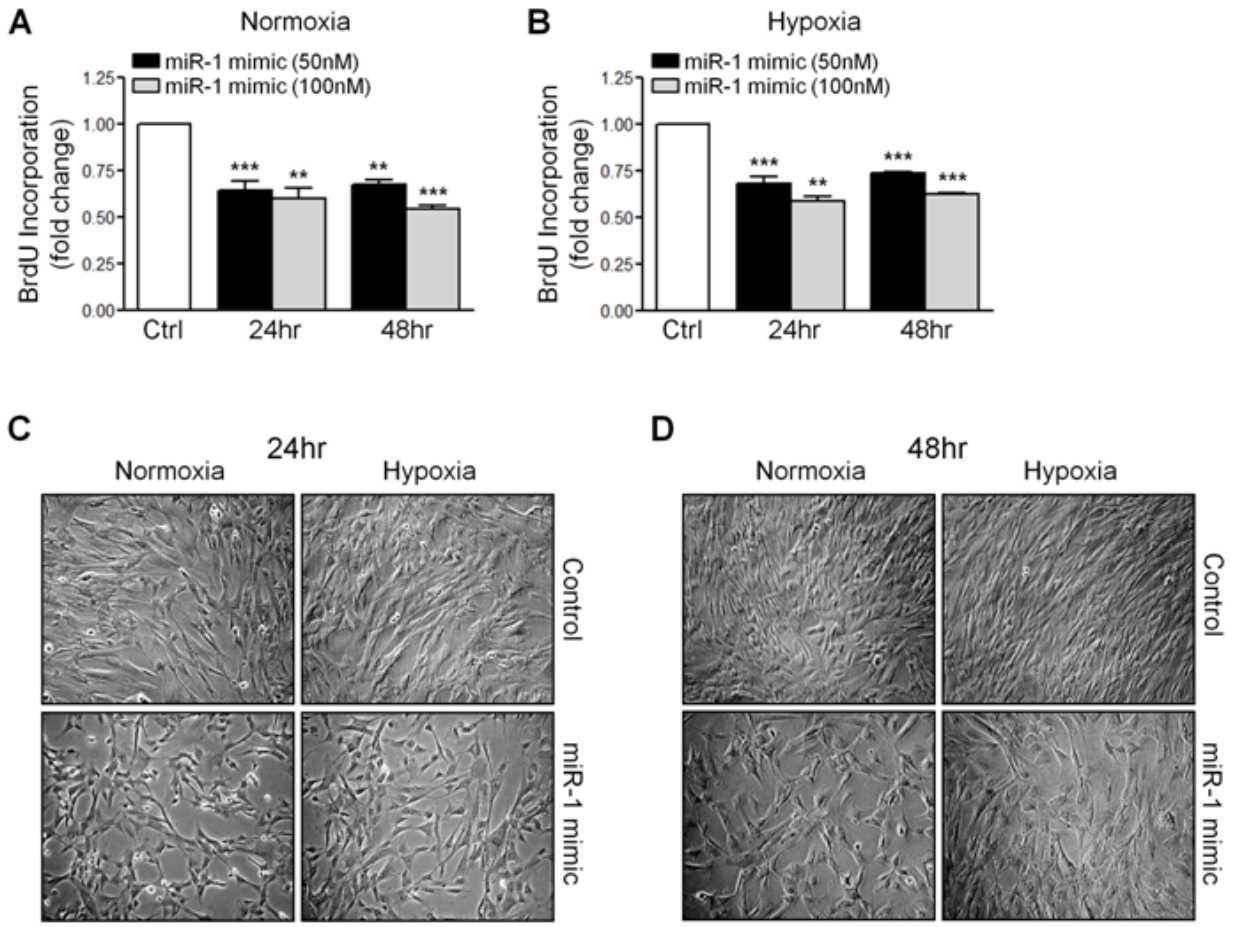

Figure 39. MiR-1 overexpression inhibits hPASMC proliferation in normoxia and hypoxia. (A) Overexpression of miR-1 mimics in hPASMCs (50-100 nM) significantly reduces proliferation in normoxia and (B) hypoxia (3% O_2, 24-48hrs). (C-D) Representative images of hPASMCs demonstrating miR-1 mimic overexpression in normoxia and hypoxia reduces the rate of proliferation (24hrs, 10x magnification). $^{**}p < 0.01$, $^{***}p < 0.001$ relative to control. Image source: Sysol JR, et al. 2017. "MicroRNA-1 Is Decreased by Hypoxia and Contributes to the Development of Pulmonary Vascular Remodeling via Regulation of Sphingosine Kinase 1." *Am J Physiol Lung Cell Mol Physiol.* 2017 Nov 22. [Epub ahead of print] doi: 10.1152/ajplung.00057.2017.

F. MiR-1 Regulates the Migration of hPASMCs

Both hPASMC proliferation and migration contribute to pathogenic pulmonary vascular remodeling in PAH. Based on the findings that miR-1 overexpression greatly reduced hPASMC proliferation *in vitro*, we tested whether miR-1 overexpression could also attenuate normoxic or

hypoxia-induced hPASMC migration. In a transwell assay, hPASMCs migrated over time under normoxic conditions, and, as expected, migration increased when these cells were exposed to hypoxia for 12-48 hrs (**Figure 40A-B**). Overexpression of miR-1 inhibited the migration of hPASMCs at normoxia, and further reduced the migration under hypoxic conditions (**Figure 40C-D**). This significant reduction in migratory capacity under hypoxia demonstrates the importance of diminished miR-1 expression during this synthetic state of hPASMCs. Thus, reduced miR-1 expression in hypoxia can enhance both proliferation and migration in this cell type during *in vitro* conditions that contribute to pulmonary vascular remodeling.

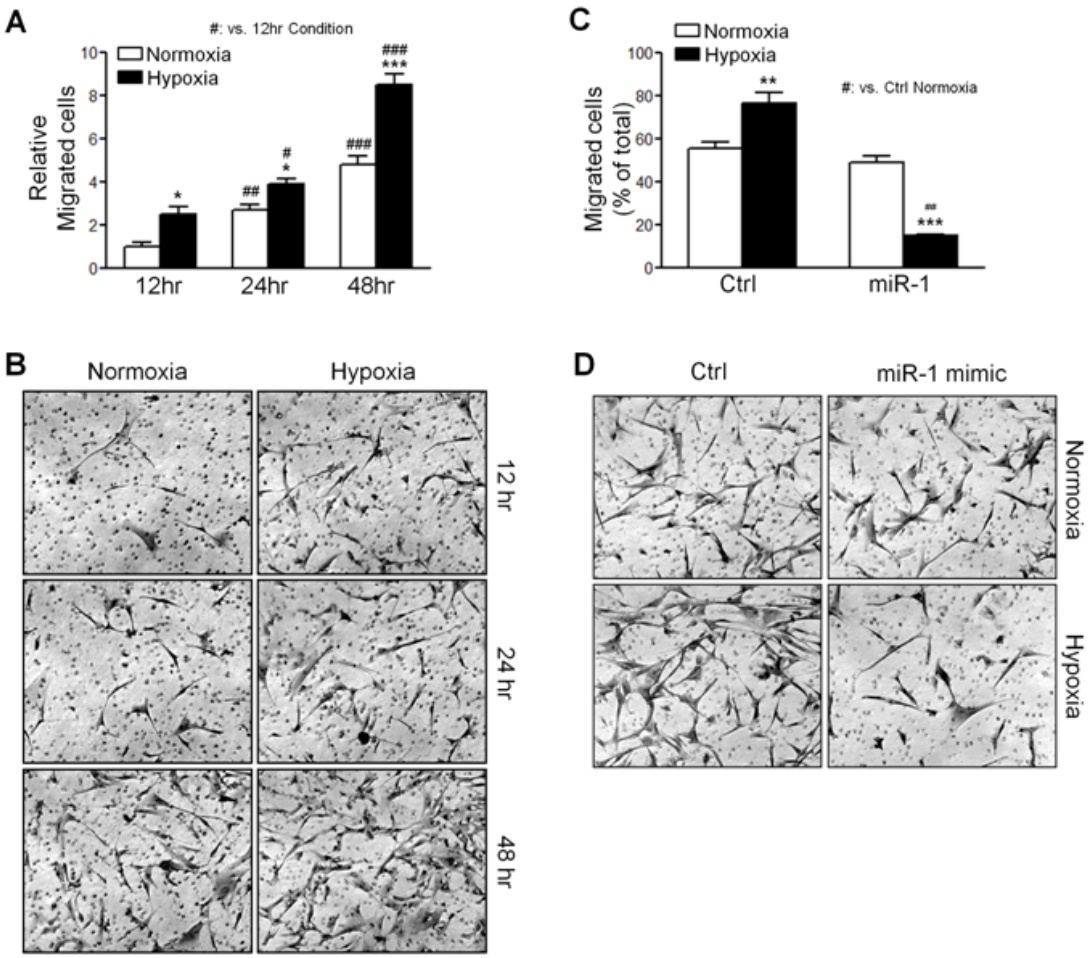

Figure 40. MiR-1 overexpression inhibits hPASMC migration in hypoxia. (A) Stimulation of hPASMCs with hypoxia induces cell migration in a transwell assay (12-48hrs, 3% O_2), with (B) representative images (10x magnification). (C) Overexpression of miR-1 mimics in hPASMCs (50 nM) significantly reduces hypoxia-induced cell migration (3% O_2, 24hrs), with (D) representative images (10x). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ relative to control, unless otherwise indicated. Image source: Sysol JR, et al. 2017. "MicroRNA-1 Is Decreased by Hypoxia and Contributes to the Development of Pulmonary Vascular Remodeling via Regulation of Sphingosine Kinase 1." *Am J Physiol Lung Cell Mol Physiol.* 2017 Nov 22. [Epub ahead of print] doi: 10.1152/ajplung.00057.2017.

G. Systemic MiR-1 Delivery Protects from the Development of HPH in Mice

To test whether increasing expression of miR-1 *in vivo* could prevent HPH development, we delivered either non-targeting or miR-1 mimics via retro-orbital injection to mice weekly throughout their 4-week hypoxia course. Systemic administration of miR-1 mimics prevented the reduction in miR-1 expression induced by hypoxia, which resulted in a 4- to 5-fold increase in expression in the

lung assessed by real-time qPCR. Compared to controls, mice receiving miR-1 mimics were protected against the development of HPH, with significant attenuation of RVSP elevation and RVH (**Figure 41A-C**). Hypoxia-induced pulmonary vascular remodeling was also reduced with miR-1 mimic treatment (**Figure 41D-F**). No changes were observed in any of these parameters under normoxic conditions with miR-1 administration. To test whether increased miR-1 expression altered SphK1 expression in this model, we measured SphK1 protein in lung tissues. Overexpression of miR-1 had no significant effect on SphK1 under normoxic conditions, but reduced SphK1 expression induction with hypoxia exposure (**Figure 42A-B**). To test whether SphK1 expression was altered in mouse PASMCs receiving miR-1 mimics, we measured both SphK1 and alpha-smooth muscle actin (α-SMA), a marker for smooth muscle cells, by immunofluorescence imaging in histological lung sections. Sphk1 expression co-localized with α-SMA and was significantly reduced in mice receiving miR-1 mimics in both normoxic and hypoxic conditions (**Figure 42C**). These results indicate that overexpression of miR-1 mimics reduces the development of experimental HPH, and together with the *in vitro* data presented in this study, suggest that altered expression of SphK1 expression by miR-1 may contribute to disease pathogenesis.

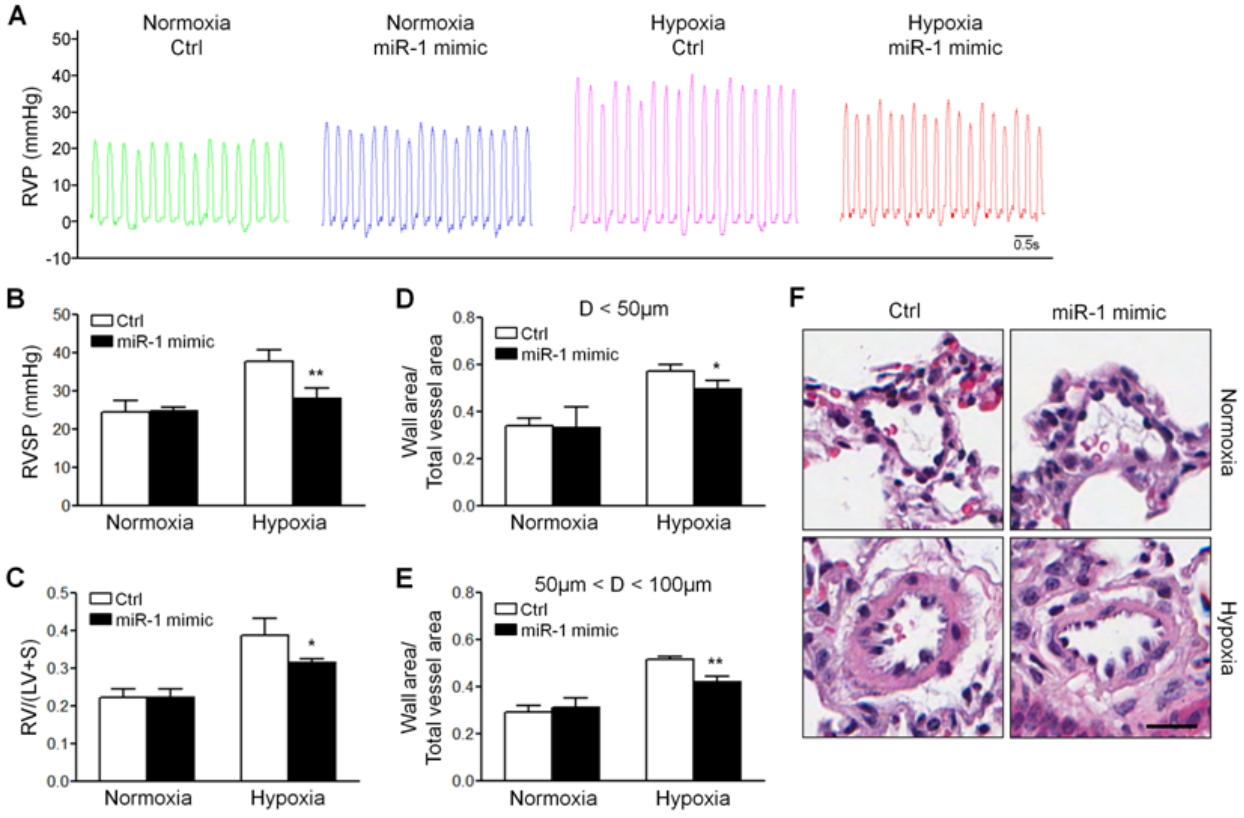

Figure 41. MiR-1 overexpression *in vivo* prevents the development of HPH in mice. Compared to controls, mice in the HPH model receiving systemic miR-1 mimics (7.8 mg/kg) develop less severe (A-B) RVSP elevation, (C) RVH, and (D-E) pulmonary vascular remodeling in arteries with diameters <50 μM and 50-100 μM, respectively. (F) Representative histological images of small pulmonary arteries in normoxia and hypoxia, with and without treatment of systemic miR-1 mimics. Scale bar = 20μm. $*p < 0.05$, $**p < 0.01$, relative to control. Image source: Sysol JR, et al. 2017. "MicroRNA-1 Is Decreased by Hypoxia and Contributes to the Development of Pulmonary Vascular Remodeling via Regulation of Sphingosine Kinase 1." *Am J Physiol Lung Cell Mol Physiol.* 2017 Nov 22. [Epub ahead of print] doi: 10.1152/ajplung.00057.2017.

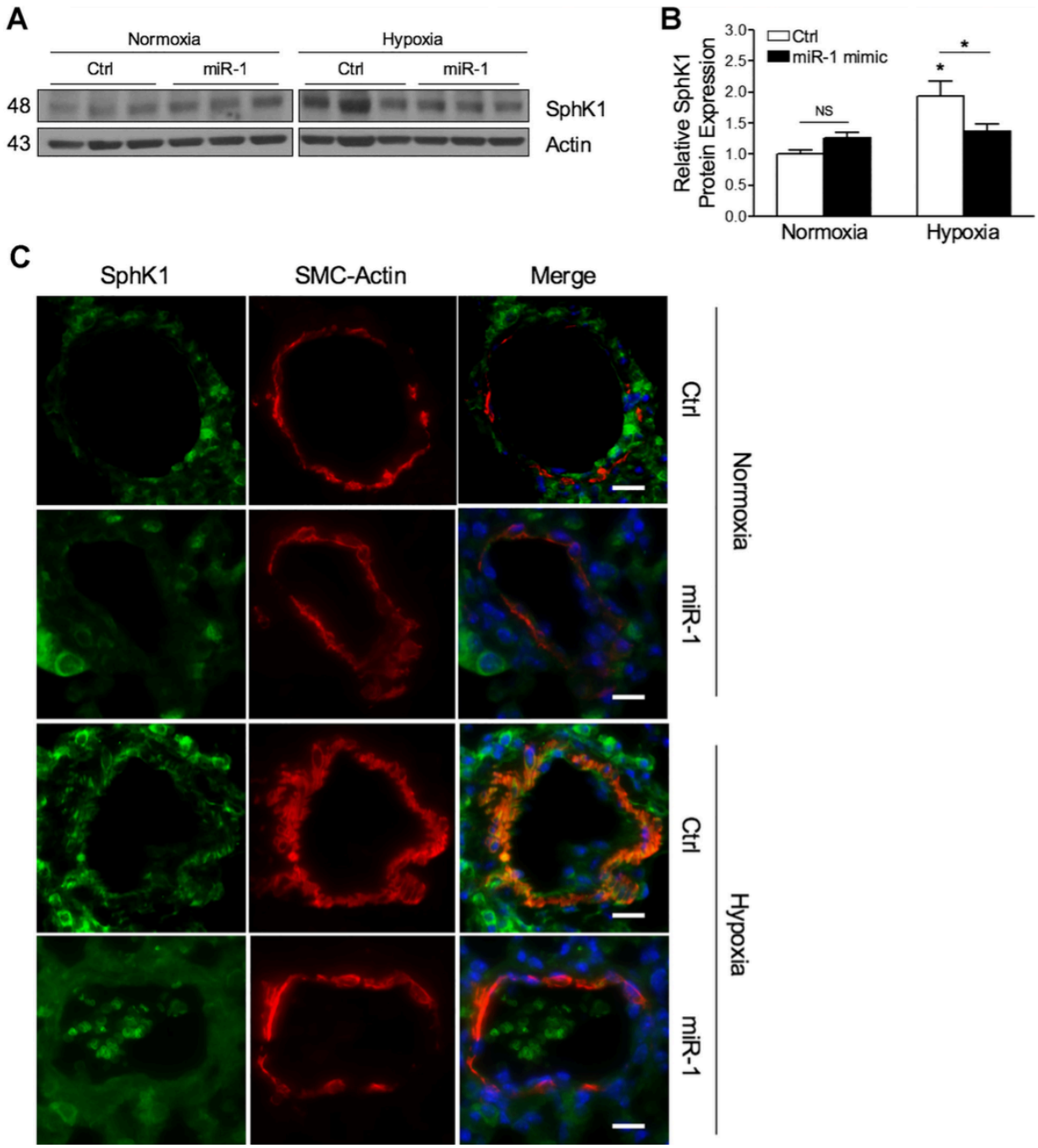

Figure 42. MiR-1 overexpression attenuates induction of SphK1 in lungs and PAs of HPH mice. (A) Representative western blotting in whole lung tissues from mice with HPH demonstrates attenuation of SphK1 expression following systemic miR-1 mimic administration (7.8 mg/kg), with (B) data quantification relative to actin expression. (C) Immunofluorescence staining demonstrates that hypoxia-induced SphK1 protein expression in small pulmonary arteries is reduced in miR-1 mimic-treated mice compared to controls. Scale bar = 20 μm. *$p < 0.05$, relative to control. Image source: Sysol JR, et al. 2017. "MicroRNA-1 Is Decreased by Hypoxia and Contributes to the Development of Pulmonary Vascular Remodeling via Regulation of Sphingosine Kinase 1." *Am J Physiol Lung Cell Mol Physiol.* 2017 Nov 22. [Epub ahead of print] doi: 10.1152/ajplung.00057.2017.

IX. DISCUSSION AND FUTURE DIRECTIONS

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1. Chen J, Tang H, Sysol JR, Moreno-Vinasco L, Shioura KM, Chen T, Gorshkova I, Wang L, Huang LS, Usatyuk PV, Sammani S, Zhou G, Raj JU, Garcia JG, Berdyshev E, Yuan JX, Natarajan V, and Machado RF. 2014. The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension. *Am J Respir Crit Care Med.* 190(9):1032-43. doi: 10.1164/rccm.201401-0121OC. (43) Reprinted with permission of the American Thoracic Society. Copyright © 2016 American Thoracic Society.
2. Sysol JR, Natarajan V, Machado RF. PDGF Induces SphK1 Expression via Egr-1 to Promote Pulmonary Artery Smooth Muscle Cell Proliferation. *Am J Physiol Cell Physiol.* 2016 Jun 1;310(11):C983-92. (44)
3. Sysol JR, Chen J, Singla S, Zhao S, Comhair SAA, Natarajan V, and Machado RF. 2017. MicroRNA-1 Is Decreased by Hypoxia and Contributes to the Development of Pulmonary Vascular Remodeling via Regulation of Sphingosine Kinase 1. *Am J Physiol Lung Cell Mol Physiol.* 2017 Nov 22. [Epub ahead of print] doi: 10.1152/ajplung.00057.2017. (45)

A. Chapter IV: SphK1 and S1P Contribute to the Development of PH

PAH is a progressive lung disease for which morbidity and mortality remains high and new therapies targeting pulmonary vascular remodeling are needed. The studies presented in Chapter IV demonstrate that the SphK1/S1P signaling axis is a novel and therapeutic target in PAH. We show that SphK1 and S1P are upregulated in patients with PAH and in experimental HPH models, and that SphK1 genetic deficiency or pharmacologic inhibition protects against the development of experimental PH. A decrease in S1P breakdown by Sgpl1 promoted the development of HPH.

S1P works through numerous mechanism both intracellularly and extracellularly which may contribute to its role in the pathogenesis of PAH. For example, the ability of S1P to induce SMC contraction and vasoconstriction has been described in isolated pulmonary arteries of rats (140) and in an isolated mouse lung model via S1PR2 (141). Similar findings of S1P-mediated vasoconstriction were observed in an isolated rat lung model (142). Other mechanism through which S1P can mediate vascular tone are increasing store-operated calcium entry into SMCs (143). S1P can also induce cell PASMC proliferation to promote pulmonary vascular remodeling, as discussed in further detail in the following section.

We demonstrated that SphK1 expression was upregulated in PAH patients and in rodent models of HPH. Hypoxia-induced upregulation of HIF1α, growth factors, and inflammatory cytokines are known to be key mediators in the pathogenesis of PAH. Interestingly, hypoxia has been shown to induce expression of SphK1 and promote proliferation in human PASMCs and PAECs (144,145), partly mediated by hypoxia-responsive elements located within SphK1 promoter. SphK1 itself has been described as an activator of HIF1α expression via the Akt/GSK-3β signaling pathway in cancer cells, which may induce a positive feedback loop to drive proliferation (103). The activation of HIF1α by the SphK1/S1P was explored in Chapter VI of this dissertation and discussed in Section C below.

The induction of growth factors, including PDGF and VEGF by hypoxia occur in PASMCs and are involved in the pathogenesis of PAH (146,147). This may be one mechanism regulating the induction of SphK1/S1P in PAH, given the significant crosstalk between growth factor and sphingolipid signaling pathways and the ability of PDGF to induce SphK1 expression in various smooth muscle cell types (126,148). Mechanistic molecular studies to describe the role of PDGF-induced SphK1 expression in PASMCs were demonstrated in Chapter VII of this dissertation and described in Section D below.

Lastly, recent data suggest that SphK1 expression can be induced by inflammatory stimuli and cytokines, such as LPS (149) and IL-1 (150). Inflammatory cells are found in and around pulmonary vascular lesions in PAH patients, and increased levels of circulating IL-1 have been reported (151). Further studies to explore the impact of hypoxia, growth factor, and inflammatory signaling on elevations of SphK1/S1P in PAH are warranted and may yield important insight into disease pathogenesis.

B. Chapter V: SphK1 and S1P Promote PASMC Proliferation via S1PR2 Signaling

The studies in Chapter V demonstrate that S1P/S1PR2 promotes PASMC proliferation via ERK signaling and can activate STAT3 signaling. In support of these findings, we showed that S1PR2 inhibition using JTE-013 prevents and reverses the development of HPH in mice. Together, these experiments highlight S1P/S1PR2 as a novel signaling pathway involved in pulmonary vascular remodeling in PAH.

The hyper-proliferation and resistance to apoptosis of cells within the pulmonary vascular wall, including PASMCs, are well known components of the pathobiology of PAH, and these features have been compared to cancer (152). In addition to the known role of the SphK1/S1P pathway in mediating pulmonary vascular tone, our results demonstrate that both SphK1 and S1P promote PASMC proliferation and contribute to pulmonary vascular remodeling associated with PAH. These data are consistent with the pro-proliferative effects of S1P described in numerous other cell types (153-155). Though not investigated in these studies, S1P is also known to inhibit apoptosis by modulating expression of key anti- and pro-apoptotic mediators, including BAX and BID (156). Changes in these factors and their association with S1P signaling could be measured in future studies to assess their role in PAH pathogenesis.

Up-regulation of SphK1 protein and mRNA levels were observed in the lungs of PAH patients and rodent PAs exposed to chronic hypoxia. We also demonstrated that overexpression of SphK1 promoted hPASMC proliferation. These findings support the model of "inside-out" signaling, where intracellular S1P produced by SphK is released from the cell via transporters, allowing for activation of nearby S1PRs. S1P could also mediate paracrine effects on neighboring cells, such as PAECs, to induce proliferation and neointimal formation.

Our data suggest that S1P promotes PASMC proliferation in an ERK-dependent manner via ligation of S1PR2. We demonstrated that S1PR2 expression is increased in PASMCs isolated from PAH patients and that inhibition of S1PR2 inhibits PASMC proliferation. S1PR2 was also shown to mediate S1P-induced ERK phosphorylation. In addition, S1PR2 inhibition via JTE-013 attenuated HPH in mice. Ligation of S1PR2 has been shown to prevent systemic vascular SMC migration (157) and to promote differentiation (158). Since our data describe the role of S1PR2 in the migration and proliferation of PASMCs, downstream signaling events of S1P/S1PR2 may differ between cell types. In muscle repair, S1P enhances satellite cell proliferation via S1PR2-dependent inhibition of RAC1 and repression of cell cycle inhibitors p21 and p27 (61). Importantly, transfection of S1PR2 and S1PR3 enhances S1P-mediated cell proliferation and inhibition of apoptosis via activation of the ERK pathway in hepatoma cells (60), consistent with our findings of S1PR2 signaling being pro-proliferative in hPASMCs.

Finally, our data suggest that S1P promotes STAT3 phosphorylation via S1PR2. STAT3 activation has been shown to be involved in the pro-survival and proliferative pulmonary vascular phenotype seen with PAH (70,72). We also demonstrated that STAT3 phosphorylation was basally increased in PASMCs of PAH patients and that STAT3 plays a role in S1P-induced hPASMC proliferation. The mechanisms of how S1P activates STAT3 and the downstream effects of this process on PASMC proliferation and pulmonary vascular remodeling are planned in future studies.

In summary, these data demonstrate that SphK1/S1P/S1PR2 signaling are important in mediating PASMC proliferation, an important process in the development of obstructive pulmonary vascular lesions in PAH. This pathway is therefore a potential therapeutic target in PAH that should be explored in future preclinical and clinical studies.

C. Chapter VI: S1P Signaling Activates HIF1α in PASMCs

S1P and hypoxia are both implicated in PAH and can stimulate hPASMC proliferation. In Chapter VI, we studied the activation of HIF1α by S1P under normoxic conditions in hPASMCs. We demonstrated that S1P/S1PR2 induces nuclear expression of HIF1α and increases expression of several of its downstream targets, including VEGFA and GLUT1. These studies identified S1P as a novel activator of HIF1α in PASMCs and suggest its importance in contributing to pulmonary vascular remodeling.

Hypoxia-inducible factor 1 (HIF1) is a heterodimeric transcription factor comprised of an α subunit (HIF1α) and a constitutively expressed β subunit (HIF1β). In hypoxic conditions, HIF1 is the master regulator of cellular responses, including modulating energy metabolism, proliferation, migration, angiogenesis, and extracellular matrix reorganization (79). HIF1α is constitutively synthesized and usually undergoes hydroxylation by prolyl hydroxylases, polyubiquitination by pVHL, proteasomal degradation under normoxic conditions. In hypoxia, prolyl hydroxylases are inactivated and HIF1α is stabilized, allowing for the induction of target gene transcription. Overexpression of HIF1α is seen in many different tumor types and contributes to cancer cell proliferation (84). Mice deficient for HIF1α have embryonic lethality, but it has been demonstrated that heterozygous HIF1α mice have impaired responses to chronic hypoxia, including attenuated pulmonary vascular remodeling and RVH (159). Recently, HIF1α in SMCs has been shown to contribute to pulmonary vascular remodeling and pulmonary hypertension in chronic hypoxia (160).

Studies suggest that positive feedback loops may sustain amplification of HIF1α in PAH pathobiology. During prolonged hypoxia exposure of PASMCs, ET-1 has been shown to induce HIF1α expression levels by up-regulating transcription and down-regulating of PHD2-mediated degradation (161). PASMCs with diminished mitochondrial ROS production have been shown to activate HIF1α in normoxia that inhibited voltage-gated potassium channels (162). Evidence also suggests that ROS can induce HIF1α levels through inhibition of prolyl hydroxylases and indirectly through activation of Akt/GSK3β signaling (100,101). Our studies demonstrate that S1P can also activate HIF1α expression in PASMCs, contributing to its accumulation in PAH. Interestingly, S1P has been shown to induce ROS in pulmonary alveolar epithelial cells, so this may be a potential mechanism of normoxic HIF1α activation in hPASMCs (163).

Mechanistically, we found that S1P induces expression of Egr-1 via ligation to S1PR2 and activation of downstream ERK signaling. Egr-1 has a binding site within the proximal HIF1α promoter, and previous studies in prostate cancer cells have shown that binding to this site is required for transactivation of HIF1α (98). The induction of Egr-1 by placenta growth factor in human pulmonary microvascular endothelial cells has also been demonstrated to regulate expression of HIF1α (164). Further studies are needed to confirm the physical binding of Egr-1 to the HIF1α promoter and the importance of this interaction in S1P-mediated HIF1α accumulation in hPASMCs.

In addition to studying the transcriptional upregulation of HIF1α by S1P, we also demonstrated that S1P induces phosphorylation of GSK-3β in hPASMCs as this effect is attenuated by inhibiting ERK signaling. This phosphorylation of GSK-3β is usually mediated by the PI3K/Akt pathway and is known to cause stabilization and nuclear accumulation of HIF1α (165). Interestingly, conflicting studies in have shown that inhibition of GSK-3β leads to accumulation of HIF1α due to enhanced mRNA stability, not protein stabilization (166). Additional studies to determine the potential role of GSK-3β phosphorylation on S1P-induced HIF1α expression are warranted and

would provide novel insight into the normoxic activation of HIF1α present in PAH and many other disorders. Studies to examine the mRNA and protein stability of HIF1α under these conditions are also important in determining possible interventions to prevent its accumulation.

HIF1α has also been shown to up-regulate cyclin D1 (CD1) and anti-apoptotic Bcl-xL and down-regulate the cell cycle inhibitors p21 and p27 (167-169), which may influence S1P-mediated hPASMC proliferation. To further explore the role of S1P-mediated HIF1α activation in modulating these targets, protein expression of these targets could be determined in S1P-treated PASMCs following silencing or inhibition of HIF1α. An alternative approach to investigating S1P-mediated HIF1α expression could be to measure expression of additional upstream mediators of HIF1α, including pVHL, Akt, and mammalian target of rapamycin (mTOR) (168,170,171). The role of other HIF family members, such as HIF1β and HIF2, and of prolyl hydroxylases could also be investigated in future studies.

D. Chapter VII: Role of PDGF in Inducing SphK1 in PASMCs

Given the important role of both PDGF and SphK1/S1P in the pathobiology of PAH (43,108), we investigated whether PDGF-induced proliferation of hPASMCs involves induction of SphK1 expression and potential mechanisms by which this may occur. In Chapter VII, we demonstrated that 1) PDGF induces SphK1 and Egr-1 transcription factor expression in hPASMCs, 2) PDGF-mediated Sphk1 transcriptional activity is mediated through binding of Egr-1 to the proximal SphK1 promoter, 3) Egr-1 and SphK1 are important for PDGF-induced hPASMC proliferation, 4) down-regulation of Egr-1 attenuates PDGF-induced expression of SphK1 in hPASMC, and 5) ERK phosphorylation is critical for induction of Egr-1 and SphK1 by PDGF. Our data describe a novel mechanism contributing to PASMC proliferation in PAH and identify the PDGF/Egr-1/SphK1 pathway as a potential therapeutic target.

PDGF-BB is released by endothelial cells and platelets during vascular injury and can stimulate hPASMC proliferation and migration (108,113). The role of PDGF and its receptors in the pathobiology of PAH is well established, and the utility of pharmacologically targeting this pathway in PAH have been proposed (108,117). One recent study found that in a rodent model of chronic hypoxia-mediated PH, mice genetically engineered with constitutively active PDGFR-β expression developed more severe pulmonary vascular remodeling than with chronic hypoxia alone (116). PDGF receptor antagonism using imatinib has also been shown to reverse advanced pulmonary vascular disease in several animal models of PH, including reversal of vascular remodeling and cor pulmonale (109). Clinically, the use of imatinib in several cases of refractory PAH has led to improvements in exercise capacity, hemodynamics, and functional class (119,120). Here, we demonstrate that PDGF can stimulate SphK1 expression in hPASMCs and that regions of the proximal SphK1 promoter are important for this induction. In other cell types, PDGF-induced SphK1 activity has been shown to increase intracellular S1P levels and is important in mediating proliferation, due in part to the ability of S1P to mobilize calcium and enhance levels of mitogenic phosphatidic acid (124,125,172). In addition, silencing of SphK1 in mouse embryonic fibroblast cells reduces PDGF-induced migration (63). We recently reported that levels of both SphK1 and S1P are elevated in patients with PAH and in rodent models of experimental PH, and that SphK1/S1P promote hPASMC proliferation (43). Genetic deletion or pharmacologic inhibition of SphK1 also protects from the development of experimental PH in several rodent models (43). The present data indicate the importance of PDGF signaling in activating SphK1 in hPASMCs, mechanistically linking these important pathways in PAH which contribute to vascular remodeling.

The SphK1 promoter deletion and ChIP analyses identified the importance of Egr-1 in mediating PDGF-induced SphK1 expression via nuclear translocation and binding to the proximal promoter. Egr-1 is a highly conserved, Cys_2His_2 type zinc-finger transcription factor known to be

induced by a variety of stimuli such as oxidative stress, shear stress, and growth factors, including PDGF (173-176). In patients with both congenital heart disease–associated PAH and idiopathic PAH, Egr-1 expression is abundant in plexiform lesions and in smooth muscle cells in vessels with severe concentric intimal fibrosis (177). Enhanced expression of Egr-1 has also been shown in the pulmonary vascular smooth muscle cell layer in human PAH and in a severe MCT-induced PAH rat model, which directly correlated with the degree of pulmonary vascular remodeling (178). Expression of Egr-1 has also been shown to be increased in the lung and pulmonary vascular cells in response to hypoxia, where it can activate several downstream targets involved in vascular remodeling in PAH (178,179). Interestingly, in a rat model of flow-associated PAH in rats, down-regulation of Egr-1 *in vivo* led to reduced expression of vascular PDGF-BB, less vascular proliferation, and increased apoptosis (178).

Here, we report that Egr-1 expression is rapidly induced in the nucleus of hPASMCs following PDGF stimulation, and that activation of Egr-1 is important for PDGF-induced SphK1 expression and cell proliferation. Using a specific SphK1 inhibitor, PF-543, we also demonstrate that SphK1 is involved in mediating PDGF-induced hPASMC proliferation. PF-543 did not alter basal proliferation, confirming previous findings that this drug does not inhibit DNA synthesis in hPASMCs (180). Our studies are also consistent with reports in other cell types that induced expression of Egr-1 is important in the control of cell proliferation, survival, and arteriogenesis (181,182). In addition to the novel activation of SphK1 by Egr-1 in hPASMCs presented here, the role of Egr-1 in activating other genes involved in regulation of vascular proliferation, inflammation, and apoptosis, including PDGF, TGF-β, IL-6, and p53, have been reported (178). These findings collectively highlight the importance of Egr-1 in key components of vascular remodeling, and suggest that targeting this pathway may be therapeutically beneficial in PAH. Several putative Egr-1 binding sites were identified within the SphK1 promoter, and this study explored the role of the

EGR1-"B" site in detail due to the importance of the promoter region containing this site in SphK1 transcriptional activation. Future studies investigating the other potential Egr-1 binding sites may be important in determining the mechanistic regulation of SphK1 in other cell types or in response to other stimuli that activate Egr-1.

To further explore the mechanisms of PDGF-induced Egr-1 and downstream SphK1 expression in hPASMCs, we investigated the influence of the ERK signaling pathway. We demonstrate that ERK1/2 phosphorylation is important for basal and PDGF-induced hPASMC proliferation and is critical for activation of Egr-1 and SphK1 expression by PDGF. The U0126 inhibitor used in these studies acts directly upon MEK1/2, so is highly specific in blocking ERK1/2 phosphorylation. The other MAPKs JNK and p38 are phosphorylated by MEK4/7 and MEK3/6, respectively (183). Our results demonstrate a nearly complete loss of PDGF-induced Egr-1 expression with U0126, suggesting that JNK and p38 are less involved in this signaling pathway in hPASMCs. Importantly, several supportive lines of evidence from the literature have demonstrated that inhibition of ERK1/2 phosphorylation can reduce hPASMC proliferation induced by a variety of stimuli *in vitro*, including hypoxia, BDNF, and peroxynitrite (66-68). Experiments to more precisely characterize the role of other downstream targets of ERK1/2 in PDGF-induced PASMC proliferation may be relevant to PAH pathobiology and could be explored in future studies.

The role of ERK-dependent activation of Egr-1 by numerous stimuli, including PDGF, has been described in other vascular smooth muscle cell types (174,175,184). In mouse macrophages, inhibition of in ERK1/2 phosphorylation and activation resulted in reduced Egr-1-induced tissue factor (TF) expression. Lysophosphatidic acid-induced Egr-1 expression has also been shown to be dependent on the MEK/ERK and JNK cascades (96). Importantly, increased levels of ERK phosphorylation have been demonstrated in mouse and rat lung tissues and in the medial layer of vascular lesions in several models of experimental PH (109,185,186). Administration of imatinib in a

monocrotaline-induced PH rat model strongly inhibited phosphorylation/activation of both PDGFR and ERK1/2 and reversed PH development (109), demonstrating an important relationship between PDGF and ERK signaling.

Notably, Egr-1 is also known to enhance expression of PDGFR (178), and S1P can induce mRNA and protein expression of PDGF-A and -B in vascular smooth muscle cells and neointimal cells from injured arteries (187). We therefore hypothesize that signaling of PDGF/ERK/Egr-1 in hPASMCs may create a positive feedback loop to generate additional PDGF/PDGFR. This would then lead to increased expression of SphK1 and other Egr-1 target genes involved in pulmonary vascular remodeling. Interestingly, the role of SphK1/S1P signaling as a positive regulator of Egr-1 has also been reported in several other cell types (95,188,189). Since our studies indicate that Egr-1 can activate expression of SphK1, which produces S1P, a positive feedback loop may be formed by the generation of additional Egr-1 expression. However, this loop may not be evident in cultured PASMCs since a biphasic induction of Egr-1 expression following PDGF stimulation has not been observed at time points as late as 48 hrs in our studies (data not shown). Future mechanistic studies to explore additional directions of the SphK1 activation pathway in PASMCs are warranted.

The process of pulmonary vascular remodeling in PAH also involves dysfunction and activation of other cell types besides smooth muscle, including endothelial cells and fibroblasts (190). During end-stage disease in a rat model of flow-associated PAH, Egr-1 expression was shown to be increased in both the medial smooth muscle and endothelial vessel layers (178). This study also demonstrated that down-regulation of Egr-1 increased vascular cell apoptosis, predominantly in the endothelial layer (178). While the present study focused on the proliferative phenotype of PASMCs, the role of PDGF activation of SphK1 in these other cells types and their crosstalk with PASMCs warrants further investigation.

Vascular smooth muscle cells, including PASMCs, have high plasticity compared to terminally-differentiated skeletal or cardiac muscle cells (191). Various environmental stimuli, including growth factors and changes in oxygen tension, can cause PASMCs to undergo a transition from a quiescent to highly proliferative phenotype (191). These changes are apparent under pathological conditions in PAH, where PASMCs demonstrate an increased proliferation and migration rate due in part to increases in cytosolic calcium concentration, ultimately leading to vessel obstruction and enhanced pulmonary vascular resistance (190,192). Here, we have demonstrated that PDGF-induced ERK/Egr-1 signaling is a novel pathway in hPASMCs that enhances SphK1 expression and cell proliferation. These studies advance our understanding of the ability of external stimuli in PAH to upregulate SphK1 expression and highlight the therapeutic potential of targeting this pathway in PAH to prevent or reverse pulmonary vascular remodeling.

E. Chapter VIII: Regulation of SphK1 by MiR-1 in PH

Despite active research in uncovering the pathogenic mechanisms resulting in PAH, therapies targeting the prevention or reversal of pulmonary vascular remodeling and subsequent elevations in pulmonary arterial pressure have not been successful. The studies presented in Chapter VIII provide evidence of the role of miR-1, a muscle-specific microRNA, and its posttranscriptional regulation of SphK1 in the pathogenesis of PAH and highlights its potential as a therapeutic target.

Using an *in silico* approach, we identify miR-1 as a potential regulator of SphK1 expression. We found that miR-1 is highly down-regulated by hypoxia in hPASMC, as well as in the lungs of mice throughout the progression of experimental HPH, coincident with increased SphK1 expression. Expression of miR-1 was also significantly reduced in PASMCs isolated from patients with PAH. Hypoxia-mediated decreases in miR-1 expression were associated with enhanced hPASMC proliferation and migration *in vitro*, and overexpression of miR-1 inhibited these effects.

Additionally, we provide *in vivo* evidence that miR-1 overexpression prevents the development of experimental HPH and reduces the overexpression of SphK1 in PASMCs in this model. Although there are likely multiple mRNAs targets of miR-1, the regulation of SphK1 expression is consistent with our findings that this kinase is highly influential in promoting PASMC proliferation and the development of experimental pulmonary hypertension (43). These experiments describe a novel role of miR-1 in regulating the intricate molecular mechanisms underlying pathogenic pulmonary vascular remodeling in PAH.

Work from our group has demonstrated that elevated levels of both S1P and SphK1 in the lung are associated with PAH (43). These studies showed that both genetic knockout of SphK1 in mice and pharmacologic SphK1 inhibition in rats could prevent the development of hypoxia-mediated PH. Inhibiting the S1PR2 in mice also prevented PH development, and mice heterozygous for S1P lyase, which catabolizes S1P, were more susceptible to PH (43). Together, these studies define the role of SphK1/S1P signaling in PAH. Here we identified miR-1 as a negative regulator of SphK1 expression. Under basal conditions, normal miR-1 expression limits SphK1 expression, whereas in hypoxia the reduction in miR-1 allows for SphK1 upregulation at both mRNA and protein levels.

Unrestrained PASMC proliferation and migration, as well are hypoxic vasoconstriction, are major contributors to pulmonary vascular remodeling in PAH, and upregulation of the SphK1/S1P signaling axis has been implicated in these pathogenic processes (43,193,194). We have previously demonstrated that both overexpression of SphK1 and stimulation with S1P promotes the proliferative phenotype of hPASMCs. Several studies have also reported that miRNAs can regulate cell proliferation and migration associated with vascular remodeling by several mechanisms, including targeting of channels, mitochondrial function, and BMPR2 signaling pathways (195). Here, we report that miR-1 is downregulated in PASMCs isolated from PAH patients, and overexpression

of miR-1 can reduce hypoxia-induced SphK1 expression and attenuate PASMC proliferation and migration. These studies indicate the critical role of miR-1 in regulating pulmonary vascular remodeling in PAH. The relevance of these findings also extends beyond PAH, as SphK1 and S1P are well-known mediators of cell proliferation, apoptosis resistance and angiogenesis in myriad cell types, including cancer cells (15,16).

Shared features of cancer development with the unrestricted vascular cell proliferation, apoptosis resistance, and glycolytic shifts of PAH have recently been appreciated (196,197). In both cases, a "pseudo-hypoxic environment" is present where glycolysis is predominant and hypoxia-inducible factor-1α is activated under normoxia. These observations are consistent with our findings of reduced miR-1 expression in PASMCs isolated from PAH patients in normoxic conditions. Importantly, inhibiting the oncogenic properties of SphK1 have shown efficacy in preclinical studies to decrease tumor growth (17,18), therefore induction of miR-1 to limit SphK1 expression may be beneficial. Recent reports have also associated downregulation of miR-1 with cancer development, including in lung squamous cell carcinoma (198), colon cancer (199), clear cell renal carcinoma (200), and prostate cancer (201), via its modulation of cell proliferation, migration, and invasion. In addition, miR-1 expression is significantly lower in colorectal carcinoma tissue compared to adjacent normal mucosa due to hyper-methylation of its upstream CpG island, consistent with reduced expression levels (202). The same study also found that liver metastatic tissues exhibited lower miR-1 expression compared to adjacent normal mucosa. These studies suggest the ubiquitous role of miR-1 in the regulation of cell proliferation and our consistent with our findings that miR-1 is anti-proliferative in PASMCs. Expression of miR-1 can also act as a tumor suppressor, as demonstrated in esophageal squamous cell carcinoma via targeting of LASP1 and TAGLN2 (203), breast cancer stem cells by inhibiting the Wnt/β-catenin pathway (204), and gastric cancer cells via targeting of

MET (205). These studies highlight the importance of downstream targets of miR-1 other than SphK1, which warrant future investigations in PAH pathobiology.

In addition to studies linking aberrant miR-1 expression to cancer, miR-1 has also been reported to regulate gene expression in SMCs. MiR-1 is an important modulator of cardiac and skeletal muscle proliferation, with excess expression leading to a reduced pool of proliferating ventricular cardiomyocytes *in vivo* (206-208). The inhibitory role of miR-1 on cell proliferation is highly evolutionarily conserved; mir-1 deletion in *Drosophila* leads to perturbed muscle cell differentiation, while mir-1 overexpression causes a reduction in the number of cardioblasts during embryogenesis (209). MiR-1 expression can be induced by myocardin, a transcriptional activator of serum response factor (SRF), to inhibit cell proliferation and contractility in human vascular SMCs (138,210), potentially through regulation of Pim-1, a recently identified biomarker in PAH (211). Down-regulation of miR-1 has also been observed in vascular SMCs isolated from spontaneously hypertensive rats and may regulate proliferation by targeting insulin-like growth factor 1 (IGF1) (212), a growth factor involved in neonatal HPH (213). In addition, genetic knockout of miR-1 in mice results in uniform lethality before weaning due to cardiac dysfunction, and hearts from these mice exhibit gene expression characteristics more similar to vascular smooth muscle (214). This suggests that the influence of miR-1 on the proliferation and migration of PASMCs likely functions through multiple parallel mechanisms to achieve the resulting cellular phenotype. Although there are likely many mRNAs targets of miR-1, the regulation of SphK1 expression is consistent with our findings that this lipid kinase is highly influential in promoting PASMC proliferation and the development of experimental pulmonary hypertension (43). In concordance, the *in vivo* data presented here demonstrate that systemic overexpression of miR-1 can target vascular SphK1 expression, reduce pulmonary vascular remodeling, and prevent the development of HPH.

MicroRNAs have shown promise as potential therapeutic targets and biomarkers for many diseases, including PAH. Interestingly, a recent study exploring the role of circulating miRNAs in human PH demonstrated reduced expression of miR-1 in the buffy coat of patients with both moderate and severe PH (215). Here, we have demonstrated that miR-1 is strongly downregulated early and throughout the course of experimental hypoxia-induced PH in the lungs, and that this down-regulation is retained in PASMCs from PAH patients. Overexpression of miR-1 can reduce the pulmonary vascular remodeling phenotype both *in vitro* and *in vivo,* therefore identifying mechanisms to elevate or sustain miR-1 expression in PAH may be beneficial. The ability of miR-1 to decrease SphK1 levels in PASMCs is one mechanism through which it regulates vascular remodeling, but other direct targets involved in PAH development may contribute to our findings here and would be important to identify in future studies.

Together, these data demonstrate that miR-1 targets SphK1 to regulate molecular mechanisms in PAH with effects on PASMCs and pulmonary remodeling. The activation of miR-1 in the pulmonary vasculature may therefore provide therapeutic benefit for PAH patients.

F. Conclusions

In conclusion, this dissertation identifies and characterizes the importance of the SphK1/S1P signaling pathway in the pathogenic process of pulmonary vascular remodeling in PAH. Several novel signaling mechanisms of S1P involved in the induction of PASMC proliferation were illustrated, including the induction of Egr-1 and STAT3 and the normoxic activation of HIF1α. The role of S1PR2 in mediating these events and in the development of experimental PH was also described. In addition, key factors regulating the overexpression of SphK1 in the context of PAH development, including PDGF and miR-1, were examined. Together, the evidence presented here highlights the SphK1/S1P signaling axis as a potential therapeutic target in PAH. Given the

progressive, fatal nature of this disease, it is imperative that novel therapies to combat pulmonary vascular remodeling are identified.

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Authors: Jiwang Chen, Haiyang Tang, Justin R. Sysol, Liliana Moreno-Vinasco, Krystyna M. Shioura, Tianji Chen, Irina Gorshkova, Lichun Wang, Long Shuang Huang, Peter V. Usatyuk, Saad Sammani, Guofei Zhou, J. Usha Raj, Joe G. N. Garcia, Evgeny Berdyshev, Jason X.-J. Yuan, Viswanathan Natarajan, and Roberto F. Machado.
Page range: 1032-43.
Volume number: 190(9).
Journal title: American Journal of Respiratory and Critical Care Medicine.
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Title: PDGF Induces SphK1 Expression via Egr-1 to Promote Pulmonary Artery Smooth Muscle Cell Proliferation

Author: Justin R. Sysol,Viswanathan Natarajan,Roberto F. Machado

Publication: Am J Physiol-Cell Physiology

Publisher: The American Physiological Society

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BACK | CLOSE WINDOW

Title: Micro-RNA-1 is decreased by hypoxia and contributes to the development of pulmonary vascular remodeling via regulation of sphingosine kinase 1

Author: Justin R. Sysol, Jiwang Chen, Sunit Singla, et al

Publication: Am J Physiol-Lung, Cellular, and Molecular Physiology

Publisher: The American Physiological Society

Date: Mar 1, 2018

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VITA

Justin R. Sysol

EDUCATION

Degree	Years	Institution	Discipline
B.S.	2005-2009	University of Rochester	Ecology and Evolutionary Biology Distinction in Research
M.D./Ph.D.	2011-2018	University of Illinois College of Medicine	Cellular and Molecular Pharmacology

PROFESSIONAL EXPERIENCE

2012 – 2018 **PhD Thesis Research**
Advisor: Roberto Machado, M.D.; Chair: Viswanathan Natarajan, Ph.D.
Departments of Pharmacology and Medicine
University of Illinois College of Medicine at Chicago

2009 – 2011 **Post-baccalaureate Intramural Research Training Award (IRTA) Fellow**
Advisors: Charles Venditti, M.D., Ph.D., Irini Manoli, M.D., Ph.D.
Genetics and Molecular Biology Branch
National Human Genome Research Institute (NHGRI), NIH

2009 **Graduate Research Assistant**
Advisor: J.H. David Wu, Ph.D.; Department of Chemical Engineering
University of Rochester

2007 – 2009 **Undergraduate Research Assistant and Senior Thesis**
Advisor: John H. Werren, Ph.D.; Department of Biology;
University of Rochester

2007 – 2008 **Undergraduate Research Assistant**
Advisor: Baek Kim, Ph.D.; Department of Microbiology and Immunology
University of Rochester

RESEARCH ACTIVITIES

Publications

1. Saraf S.L., **Sysol J.R.**, Susma A., Setty S., Zhang X., Gudehithlu K.P., Arruda J.A.L., Singh A.K., Machado R.F., Gordeuk V.R. Progressive glomerular and tubular damage in sickle cell

trait and sickle cell anemia mouse models. *Transl Res.* 2017 Feb https://doi.org/10.1016/j.trsl.2018.01.007. [Epub ahead of print]

2. **Sysol J.R.**, Chen J., Singla S., Zhao S., Comhair S.A.A., Natarajan V., Machado R.F. MicroRNA-1 Is Decreased by Hypoxia and Contributes to the Development of Pulmonary Vascular Remodeling via Regulation of Sphingosine Kinase 1. *Am J Physiol Lung Cell Mol Physiol.* 2017 Nov 22:ajplung000572017. doi: 10.1152/ajplung.00057.2017. [Epub ahead of print]

3. Singla S., **Sysol J.R.**, Dille B., Jones N., Chen J., Machado R.F. Hemin Causes Lung Microvascular Endothelial Barrier Dysfunction by Necroptotic Cell Death. *Am J Respir Cell Mol Biol.* 2017;57(3):307-14.

4. Singla S., Chen J., Sethuraman S., **Sysol J.R.**, Gampa A., Zhao S., Machado R.F. Loss of Lung WWOX Expression Causes Neutrophilic Inflammation. *Am J Physiol Lung Cell Mol Physiol.* 2017;312(6):L903-11.

5. Chen J., **Sysol J.R.**, Singla S., Zhao S., Yamamura A., Valdez-Jasso D., Abbasi T., Shioura K.M., Sahni S., Reddy V., Sridhar A., Gao H., Torres J., Camp S.M., Tang H., Ye S.Q., Comhair S., Dweik R., Hassoun P., Yuan J.X.J., Garcia J.G.N., Machado R.F. Nicotinamide Phosphoribosyltransferase Promotes Pulmonary Vascular Remodeling and is a Therapeutic Target in Pulmonary Arterial Hypertension. *Circulation.* 2017;135(16):1532-46.

6. Lerin C., Goldfine A.B., Boes T., Liu M., Kasif S., Dreyfuss J., De Sousa-Coelho A.L., Daher G., Manoli I., **Sysol J.R.**, Isganaitis E., Jessen N., Goodyear L.J., Beebe K., Gall W., Venditti C.P., Patti M.E. Defects in Muscle Branched-Chain Amino Acid Oxidation Contribute to Impaired Lipid Metabolism. *Mol Metab.* 2016;5(10):926–36.

7. Duarte J.D.*, Desai A.A.* (*Equal contribution), **Sysol J.R.**, Abbasi T., Patel A.R., Lang R.M., Gupta A., Garcia J.G., Gordeuk V., Machado R.F. Genome-Wide Analysis Identifies IL-18 and FUCA2 as Novel Genes Associated with Diastolic Function in African Americans with Sickle Cell Disease. *PLoS One.* 2016;11(9):e0163013.

8. **Sysol J.R.**, Natarajan V., Machado, R.F. PDGF Induces SphK1 Expression via Egr-1 to Promote Pulmonary Artery Smooth Muscle Cell Proliferation. *Am J Physiol Cell Physiol.* 2016;310(11):C983-92.

9. Tang H., Chen J., Fraidenburg D.R., Song S., **Sysol J.R.**, Drennan A.R., Offermanns S., Ye R.D., Bonini M.G., Minshall R.D., Garcia J.G., Machado R.F., Makino A., Yuan J.X. Deficiency of Akt1, but not Akt2, Attenuates the Development of Pulmonary Hypertension. *Am J Physiol Lung Cell Mol Physiol.* 2015;308(2):L208-220.

10. Chen J.*, Tang H.*, **Sysol J.R.* (*Equal contribution)**, Moreno-Vinasco L., Shioura K.M., Chen T., Gorshkova I., Wang L., Usatyuk P.V., Huang L., Sammani S., Zhou G., Raj J.U., Garcia J.G., Berdyshev E., Yuan J.X., Natarajan V., Machado R.F.. The sphingosine kinase 1/sphingosine-1-phosphate pathway in pulmonary arterial hypertension. *Am J Respir Crit Care Med.* 2014;190(9):1032-1043.

11. Manoli I.*, **Sysol J.R.* (*<u>Equal contribution</u>)**, Li L., Houillier P., Garone C., Wang C., Zerfas P.M., Cusmano-Ozog K., Young S., Trivedi N.S., Cheng J., Sloan J.L., Chandler R.J., Abu-Asab M., Tsokos M., Elkahloun A.G., Rosen S., Enns G.M., Berry G.T., Hoffamnn V., DiMauro S., Schnermann J., Venditti C.P.. Targeting proximal tubule mitochondrial dysfunction attenuates the renal disease of methylmalonic academia. *Proc Natl Acad Sci U S A*. 2013;110(33):13552-13557.

12. **Sysol J.R.**, Kempf C., Helton M.N., Dong Y., Zhu D., Sun H., Garcia J.G., Machado R.F., Chen J. Evaluation of a reliable and cost-effective method of DNA isolation for mouse genotyping. *Biotechnol Lett*. 2013;35(4):509-514.

13. Senac J.S., Chandler R.J., **Sysol J.R.**, Li L., Venditti C.P. Gene therapy in a murine model of methylmalonic acidemia using rAAV9-mediated gene delivery. *Gene Ther*. 2012;19(4):385-391.

14. Sloan J.L., Johnston J.J., Manoli I., Chandler R.J., Krause C., Carrillo-Carrasco N., Chandrasekaran S.D., **Sysol J.R.**, O'Brien K., Hauser N.S., Sapp J.C., Dorward H.M., Huizing M., NISC, Barshop B.A., Berry S., James P.M., Champaigne N.L., de Lonlay P., Valayannopoulos V., Geschwind M.D., Gavrilov D.K., Nyhan W.L., Biesecker L.G., Venditti C.P. Whole exome sequencing identifies ACSF3 as a putative human malonyl-CoA synthetase and the gene responsible for Combined Malonic and Methylmalonic Aciduria. *Nat Genet*. 43(9):883-6 (2011).

<u>Book Chapters</u>

1. **Sysol J.R.**, Machado R.F. Sickle Cell Disease and Acute Chest Syndrome: Epidemiology, Diagnosis, Management, Outcomes. *Respiratory Medicine: Hematologic Abnormalities and Acute Lung Syndromes*. Springer International Publishing, Switzerland. pp 67-87 (2017).

<u>Selected Abstracts</u>

1. **JR Sysol**, J Chen, S Singla, S Comhar, SC Erzurum, V Natarajan, RF Machado. "Role of MicroRNA-1 in Regulating Pulmonary Vascular Remodeling in Pulmonary Hypertension." Central Society for Clinical and Translational Research (CSCTR) Annual Meeting, Chicago, IL (2016). *J Investig Med*, 64(4):969.

2. SL Saraf, **JR Sysol**, A Susma, S Setty, KP Gudehithlu, JAL Arruda, AK Singh, RF Machado, VR Gordeuk. "Progressive Glomerular Damage in Sickle Cell Trait and Sickle Cell Anemia Mouse Models." Central Society for Clinical and Translational Research (CSCTR) Annual Meeting, Chicago, IL (2016). *J Investig Med*, 64(4), 957-958.

3. J Chen, **JR Sysol**, KM Shioura, S Singla, H Yamamura, A Yamamura, V Reddy, J Torres, A Sridhar, H Tang, JX Yuan, JG Garcia, RF Machado. "Inhibition of Nicotinamide Phosphoribosyltransferase (NAMPT) Attenuates Experimental Pulmonary Hypertension."

Central Society for Clinical and Translational Research (CSCTR) Annual Meeting, Chicago, IL (2016). *J Investig Med,* 64(4)912-913.

4. **JR Sysol**, J Chen, S Singla, V Natarajan, RF Machado. "MicroRNA-1 is Decreased by Hypoxia and Contributes to the Development of Experimental Pulmonary Hypertension via Regulation of Sphingosine Kinase 1 (SphK1)." *Accepted for poster discussion*, American Thoracic Society International Conference, San Francisco, CA (May 13-18, 2016).

5. J Chen, S Singla, **JR Sysol**, S Ye, S Erzurum, RF Machado. "Autocrine and paracrine nicotinamide phosphoribosyltransferase (NAMPT) activity promote proliferation of human pulmonary artery smooth muscle cells." *Accepted for poster presentation*, American Thoracic Society International Conference, San Francisco, CA (May 13-18, 2016).

6. S Singla, J Chen, **JR Sysol**, RF Machado. "Lung WWOX knockdown causes neutrophilic inflammation associated with increased epithelial IL-8 secretion." *Accepted for poster discussion*, American Thoracic Society International Conference, San Francisco, CA (May 13-18, 2016).

7. **JR Sysol**, J Chen, JGN Garcia, V Natarajan, RF Machado. "Sphingosine Kinase 1 (SphK1) Promoter is Regulated by Hypoxia and Platelet-Derived Growth Factor (PDGF) in Pulmonary Artery Smooth Muscle Cells." American Thoracic Society International Conference, Denver, CO (May 15-20, 2015). *Am J Respir Crit Care Med*, 191;2015:A1987.

8. **JR Sysol**, J Chen, P Fu, JGN Garcia, V Natarajan, RF Machado. "Sphingosine-1-Phosphate (S1P) Induces Hypoxia-Inducible Factor-1 Alpha (HIF1-α) Activation in Human Pulmonary Artery Smooth Muscle Cells." American Thoracic Society International Conference, Denver, CO (May 15-20, 2015). *Am J Respir Crit Care Med*, 191;2015:A5957.

9. **JR Sysol**, S Sammani, E Letsiou, S Singla, SM Dudek, RF Machado. "Mice with Sickle Cell Disease are Protected from Ventilator-Induced Lung Injury but not LPS-Induced Lung Injury." American Thoracic Society International Conference, Denver, CO (May 15-20, 2015). *Am J Respir Crit Care Med*, 191;2015:A2060.

10. J Chen, **JR Sysol**, LS Huang, AS Chehtha, Y Dong, H Tang, JXJ Yuan, V Natarajan, RF Machado. "Conditional SphK1 Deficiency in Smooth Muscle Cells, but Not Endothelial Cells, Protects Against Hypoxia-mediated Pulmonary Hypertension in Mice." American Thoracic Society International Conference, Denver, CO (May 15-20, 2015). *Am J Respir Crit Care Med*, 191;2015:A1919.

11. S Singla, **JR Sysol**, P Belvitch, J Chen, RF Machado. "Hemin induces pulmonary endothelial cell barrier dysfunction via NF-kB." American Thoracic Society International Conference, Denver, CO (May 15-20, 2015). *Am J Respir Crit Care Med*, 191;2015:A2075.

12. S Sammani, E Letsiou, A Rizzo, **JR Sysol**, L Meliton, RF Machado, SM Dudek. "Hemin-induced acute lung injury is mediated by GVPLA2 in mice." Central Society for Clinical and Translational Research (CSCTR) Annual Meeting, Chicago, IL (2015). *J Investig Med*, 63(4):699-700.

13. J Chen, **JR Sysol**, H Tang, L Moreno-Vinasco, KM Shioura, JXJ Yuan, JGN Garcia, V Natarajan, RF Machado. "Transgenic or pharmacological inhibition of SphK1 and S1PR2 prevents hypoxia-mediated pulmonary hypertension." Central Society for Clinical and Translational Research (CSCTR) Annual Meeting, Chicago, IL (2015). *J Investig Med*, 63(4):660.

14. **JR Sysol**, J Chen, H Tang, V Reddy, JXJ Yuan, V Natarajan, RF Machado. "Sphingosine-1-phosphate receptor 2 is elevated in patients with pulmonary arterial hypertension and regulates S1P-induced pulmonary artery smooth muscle cell proliferation." American Thoracic Society International Conference, San Diego, CA (May 16-21, 2014). *Am J Respir Crit Care Med*, 189;2014:A4776.

15. J Chen, H Tang, **JR Sysol**, A Sridhar, JXJ Yuan, V Natarajan, RF Machado. "S1P lyase deficiency in mice enhances susceptibility to hypoxia-mediated pulmonary hypertension." American Thoracic Society International Conference, San Diego, CA (May 16-21, 2014). *Am J Respir Crit Care Med*, 189;2014:A3298.

16. X Sun, B Liu, **JR Sysol**, V Natarajan, RF Machado. "Promoters of sphingosine-1-phosphate receptors are differentially regulated by growth factors and hypoxia." American Thoracic Society International Conference, San Diego, CA (May 16-21, 2014). *Am J Respir Crit Care Med*, 189;2014:A4813.

17. H Tang, J Chen, AR Drennan, DR Fraidenburg, S Song, **JR Sysol**, KA Smith, RF Machado, A Makino, JXJ Yuan. "Akt/mTOR signaling contributes to the development of pulmonary arterial hypertension." American Thoracic Society International Conference, San Diego, CA (May 16-21, 2014). *Am J Respir Crit Care Med*, 189;2014:A5559.

18. H Tang, J Chen, DR Fraidenburg, **JR Sysol**, RF Machado, A Makino, JXJ Yuan. "Conditional deletion of beta-catenin in smooth muscle cells fails to affect the development of pulmonary hypertension." Central Society for Clinical and Translational Research (CSCTR) and the Midwestern Section of the American Federation for Medical Research (MWAFMR) Combined Annual Meeting (Apr 24-25, 2014). *J Investig Med.* 62(4):743-744 (2014).

19. I Manoli, **JR Sysol**, MK Crocker, G Niu, J Storrar, S Mendelson, J Sloan, C Wang, Y Ktena, PM Zerfas, V Hoffman, HJ Vernon, A Hamosh, JC Reynolds, X Chen, O Gavrilova, JA Yanovski, CP Venditti. "A lipomatosis endophenotype in methylmalonic acidemia: evidence from patients and mice." American Society of Human Genetics Meeting, Boston, MA (Oct 22-26, 2013).

20. JS Senac, VH Aswani, **JR Sysol**, I Manoli, CP Venditti. "A Partial Deficiency Model of MUT Methylmalonic Acidemia (MMA) Displays Diet Inducible Disease and Sensitivity to Acetaminophen (APAP). American Society of Gene and Cell Therapy, 16th Annual Meeting, Salt Lake City, UT (May 15-18, 2013). *Molecular Therapy*, Vol 21, Sup 1: S107 (2013).

21. J Chen, S Shahrara, A Yamaura, **JR Sysol**, H Tang, A Fahs, L Xie, Y Dong, S Song, JGN Garcia, JXJ Yuan, RF Machado. "Interleukin 7 (IL7) And IL7 Receptor (IL7R) Are Novel Mediators In The Development Of Pulmonary Hypertension In Humans And Mice."

American Thoracic Society International Conference, Philadelphia, PA (May 17-22, 2013). *Am J Respir Crit Care Med*, 187; 2013.

22. J Chen, **JR Sysol**, T Chen, H Tang, E Berdyshev, I Gorshkova, G Zhou, Y Dong, A Fahs, JU Raj, JGN Garcia, JXJ Yuan, V Natarajan, RF Machado. "Sphingosine Kinase 1 Is Upregulated In Pulmonary Artery Smooth Muscle Cells (PASMCs) From Patients With Pulmonary Arterial Hypertension And Modulates Human PASMC Proliferation." American Thoracic Society Conference, Philadelphia, PA (May 17-22, 2013). *Am J Respir Crit Care Med*, 187; 2013.

23. JS Senac, VH Aswani, **JR Sysol**, I Manoli, CP Venditti. "A mouse model of Mut$^-$ methylmalonic acidemia (MMA)." American Society of Human Genetics, 62st Annual Meeting, San Francisco, CA (Nov 6-10, 2012).

24. **JR Sysol**, J Chen, Wade MS, T Abbasi, A Fahs, JGN Garcia, JXJ Yuan, RF Machado. "Pre-B Cell Colony-Enhancing Factor (PBEF) is elevated in plasma and lung tissue of patients with pulmonary hypertension and promotes pulmonary artery smooth muscle cell proliferation." American Thoracic Society International Conference, Philadelphia, PA (May 17-22, 2013). *Am J Respir Crit Care Med*, 187; 2013:A1744.

25. J Chen, **JR Sysol**, H Yamamura, KM Shioura, S Sahni, J Torres, A Yamamura, Q Guo, S Camp, C Evenoski, Y Zhao, A Desai, L Moreno-Vinasco, JXJ Yuan, JGN Garcia, RF Machado. "Inhibition of Pre-B Cell Colony-Enhancing Factor (PBEF) Prevents And Reverses Monocrotaline-Induced Pulmonary Hypertension." Translational Research Approaches to Reduce Health Disparities in Lung Disease Symposium, Chicago, IL (Sept 10-11, 2012).

26. J Chen, L Moreno-Vinasco, **JR Sysol**, KM Shioura, S Sammani, L Huang, S Pendyala, JXJ Yuan, JGN Garcia, V Natarajan, RF Machado. "Sphingosine Kinase 1 Deficiency Protects Rodents from Chronic Hypoxia-mediated Pulmonary Hypertension." Translational Research Approaches to Reduce Health Disparities in Lung Disease Symposium, Chicago, IL (Sept 10-11, 2012).

27. JS Senac, **JR Sysol**, CP Venditti. "Hepatocyte therapy for MUT methylmalonic academia." American Society of Gene and Cell Therapy, 15th Annual Meeting, Philadelphia, PA (May 16-19, 2012). *Molecular Therapy*, Vol 20, Sup 1: S59 (2012).

28. J Chen*, L Moreno-Vinasco*, **JR Sysol**$^{\#}$, KM Shioura, S Sammani, V Mohan, L Huang, S Pendyala, J Yuan, JG Garcia, V Natarajan, RF Machado. "Sphingosine Kinase 1 deficiency protects rodents from chronic hypoxia-mediated pulmonary hypertension." American Thoracic Society International Conference, San Francisco, CA (May 18-23, 2012). *Am J Respir Crit Care Med*, 185; 2012:A3440. $^{\#}$Name added to poster only.

29. J Chen, H Yamamura, S Sahni, KM Shioura, J Torres, **JR Sysol**, BY Dong, A Yamaura, J Wan, Q Guo, S Camp, C Evanoski, Y Zhao, A Desai, L Moreno-Vinasco, J Yuan, JG Garcia, RF Machado. "Inhibition of pre-B cell colony-enhancing factor (PBEF) prevents and reverses monocrotaline-induced pulmonary hypertension." Central Society for Clinical Research Conference, Chicago, IL (Apr. 2012).

30. I Manoli, **JR Sysol**, L Li, C Garone, S Young, RJ Chandler, V Hoffmann, P Zerfas, S DiMauro, J Schnerman, CP Venditti. "A liver-specific transgenic mouse model identifies new disease-associated biomarkers and establishes antioxidants as an ameliorative treatment for the renal disease of methylmalonic acidemia (MMA)." American Society of Human Genetics, 61st Annual Meeting (12th International Congress of Human Genetics), Montreal, Canada (Oct 11-15, 2011).

31. JL Sloan, JJ Johnston, I Manoli, RJ Chandler, C Krause, N Carrillo-Carrasco, SD Chandrasekaran, **JR Sysol**, K O'Brien, NS Hauser, JC Sapp, HM Dorward, M Huizing, NISC, BA Barshop, S Berry, PM James, NL Champaigne, P de Lonlay, V Valayannopoulos, MD Geschwind, DK Gavrilov, WL Nyhan, LG Biesecker, CP Venditti. "Whole exome sequencing identifies ACSF3 as a putative human malonyl-CoA synthetase and the gene responsible for Combined Malonic and Methylmalonic Aciduria." American Society of Human Genetics, 61st Annual Meeting (12th International Congress of Human Genetics), Montreal, Canada (Oct 11-15, 2011).

32. JS Senac, **JR Sysol**, I Manoli, RJ Chandler, CP Venditti. "A mouse model to test liver-targeted therapies for MUT methylmalonic acidemia." American Society of Gene & Cell Therapy, 14th Annual Meeting, Seattle, WA (May 18-21, 2011). *Molecular Therapy*, Vol 19, Sup 1: S174 (2011).

33. I Manoli I, **JR Sysol**, L Li, RJ Chandler, J Senac, V Hoffmann, P Zerfas, J Schnermann, CP Venditti. "Muscle targeted transgene expression rescues the lethal phenotype of Mut knockout mice." Annual Meeting of the Society of Inherited Metabolic Disorders, Pacific Grove, CA (Feb 27 – Mar 2, 2011). *Mol. Genetics and Metabolism,* Vol 102 (3), (2011).

34. **JR Sysol**, I Manoli, L Li, J Senac, RJ Chandler, V Hoffmann, P Zerfas, J Schnermann, CP Venditti. "Metabolic sink therapy in methylmalonic acidemia using a novel muscle-specific transgenic mouse model." American Society of Human Genetics, 60th Annual Meeting, Washington, DC (Nov 2-6, 2010). Selected for oral presentation with Travel Award.

35. JS Senac, RJ Chandler, I Manoli I, **JR Sysol**, CP Venditti. "Long-term phenotypic correction of a lethal mouse model of methylmalonic acidemia using rAAV9-mediated gene therapy and metabolic improvement after re-boosting at one year." American Society of Human Genetics, 60th Annual Meeting, Washington, DC (Nov 2-6, 2010).

36. I Manoli, **JR Sysol**, RJ Chandler, J Sloan, K Cusmano-Ozog, P Zerfas, V Hoffmann, M Abu-Asab, M Tsokos, GM Enns, CP Venditti. "Insights into the pathophysiology of methylmalonic acidemia (MMA) from tissue-specific transgenic mouse models." Annual Meeting of the Society for the Study of Inborn Errors of Metabolism, Istanbul, Turkey (Sep 2, 2010). *J of Inherited Met. Disease*, Vol 33, Sup 1 (2010).

37. I Manoli, **JR Sysol**, RJ Chandler, J Sloan, P Zerfas, V Hoffmann, M Abu-Asab, M Tsokos, CP Venditti. "Cell autonomy in methylmalonic acidemia: Implications for gene and cell therapy." American Society of Gene & Cell Therapy, 13th Annual Meeting, Washington, DC (May 17-22, 2010). *Molecular Therapy*, Vol 18, Sup 1: 135 (2010).

38. JS Senac, RJ Chandler, I Manoli, **JR Sysol**, CP Venditti. "Rapid transgene expression and long-term phenotypic correction of a lethal mouse model of methylmalonic acidemia using rAAV9-mediated gene delivery." American Society of Gene & Cell Therapy, 13th Annual Meeting, Washington, DC (May 17-22, 2010). *Molecular Therapy*, Vol 18, Sup 1: 701 (2010).

39. I Manoli I, **JR Sysol**, RJ Chandler, J Sloan, K Cusmano-Ozog, P Zerfas, V Hoffmann, M Abu-Asab, M Tsokos, G Enns, CP Venditti. "The renal disease of methylmalonic academia: Insights into pathophysiology using mouse models and human studies." Annual Meeting of the Society of Inherited Metabolic Disorders, Albuquerque, NM (Mar. 2010). *Mol Genetics and Metabolism,* Vol 99 (3), (2010).

40. **JR Sysol**, JH Werren. "Hybrid incompatibility between two species of *Nasonia* wasps: Is the mitochondrial ribosome involved?" National Conference on Undergraduate Research (NCUR), La Crosse, WI (Apr 16-18, 2009).

Invited Presentations

2016 UIC Medical Scientist Training Program, Graduate Research Dinner Seminar.

2014, 2015 UIC Department of Pharmacology, Graduate Student Research Seminar.

2014 UIC Department of Medicine, Pulmonary Research Conference. "Role of the SphK1/S1P Signaling Axis in the Pathogenesis of PAH." Dec 18, 2014.

2013 UIC Department of Medicine, Pulmonary Research Conference. "The Role of S1P Signaling in the Development of Pulmonary Hypertension." Dec 19, 2013.

EXTRAMURAL FUNDING

Current

2015 – 2018 **F30 National Research Service Award (NRSA); Project: HL128034**
Funding Agency: National Heart Lung and Blood Institute (NHLBI), NIH
Project Title: "Role of the SphK1/S1P Signaling Axis in the Pathogenesis of Pulmonary Arterial Hypertension" [PI: Justin R. Sysol]
Impact Score: 19 (Scale of 10-90, with 10-20 considered 'exceptional')
Award Amount: $192,480

Completed

2015 - 2016 **Pre-doctoral Fellowship, Midwest Affiliate; Project: 15PRE21900004**
Funding Agency: American Heart Association
Project Title: "The Roles of Sphingosine Kinase 1 (SphK1) and Sphingosine-1-phosphate (S1P) in Pulmonary Vascular Remodeling" [PI: Justin R. Sysol]
Priority Score: 1.27 (Scale of 1.0-5.0, with 1.00-1.40 considered 'excellent')
Award Amount: $52,000

INTRAMURAL FUNDING

Completed

2015 - 2017 **Chancellor's Graduate Research Award (Biomedical Deiss Fund)**
Funding Agency: UIC Graduate College; Award Amount: $8,000
Project Title: "Regulation of the Sphingosine Kinase 1 / Sphingosine-1-Phosphate Pathway in Pulmonary Arterial Hypertension"
Award Amount: $8,000

2015 - 2016 **CCTS Pre-doctoral Education for Clinical and Translational Scientists (PECTS) Program Award**
Funding Agency: UIC CCTS/College of Medicine
Award Amount: $9,500

2014 – 2015 **Graduate Student Position; T32 Institutional Pre-doctoral NRSA Training Grant (#5T32HL007829-20)**
Funding Agency: National Heart Lung and Blood Institute (NHLBI), NIH Training Program in Lung Biology and Pathobiology, University of Illinois at Chicago, Department of Pharmacology [PI: Asrar B. Malik, Ph.D.]

EDUCATIONAL ACTIVITIES

Teaching

2015 Research Mentor, UIC Urban Health Program/Center for Clinical and Translational Science (UHP/CCTS) Summer Research Fellowship Program.

2009 – present Laboratory training for undergraduate and summer research students (10 total)

2009 Teaching Assistant, Department. of Biology, University of Rochester

Activities

2016 – present Peer Reviewer; American Thoracic Society Early Career Group
American Journal of Respiratory and Critical Care Medicine Journal

2014 – present Gold Humanism Honor Society (GHHS) Selection Committee Member
University of Illinois College of Medicine

2011 – 2013 Bloodsucker's Phlebotomy Program Volunteer
University of Illinois College of Medicine; Community Health Free Clinic

2010 – 2011 D.C. Autism Buddies Program Volunteer

2009 – 2011 Clinical Rounds of the Undiagnosed Diseases Program (UDP) Volunteer
National Human Genome Research Institute (NHGRI), NIH

2009 – 2011 ICU Rounds Shadowing Program, NIH Clinical Center

Society Memberships

2015 – present American Society for Cell Biology (ASCB)

2014 – present American Thoracic Society (ATS)

2012 – present American Physician Scientists Association (APSA)

2015 – present American Medical Student Association (AMSA)

2011 – 2014 American Medical Association (AMA)

2010 – 2012 American Society of Human Genetics (ASHG)

2009 – 2011 American Association for the Advancement of Science (AAAS)

2007 – 2009 Society of Undergraduate Biology Students, University of Rochester

2009 Charles Drew Pre-Health Society, University of Rochester

2008 Undergraduate Student Geological Organization, University of Rochester

HONORS AND AWARDS

2018 *Ervin G. Erdös, MD and Sara F. Rabito Erdös, MD Prize for Excellence in Basic Sciences*, Commencement Award, UIC College of Medicine. $1000 award.

2017 *Dr. Erminio Costa Scholarship Award*, UIC College of Medicine, granted annually to a medical student in the MSTP who intends to pursue an academic career as a physician-scientist in teaching, research, and patient care. $950 award.

2017 *Surgical Scholars in Technology Research Paper, 2nd place*, M3 Surgery Clinical Clerkship, UIC College of Medicine. Title: "Role of Ex Vivo Lung Perfusion in Lung Transplantation."

2016 *Dr. Edward P. Cohen Medical Scientist Training Award*, UIC MSTP, presented on behalf of the UIC MSTP alumni to the most outstanding M.D./Ph.D. student who completed their Ph.D. in 2016. $100 award.

2016 *Trainee Travel Award*, Central Society for Clinical and Translational Research (CSCTR) and the Midwestern Section of the American Federation for Medical Research (MWAFMR) Combined Annual Meeting (Chicago, IL). $300 award.

2015 *Multidisciplinary Team Science Award*, *Honorable Mention*, UIC Center for Clinical and Translational Science (CCTS), College of Medicine Research Forum.

2015 *Honorable Mention Certificate,* Graduate Student Category, UIC College of Medicine Research Forum poster presentation.

2015 *Albert and Doris Woeltjen Student Achievement Award*, UIC Department of Pharmacology, granted annually to the graduate student achieving the most in the area of research in the last academic year.

2015 *W.C. and May Preble Deiss Fund Award for Biomedical Graduate Research*, UIC Graduate College, to attend "The Genome Access Course"; Cold Spring Harbor Laboratory at the New York Genome Center, New York, NY (Sept 2-4, 2015). $1629 award.

2015 *Trainee Travel Award,* Graduate Student Council of the UIC Graduate College, to help defray the cost of travel to the American Thoracic Society International Conference for graduate research presentation. $275 award.

2015 *Junior Trainee Scholar's Program Award*, Midwestern Section of the American Federation for Medical Research (MWAFMR). $250 award.

2015 *Trainee Travel Award*, Central Society for Clinical and Translational Research (CSCTR) and the Midwestern Section of the American Federation for Medical Research (MWAFMR) Combined Annual Meeting (Chicago, IL). $300 award.

2014 *LEARN (Lippincott Editorial AMSA Review Network) Program Certificate of Achievement* for exemplary editorial reviews of education products and/or content, Wolters Kluwer Health, Lippincott Williams & Wilkins.

2013 *Honorable Mention Certificate,* Graduate Student Category, UIC College of Medicine Research Forum poster presentation.

2013 *Research Achievement Award for Scholarly Activities* University of Illinois Hospital & Health Sciences System, Department of Medicine Research Day. $100 award.

2013 *Trainee Travel Award*, UIC MSTP, to present research poster at the American Physician Scientist Association (APSA) Conference, Chicago, IL (April 26-28, 2013).

2013 *Team Member of the Week*, University of Illinois Hospital & Health Sciences System, Department of Medicine, June 14, 2013 Newsletter.

2012 *Trainee Travel Award*, UIC MSTP, to attend and present research poster at Translational Research Approaches to Reduce Health Disparities in Lung Disease Symposium, Chicago, IL (Sept 10-11, 2012).

2010 *Trainee Research Award,* American Society of Human Genetics (ASHG), Semifinalist in Pre-doctoral Translational Research. Oral presentation with $400 Travel Grant.

2009 *Trainee Travel Award*, University of Rochester, to give oral research presentation at the National Conference of Undergraduate Research, University of Wisconsin (La Cross

2009 *Distinction in Research in Biological Sciences* Diploma, University of Rochester.

2007 – 2009 *Dean's List*, University of Rochester (Requirement of grade-point average ≥ 3.4).