Pathogenic Roles of STIM2 and Orai2 in the Development of Pulmonary Arterial Hypertension

BY

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THESIS

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LIST OF ABBREVIATIONS

AVD	Apoptotic Volume Decrease
ATP	Adenosine Triphosphate
$[Ca^{2+}]_{cyt}$	Cytosolic Free Ca^{2+} Concentration
$[Ca^{2+}]_{SR}$	(Intracellular Stored) Ca^{2+} Concentration in the Sarcoplasmic Reticulum
BMP	Bone Morphogenetic Protein
BMPR2	Bone Morphogenetic Protein Receptor Type II
BSA	Bovine Serum Albumin
CaM	Calmodulin
CCE	Capacitive Ca^{2+} Entry
cDNA	Complementary Deoxyribonucleic Acid
COPD	Chronic Obstructive Pulmonary Disease
CREB	cAMP Response Element Binding Protein
CPA	Cyclopiazonic Acid
DAG	Diacylglycerol
DAPI	4’6-diamindino-2-phenylindole
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
E_m	Membrane Potential
FPAH	Familial Pulmonary Arterial Hypertension
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
GPCR	G Protein-Coupled Receptor
H&E	Hematoxylin and Eosin

LIST OF ABBREVIATIONS (continued)

HEK	Human Embryonic Kidney
Hg	Mercury
HPH	Hypoxia-Induced Pulmonary Hypertension
IPAH	Idiopathic Pulmonary Arterial Hypertension
IP_3	Inositol 1,4,5-Trisphosphate
IP_3R	Inositol Triphosphate (IP_3) Receptor
LV	Left Ventricle
MAPK	Mitogen-Activate Protein Kinase
MCT	Monocrotaline
MCT-PH	Monocrotaline (MCT)-Induced Pulmonary Hypertension
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain Phosphatase
mRNA	Messenger Ribonucleic Acid
NFAT	Nuclear Factor of Activated T-Cell
PA	Pulmonary Artery
PAEC	Pulmonary Arterial Endothelial Cell
PAH	Pulmonary Arterial Hypertension
PAP	Pulmonary Arterial Pressure
PASMC	Pulmonary Arterial Smooth Muscle Cell
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction

LIST OF ABBREVIATIONS (continued)

PDGF	Platelet-Derived Growth Factor
PH	Pulmonary Hypertension
PIP_2	Phosphatidylinositol 4,5 bisphosphate
PLC	Phospholipase C
PPH	Primary Pulmonary Hypertension
PTK	Protein Tyrosine Kinase
PVR	Pulmonary Vascular Resistance
RIPA	Radio Immunoprecipitation Assay
ROC	Receptor-Operated Ca^{2+} Channel
ROS	Reactive Oxygen Species
RV	Right Ventricular
RTK	Receptor Tyrosine Kinase
SDS-PAGE	Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis
SERCA	Sarco/Endoplasmic Reticulum Ca^{2+}-Mg^{2+} ATPase
SR	Sarcoplasmic Reticulum
SRF	Serum Response Element
SU5416	Sugen 5416
TBS	Trisbuffered Saline
TBS-T	TBS with 0.05% Tween 20
TGF-β	Transforming Growth Factor-β
TRPC	Canonical Transient Receptor Potential Channel
VDCC	Voltage-Dependent Ca^{2+} Channel
VEGF	Vascular Endothelial Cell Growth Factor

SUMMARY

Pulmonary arterial hypertension (PAH) is a fatal and progressive disease that predominantly affects young women; however, the pathogenic mechanisms of the disease remain unclear. Sustained pulmonary vasoconstriction and excessive pulmonary vascular remodeling are two major causes for the elevated pulmonary vascular resistance and pulmonary arterial pressure in patients with idiopathic PAH and pulmonary hypertension associated with other cardiopulmonary diseases. Under physiological conditions, most (if not all) of smooth muscle cells in the pulmonary vasculature are in contractile phenotype; a transition from the contractile phenotype to proliferative phenotype of pulmonary vascular smooth muscle cells is potentially a critical pathogenic mechanism for the development and progression of pulmonary vascular remodeling in patients with PAH and associated PAH.

Increased cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in pulmonary arterial smooth muscle cells (PASMC) is a major factor for pulmonary vasoconstriction and an significant catalyst for PASMC proliferation leading to pulmonary arterial wall thickening, pre-capillary and capillary muscularization (important pathological changes observed in patients with PAH). Increased $[Ca^{2+}]_{cyt}$ In PASMC, is caused by Ca^{2+} influx through Ca^{2+}-permeable channels on the plasma membrane and Ca^{2+} mobilization, or release of Ca^{2+} from the intracellular store, the sarcoplasmic reticulum (SR). When PASMC is exposed to a vasoconstrictive agonist (e.g., endothelin-1, ET-1) or a mitogenic factor, binding of the ligand to the receptor in the plasma membrane leads to increases in synthesis of the second messengers, inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 then activates IP_3 receptors (IP_3R), a family of Ca^{2+} release

channels in the ER/SR membrane, and causes Ca^{2+} mobilization or release from the ER/SR to the cytosol raising $[Ca^{2+}]_{cyt}$. Depletion or significant reduction of Ca^{2+} levels in the ER/SR due to Ca^{2+} mobilization or release leads to Ca^{2+} influx through store-operated Ca^{2+} channels (SOC), commonly referred to as store-operated Ca^{2+} entry (SOCE). DAG can directly and indirectly open another family of Ca^{2+}-permeable channels, termed receptor-operated Ca^{2+} channels (ROC), and induce receptor-operated Ca^{2+} entry (ROCE) further contributing to raising $[Ca^{2+}]_{cyt}$.

It has recently been demonstrated that STIM proteins (STIM1 and STIM2) function as Ca^{2+} sensor in the ER/SR. When Ca^{2+} in the ER/SR is depleted or significantly reduced, STIM1 and STIM2 are activated and translocate to the ER/SR-plasma membrane junction, recruit Orai proteins in the plasma membrane, facilitate to form Ca^{2+}-permeable channels, and induce SOCE. In addition, it has been reported that some of the TRP channels identified in vascular smooth muscle and endothelial cells, e.g., TRPC1, TRPC3 and TRPC6, participate in the formation of SOC in the plasma membrane. Indeed, our lab has previously showed that genetic deletion of TRPC6 significantly inhibited SOCE in lung vascular endothelial and smooth muscle cells, indicating that TRPC channels, along with Orai channels, contribute to forming SOC and play an important role in SOCE and regulation of $[Ca^{2+}]_{cyt}$.

In the present study, we tested the hypothesis that upregulation of STIM2 contributes to the phenotypic transition of pulmonary arterial smooth muscle cells (PASMC) from the contractile phenotype to the proliferative phenotype, as observed in patients with severe pulmonary hypertension, such as those with idiopathic pulmonary

arterial hypertension (IPAH). We demonstrated that STIM2 upregulation may play an important role in the early transition of the disease (i.e., IPAH) pathogenesis from the sustained vasoconstrictive stage to highly proliferative stage due to the switch of PASMC from the contractile phenotype to the highly proliferative phenotype. The phenotypic transition of PASMC may be involved not only in the initiation and development, but also in the progression, of pulmonary vascular remodeling or pulmonary arterial wall thickening, the major cause for the elevated pulmonary vascular resistance (PVR) in patients with IPAH.

We determined that the proliferative phenotype of PASMC utilizes store-operated Ca^{2+} channels (SOC) for increased store-operated Ca^{2+} entry (SOCE), causing increased cytosolic Ca^{2+} concentration $[Ca^{2+}]_{cyt}$. It has been previously demonstrated that STIM2 expression levels, and not STIM1, are increased, and also Orai2 and TRPC6 expressions are increased in PASMC isolated from IPAH patients, providing an underlying mechanism for enhanced SOCE.

PASMC can undergo a phenotypic switch in response to vascular injury, a process that contributes to vascular wall remodeling in late stage of IPAH. Under pathological conditions or vascular injury, PASMC undergo morphological and functional alterations (or reprogramming) that enable the cells to phenotypically change to a highly proliferative state for “wound healing” or repair. Different protein markers are often used to characterize smooth muscle cell phenotype. PASMC switch or transition from a contractile phenotype to a proliferative phenotype is characterized by a decrease

in markers like smooth muscle 22α (SM22α), calponin, and myosin heavy chain (MYH); these proteins are involved in triggering and regulating smooth muscle cell contraction.

Furthermore, our lab previously demonstrated that SOCE was significantly enhanced in PASMC from IPAH patients compared to PASMC from normal subjects and patients without pulmonary hypertension. Enhanced SOCE plays an important role in the pathophysiological changes in PASMC associated with increased proliferation and migration in IPAH. Augmented Ca^{2+} entry through SOC or enhanced SOCE, due potentially to upregulation of Orai/TRPC channels and STIM proteins, is an important pathway to maintain a sufficiently high $[Ca^{2+}]_{cyt}$ in PASMC to consistently stimulate cell proliferation and migration. Many investigators have focused on the functional role of STIM1 and Orai1 in regulating SOCE; however, whether STIM2 (and Orai2) upregulation and enhanced SOCE contributes to the phenotypic switch leading to proliferation in PASMC has not been elucidated, and may play an important role in PASMC phenotypic transition from a contractile to a proliferative phenotype. This study aims at revealing *a*) whether upregulation of STIM2 or STIM2-mediated activation of SOCE in PASMC is involved in dedifferentiation (or phenotypic transition) of PASMC from a contractile phenotype to a proliferative phenotype; and *b*) whether upregulation of STIM2, Orai2 and TRPC6 in PASMC is involved in enhanced SOCE and, ultimately, the development and progression of pulmonary hypertension. Our experimental data demonstrated that:

i) Protein expression levels of STIM2, Orai2 and TRPC6 were all upregulated in proliferative PASMC compared to contractile PASMC.

ii) The upregulated STIM2, Orai2 and TRPC6 were associated with a significant enhancement of SOCE in proliferative PASMC compared to contractile PASMC.

iii) The transition of contractile phenotype of PASMC to proliferative phenotype was characterized by a significant decrease in protein expression of contractile markers, such as MHC, SM22α and calponin, and a significant increase in protein expression of cell proliferation markers, such as proliferation cell nuclear antigen (PCNA).

These data indicate an important role of upregulated STIM2, Orai2 and TRPC6 in the phenotypic transition of PASMC from a contractile state to a proliferative state, and in proliferation of PASMC.

iv) Induction of PASMC differentiation from a highly proliferative phenotype to a less-proliferative state by serum-starvation, TGF-β or heparin treatment resulted in a significant decrease in protein expression levels of STIM2, Orai2 and TRPC6, which was associated with a decrease in proliferation markers (PCNA) and an increase in contractile phenotype markers (MHC, SM22α and calponin).

v) Knockdown of Orai2 with siRNA significantly inhibited SOCE induced by passive deletion of intracellularly stored Ca^{2+} in the SR using cyclopiazonic acid (CPA, a blocker of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-Mg^{2+} ATPase) in proliferative PASMC.

vi) Overexpression of STIM2 in proliferative PASMC significantly enhanced the increase in $[Ca^{2+}]_{cyt}$ due to SOCE, suggesting that STIM2 is sufficient to enhance SOCE in PASMC.

These data indicate that STIM2 and Orai2 all contribute to form functional SOC in proliferative PASMC; upregulated STIM2 is necessary and sufficient for the enhanced SOCE required for the growth factor- and serum-mediated PASMC proliferation. In this study increased STIM2 expression levels, along with increased Orai2 and TRPC6, may be responsible for maintaining a proliferative phenotype by sustaining an increase in $[Ca^{2+}]_{cyt}$ and thus promoting proliferation of PASMC.

In the future, we plan to determine whether Orai2 and/or TRPC6 channels are upregulated in PASMC from animals with hypoxia-induced pulmonary hypertension (HPH) and monocrotaline-induced pulmonary hypertension (MCT-PH). Both HPH and MCT-PH are well-established models of PH. We would also like to determine *a*) whether these channels are responsible for enhanced SOCE in PASMC from these animal models; *b*) whether pharmacological blockade of STIM2 and/or Orai2/TRPC6 attenuate the development and progression of HPH and MCT-PH; and *c*) whether conditional and inducible knockout of STIM2, Orai2 or TRPC6 (in smooth muscle cells or endothelial cells) can prevent the development of HPH in mice. These studies will provide evidence showing inhibition of SOCE through blockade of STIM2 (or Orai2/TRPC6) is potentially a novel therapeutic target in preventing the development and progression of PH.

I. INTRODUCTION

*Portions of the text and/or figures were reprinted with permission from: Fernandez RA et al. Pathogenic role of store-operated and receptor-operated Ca^{2+} channels in pulmonary arterial hypertension. *J Signal Transduct*. 2012:951497, 2012.

A. **Background**

Pulmonary arterial hypertension (PAH) is a progressive and fatal disease that predominantly affects women. The initial genetic or pathogenic cause for PAH is still unknown, however the elevated pulmonary arterial pressure (PAP) in patients with PAH is mainly caused by increased pulmonary vascular resistance (PVR) resulting from sustained pulmonary vasoconstriction; excessive concentric pulmonary vascular remodeling (due primarily to increased proliferation and decreased apoptosis of pulmonary arterial smooth muscle and endothelial cells and fibroblasts); *in situ* thrombosis; and increased pulmonary vascular wall stiffness (Morrell et al., 2009). An increase in PAP and PVR leads to an increase of the right ventricular afterload and, if untreated, right heart failure.

The treatment algorithm or therapeutic approaches for PAH has progressively increased over the years. Currently, calcium channel blockers (CCB) and other vasodilators (e.g., nitric oxide, adenosine and prostacyclin) are used to treat vasoreactive patients; however, only a small percentage of patients with PAH are acutely vasoreactive, and only a small portion respond positively to CCB and other vasodilators. Acute vasoreactivity testing is a test performed on patients with idiopathic

PAH (IPAH), to determine if they will respond favorably to long-term treatment of CCB. However, at advanced or late stages of PAH, most patients have a negative response to the acute vasoreactivity test. Therefore, treatment of PAH, especially for those who do not respond to acute vasodilators (or non-responders), should be focused on using drugs that have antiproliferative effects on highly proliferative vascular cells (e.g., fibroblasts, PASMC and PAEC). Considering that PAH is not only attributed to sustained pulmonary vasoconstriction, but also to excessive pulmonary vascular remodeling due primarily to enhanced vascular-wall cell proliferation, therapy targeting increased cell proliferation by inhibiting PASMC phenotypic transition from a contractile phenotype to a highly proliferative phenotype could be of great interest.

In general, vascular smooth muscle cells (SMC) including PASMC are extremely plastic and can dedifferentiate in response to various environmental stimuli from a contractile, quiescent or fully differentiated phenotype to a synthetic, dedifferentiated or highly proliferative phenotype (Owens et al., 2004; Rensen et al., 2007). A phenotypic switch or transition of PASMC is inevitable for any pathological and physiological vascular remodeling process to occur. Contractile PASMC represent the majority of PASMC in a healthy vessel; they are responsible for maintenance of pulmonary arterial tone and thus manage blood pressure and blood flow by controlling the blood vessel diameter. A contractile phenotype of PASMC has higher expression of SMC differentiation marker genes, for example, smooth muscle myosin heavy chain (MYH), smooth muscle 22-alpha (SM22α), and calponin (Chamley-Campbell et al., 1979). However, under pathological conditions or during vascular injury, PASMC undergo a switch or transition in phenotype, characterized by a significantly increased proliferation

rate, to enable the vessel to repair. The phenotypic transition is controlled and regulated by multiple mechanisms including an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) (Marchand et al., 2012).

Of note, an increase in $[Ca^{2+}]_{cyt}$ PASMC is not only a major trigger for pulmonary vasoconstriction, but also an important stimulus for causing and maintaining proliferation (Morrell et al., 2009; Reid, 1990). $[Ca^{2+}]_{cyt}$ is increased by: 1) Ca^{2+} influx through Ca^{2+}-permeable channels in the plasma membrane including voltage-dependent Ca^{2+} channels (VDCC) (e.g., $Ca_v1.2$), receptor-operated Ca^{2+} channels (ROC) (e.g., TRPC), and store-operated Ca^{2+} channels (SOC) (e.g., Orai and TRPC) (Putney, 1986; Putney, 1990); and 2) Ca^{2+} release or mobilization from the SR (intracellular stores) through inositol 1,4,5-trisphosphate receptors (IP_3R) (Ca^{2+} releasing channels).

A rise in cytosolic $[Ca^{2+}]$ rapidly causes an increase in nuclear $[Ca^{2+}]$ because of small resistance of the nuclear envelope to Ca^{2+}. Nuclear and cytosolic Ca^{2+} pools stimulate cell proliferation by activating Ca^{2+}-dependent kinases (e.g., Ca^{2+} calmodulin kinase (CaMK)) and other transcription factors (e.g., c-Fos, nuclear factor of activated T-cells (NFAT), cAMP response element binding protein (CREB)), which are necessary for causing and maintaining cell growth (Berridge, 1995; Graef et al., 2001; Sheng et al., 1990). Ca^{2+} can also impact gene expression by interacting with protein kinase C (PKC) and calmodulin (CaM), and activating proteins involved in the cell cycle (e.g., cyclins and cyclin-dependent kinases). In addition to stimulating quiescent cells (cells in the G_0 phase) to enter the cell cycle, the transition from Gap 0 (G_0 phase) to Gap 1 (G_1 phase), Ca^{2+}/CaM is also required for progression from the G_1 phase to the DNA synthesis

phase (S), and from Gap 2 (G_2 phase) through mitosis checkpoints, and the whole mitosis process (Berridge, 1995; Cahalan, 2009; Means, 1994; Short et al., 1993). In PASMC specifically, both an increase in $[Ca^{2+}]_{cyt}$ and intracellularlly stored Ca^{2+} are essential for cell proliferation (Golovina et al., 2001). In the presence of serum and growth factors, removal of extracellular Ca^{2+} or depletion of intracellularlly stored Ca^{2+} significantly inhibits proliferation of PASMC, further establishing the role of Ca^{2+} for cell cycle progression and cell growth (Golovina et al., 2001).

Stromal interaction molecule (STIM) proteins are newly identified intracellular regulatory proteins that sense the level of intracellular-stored $[Ca^{2+}]$ and regulates the level of cytosolic $[Ca^{2+}]$ by controlling Ca^{2+} influx through store-operated Ca^{2+} channels in the plasma membrane. Therefore, the role of STIM proteins in PASMC phenotypic switches from a contractile phenotype to a proliferative phenotype is of high interest.

STIM proteins are critical Ca^{2+}-signal coordinators responsible for sensing small changes in SR Ca^{2+} concentration ($[Ca^{2+}]_{SR}$) and activation of STIM results in increased $[Ca^{2+}]_{cyt}$ and plays an important role in refilling Ca^{2+} into the SR. STIM dimers translocate to the plasma membrane-SR junction where they form clusters, activate the highly Ca^{2+}-selective Orai (and certain TRPC) channels, and mediate store-operated Ca^{2+} entry (SOCE). SOCE is important for maintenance of sustained increase in $[Ca^{2+}]_{cyt}$ and long-term cytosolic Ca^{2+} signaling; SOCE is also important to replenish (or refill) Ca^{2+} into the SR stores after depletion (Cahalan, 2009; Roos et al., 2005). Influx of Ca^{2+} provides precise local Ca^{2+} pools crucial for regulating long-term cellular responses triggered by Ca^{2+}/CaM and Ca^{2+}-sensitive signaling pathways, including PASMC proliferation (Soboloff et al., 2012).

STIM1 and STIM2 proteins have similar domain architecture, however STIM2 plays a more significant role in maintaining Ca^{2+} levels in the SR under basal conditions than STIM1, by activating Ca^{2+} influx in response to smaller decreases in $[Ca^{2+}]_{SR}$ (Brandman et al., 2007). A depletion of Ca^{2+} from the SR is required to activate STIM1 (Luik et al., 2008); thus it is accepted that STIM2 is necessary to tightly regulate $[Ca^{2+}]_{SR}$ before STIM1 is activated. A small decrease in $[Ca^{2+}]_{SR}$ (prior to depletion of $[Ca^{2+}]_{SR}$) would first activate STIM2, induce SOCE and maintain a high level of cytosolic $[Ca^{2+}]$, and start refilling Ca^{2+} into the partially depleted SR. A significant decrease (or depletion) in $[Ca^{2+}]_{SR}$, however, would activate STIM1, induce a large scale of SOCE, and contribute to a large increase in $[Ca^{2+}]_{cyt}$, and further activation of transcription factors, leading to SMC proliferation and growth (Brandman et al., 2007; Mancarella et al., 2013; Parekh, 2011; Soboloff et al., 2012). Enhanced SOCE in PASMC is evident in patients with idiopathic PAH and animals with experimental PH (Song et al., 2011; Yu et al., 2004). Even more interesting, STIM2, and not STIM1, was found to be upregulated in PASMC isolated from patients with idiopathic PAH (Song et al., 2011). We also found that Orai2 and TRPC6 channels were upregulated in PASMC from patients with idiopathic PAH (Song et al., 2011; Yu et al., 2004). Furthermore, TRPC6 not only plays a role in ROCE, but data has demonstrated its role in SOCE (Yu et al., 2003b). These preliminary and previous data from our laboratory direct us to study the potential pathogenic role of STIM proteins (especially STIM2) in *a*) the phenotypical transition of PASMC from a contractile phenotype to a proliferative phenotype, and *b*) the development and progression of pulmonary hypertension using cells and tissues from

normal subjects and patients with pulmonary hypertension and animal models of pulmonary vascular disease.

B. Statement of Hypothesis

In this study, we hypothesized that *i*) upregulation of STIM2 in PASMC plays an important role in the early transition of the disease (PAH) pathogenesis from the sustained vasoconstrictive stage to highly proliferative stage by facilitating the phenotypical transition of PASMC from a contractile phenotype to highly proliferative phenotype; *ii*) upregulated STIM2 expression in proliferative PASMC contributes to the enhanced SOCE required for excessive PASMC proliferation; and *iii*) upregulation of STIM2 expression is associated with upregulation of Orai2 and TRPC6 channels in PASMC from patients with idiopathic PAH and animals with experimental pulmonary hypertension and contributes to the enhanced SOCE and sustained increase in $[Ca^{2+}]_{cyt}$ and, ultimately, to pulmonary vascular remodeling characterized by a significant vascular wall medial hypertrophy.

C. Significance of the Study

Idiopathic and heritable PAH is still a fatal disease that predominantly affects women, and the pathogenic mechanisms of the disease remain unclear. This study links upregulation of STIM2, a newly identified intracellular regulatory protein that senses Ca^{2+} level in the SR and stimulates SOCE, to the transition or switch of PASMC from a contractile phenotype to a highly proliferative phenotype, which is potentially an early

step of the pathogenic process for excessive concentric pulmonary vascular-wall thickening. Upregulation of STIM2, in association with upregulation of Orai2 and TRPC6, enhances SOCE, increases $[Ca^{2+}]_{cyt}$ (and $[Ca^{2+}]_{SR}$), maintains a sustained high level of $[Ca^{2+}]_{cyt}$, stimulates PASMC proliferation (and migration) and, ultimately, contributes to causing pulmonary vascular remodeling (a major causing factor for the elevated pulmonary vascular resistance and pulmonary arterial pressure in patients with idiopathic and heritable PAH). This study also provides compelling evidence that enhanced Ca^{2+} signaling observed in PASMC from PAH patients is likely due to "abnormal" upregulation of multiple proteins or channels and channel regulators. Combination therapy targeting all these proteins or channels and channel regulators would prove to be more effective and efficient for attenuating (and/or reversing) the development and progression of PAH.

II. OVERVIEW OF THE LITERATURE

A. Pulmonary Circulation and Pulmonary Hypertension

The only organ in the body to receive the entire cardiac output (CO) at one time is the lungs. A normal pulmonary circulatory system maintains a high-flow, low-resistance, low-pressure system that allows for gas exchange. The pulmonary circulation transports the deoxygenated venous blood from the right ventricle to the pulmonary artery into the pulmonary capillaries where the deoxygenated blood takes up oxygen and unloads excess carbon dioxide. Pulmonary arterial hypertension (PAH) is a severe chronic disorder that affects the pulmonary circulatory system, increased right ventricular afterload and, if untreated, causes right heart failure.

PAH is a pulmonary vascular disease that may be idiopathic, heritable, or secondary to other diseases such as chronic obstructive pulmonary disease (COPD). Even though various diagnoses exist, PAH is often misdiagnosed for other related diseases, such as, congenital heart disease, emphysema, or pulmonary embolism. It is also typically determined from elimination of these other disorders. Pathobiology understanding of PH is becoming more known; conversely, there remain crucial elementary gaps in our understanding of the basic pathological changes in pulmonary arteries and veins in this disease. It is increasingly clear that although vasoconstriction plays a contributing role, PH is an obstructive lung disorder of the blood vessels. Disordered metabolism, inflammation, and alterations of growth factors lead to a proliferative or a state which is resistant to apoptosis.

There is a need to further understand how the pathobiology leads to a more severe PH in some patients while other patients only present with mild PH. Recent appreciation for the potential role of cell differentiation and phenotypic transition of PASMC suggests new therapeutic approaches, diagnostic methods, and specific biomarkers. Early pathogenesis of PH is attributed to an increase in vasoconstriction and patients are more responsive to calcium channel blockers (CCB). We are learning that severe PH patients are not responding to the traditional CCB treatments and a new method of studying PH needs to be considered. Hence, examining the role of proliferation mediators and progression of PH has provided an alternative direction in finding a cure for PH. Understanding the mechanism involved with pulmonary vascular remodeling will hopefully lead to novel therapeutics and diagnostics.

Pulmonary arterial pressure (PAP) varies over a lifetime. During early childhood through the age of about 50, maximum mean PAP (mPAP) is 10–20 mm Hg, whereas mean systemic arterial pressure is 70–105 mm Hg. Pulmonary arterial hypertension (PAH) is clinically defined as a resting mPAP greater than or equal to 25 mm Hg at rest, (Badesch et al., 2009). PAP is the product of CO and PVR; the equation is as follows PAP = CO X PVR. In this equation, PVR is the vascular resistance from the whole lung ($PVR_{arteries}$ + $PVR_{capillaries}$ + PVR_{veins}) (Mandegar et al., 2004). In healthy individuals the arteries, capillaries, and veins have a compensative elastic mechanism, which can result in an increase in the cross-sectional area of the pulmonary vascular bed when needed. Due to the pulmonary arteries' high elasticity, exercise causes only a marginal change in PAP, following increased CO. Conversely, patients with PH, at rest with no increased CO, have increased levels of PAP due to an increase in PVR from the

arteries, capillaries, and veins. PVR is related to the flow of liquid through a cylindrical structure using the Poiseuille equation, PVR = ($8L\eta/\pi$) x ($1/r^4$), (*L* represents the length of the artery, η represents the viscosity coefficient of blood, and *r* represents the inner radius). Consequently, any small decrease of the inner radius of the blood vessel results in a significant increase of pulmonary vascular resistance, and, therefore, PAP. Increased PVR is the major cause for the development of PH. An increased PVR results in increased RV afterload, which leads to right heart failure and death (Firth et al., 2010). Although the initial culprit in PAH involves the pulmonary vasculature, survival of patients with PAH is closely related to right ventricular dysfunction (Ghio et al., 2010; van Wolferen et al., 2007). The right ventricle adapts to the increased afterload by increasing its wall thickness and contractility. Right heart failure in patients with PAH can be defined as a complex clinical syndrome due to decreased delivery of blood and/or elevated systemic venous pressure at rest or exercise as a consequence of elevated RV afterload.

PH is a disease that is often misdiagnosed during routine medical examination. PH was previously classified into 2 categories: 1) primary pulmonary hypertension, or 2) secondary pulmonary hypertension according to the presence of identified causes or risk factors (Simonneau et al., 2013). At the second World Symposium on Pulmonary Hypertension (WSPH) held in Evian, France, in 1998, a clinical classification was established in order to customize different categories of PH that shared similar pathological findings, hemodynamic characteristics, and management. Five groups of disorders that cause PH were identified: pulmonary arterial hypertension (Group 1); pulmonary hypertension due to left heart disease (Group 2); pulmonary hypertension

due to chronic lung disease and/or hypoxia (Group 3); chronic thromboembolic pulmonary hypertension (Group 4); and pulmonary hypertension due to unclear multifactorial mechanisms (Group 5). During the following world meetings, changes were made reflecting the progress of further understanding of the disease. The current clinical classification of pulmonary hypertension is now well accepted and widely used in the field by pulmonary hypertension experts. The Guidelines Committee of the Societies of Cardiology and Pneumology adopted these clinical classifications. Furthermore, the U.S. Food and Drug Administration (FDA) and the European Agency currently use these classifications for Drug Evaluation for the labeling of new drugs approved for pulmonary hypertension.

During the fifth WSPH held in 2013 in Nice, France, a consensus was made to maintain the general outlook of previous clinical classifications. Some modifications and updates, especially for Group 1, were proposed according to new data published in recent years. It was also agreed upon to add some specific items related to pediatric PH in order to have a more comprehensive classification encompassing both adults and children (Table 1) (Galie and Simonneau, 2013).

A noninvasive initial screening is achieved by estimating PAP levels through transthoracic echocardiography, however, right heart catheterization is the standard and required screening tool for clinical diagnosis of PH (Simonneau et al., 2013). Right heart catheterization is the process of placing a long thin hollow tube that is then placed through a central venous catheter and is guided through the right chambers of the heart and into the pulmonary artery. This catheter measures pressures in the heart and large blood vessels and determines the function of the heart. Although PH is defined as a

mPAP of >25 mm Hg with a normal pulmonary capillary wedge pressure, it is well known that, on the basis of clinical and hemodynamic findings, PH can range from mild to severe, even in the presence of the same stimulus. This spectrum of severity raises several critical pathogenic and clinical questions in terms of the distinctive features that may differentiate the severity of the stages.

Screening done by the recent REHAP Registry in Spain showed a 3.4:1 incidence of PAH in women to men, with an average age of 45 ± 17 years (Escribano-Subias et al., 2012). The U.S. Registry to Evaluate Early and Long-Term PAH Disease Management (REVEAL) uses a protocol with an approach to analyzing survival from diagnosis, using both newly diagnosed and previously diagnosed patients. The French Registry is very similar in that they analyze the survival from time of diagnosis, by using data from both incident and prevalent patients, and is comparable to survival estimates that are restricted to incident patients (McGoon et al., 2013). These registries have provided important information on the epidemiology and phenotype of patients with PAH.

Extensive changes in the PAH phenotype have been observed over the past years. These changes include substantial variations in age, sex, and survival (McGoon et al., 2013). Although the mean age of patients with idiopathic PAH in the first registry created in 1981 (U.S. NIH Registry) was 36 ± 15 years (Rich et al., 1987), now elderly patients are more frequently diagnosed with PAH, thus a mean age at diagnosis between 50 ± 14 and 65 ± 15 years has emerged. Furthermore, the female PH prevalence is relatively variable among the registries and may not be evident in elderly patients (Hoeper et al., 2013). To determine whether these differences reflect a variation in the disease, one must examine all of the biases that are present within PH

registries before any conclusions suggesting changes in the PAH characterization and phenotype can be made that would alter the understanding of the disease. In the following section, we will discuss the pathology and pathophysiology of PAH.

TABLE 1

THE CURRENT PULMONARY HYPERTENSION CLASSIFICATIONS

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

BMPRT = bone morphogenic protein receptor type II; CAV1 = caveolin-1; ENG = endoglin; HIV = human immunodeficiency virus; PAH = pulmonary arterial hypertension. Reprinted with permission from Elsevier Ltd (Simonneau et al., 2013).

B. Pathology and Pathophysiology of Pulmonary Arterial Hypertension

There are 15 orders of branching in the pulmonary arteries between the main pulmonary artery and the capillaries in the human lung, according to the diameter-defined Strahler system (Huang et al., 1996). The pulmonary artery is comprised of three layers, which include the inner intima, consisting of pulmonary arterial endothelial cells (PAEC), the media layer, consisting of pulmonary arterial smooth muscle cells (PASMC), and the outer adventitia layer, consisting of fibroblasts (Fig. 1). As the orders of branching increases, the diameter of the artery decreases.

The pathogenesis of PAH is attributed to the collective effects of vascular remodeling, persistent vasoconstriction, *in situ* thrombosis, and arterial wall stiffening; together these attributes increase PVR leading to right heart failure (Morrell et al., 2009; Yuan and Rubin, 2005). Vascular remodeling occurs throughout all branches resulting in a decreased radius and thus increased PAP (Fig. 2B). The adventitial layer of the vessel wall has been shown to undergo early structural changes following exposure to hypoxia. Proliferation of fibroblasts sustains and exceeds that of PAEC or PASMC in these models (Belknap et al., 1997). Endothelial cells, smooth muscle cells, and fibroblasts in the vascular wall play a specific role in response to injury.

The factors responsible for the aggravation or acceleration of PH remain poorly defined. The contributing factors likely involve accumulation of multiple genetic predispositions. Some of these factors involve vasoconstriction and increased remodeling, through the action of inflammatory, procoagulant and antiapoptotic autoimmune mediators, cell–cell and cell–matrix interactions, and environmental factors over a period of time (Archer et al., 2010). Although at advanced stages of PH, the

pulmonary artery seems to be unreactive to vasodilators, vasoreactivity and remodeling both lead to progression of the disease and need to be studied.

Early stages of PH are also characterized by increased vasoconstriction. Vasoconstriction is defined by increased tensile force, leading to a narrowed lumen of the blood vessel. Pulmonary vasoconstriction is triggered by a transient rise in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in PASMC, while sustained vasoconstriction is maintained by both activation of Ca^{2+}-dependent and Ca^{2+}-independent mechanisms. Increased $[Ca^{2+}]_{cyt}$ directly activates myosin light chain kinase (MLCK) leading to contraction and migration. Additionally, an increase in $[Ca^{2+}]_{cyt}$ stimulates Ca^{2+}-dependent signal transduction proteins. It is well understood that the amplitude of contraction could be affected by change in the ratio of MLCK: MLCP activity. MLCK activity is dependent on Ca^{2+}-calmodulin, hence, a rise in $[Ca^{2+}]_{cyt}$ is the primary determinant of smooth muscle contraction. The increase in sensitivity of smooth muscle contractility to $[Ca^{2+}]_{cyt}$ (i.e., Ca^{2+} sensitization) may be caused by inhibition of MLCP activity. It has been shown that one of the pathological causes in idiopathic pulmonary arterial hypertension (IPAH) in patients is a loss of vascular compliance and an increase in PVR due to pulmonary vascular remodeling and vasoconstriction (Rubin, 1997).

As the disease progresses, features of pulmonary vascular remodeling in PAH include medial cell layer thickening, which is attributed to PASMC abnormalities such as increased PASMC proliferation (Reid, 1990). Angiograms and histology images from PAH patients reveal both remodeling and thrombosis in the pulmonary arteries (Fig. 2). The angiogram in Figure 2 depicts narrowing or occlusion of the arteries. These images were obtained by injection of silicone into the main pulmonary artery, of *en bloc*

removed heart and lungs. In the angiograms of PAH patients, the silicon is unable to reach smaller arteries due to occlusion and are thus not depicted in this Figure 2A. This is not a loss of arteries per se, but rather a representation of the vascular remodeling, vasoconstriction, and *in situ* thrombosis resulting in occlusions of the pulmonary artery (Bedford et al., 1957). *In vivo*, a balance of apoptosis and proliferation of pulmonary vascular wall cells sustains a normal thickness and tissue mass of the pulmonary arterial walls. Disturbance of this balance in favor of proliferation results in abnormal pulmonary arterial wall thickening, intraluminal narrowing, and eventually leads to increased PVR and elevated PAP.

In situ thrombosis may partially or completely occlude the pulmonary artery contributing to increased PVR in patients with PAH. Endothelial cell dysfunction, as well as interaction with growth factors and platelets, causes obliterative pulmonary hypertension. This type of pulmonary hypertension is caused by a procoagulant environment within the pulmonary vascular bed (Mandegar et al., 2004). Additionally, an important contributor to PAH is increased pulmonary vascular wall stiffness due to increased extracellular matrix. Hypoxic animal models have increased adventitial thickening, excessive extracellular matrix proteins (type I collagen and cellular fibronectin) and myofibroblast accumulation/differentiation compared to their normoxic control (Stenmark et al., 2006).

In summary, "sustained pulmonary vasoconstriction, excessive pulmonary vascular remodeling, *in situ* thrombosis, and increased pulmonary vascular stiffness are the four major causes of elevated PVR and PAP, which lead to PAH" (Fernandez et al., 2012). The following sections review the important role of Ca^{2+} and specific Ca^{2+}

channels that are involved in the Ca^{2+} regulation in PASMC leading to the initiation and progression of PAH.

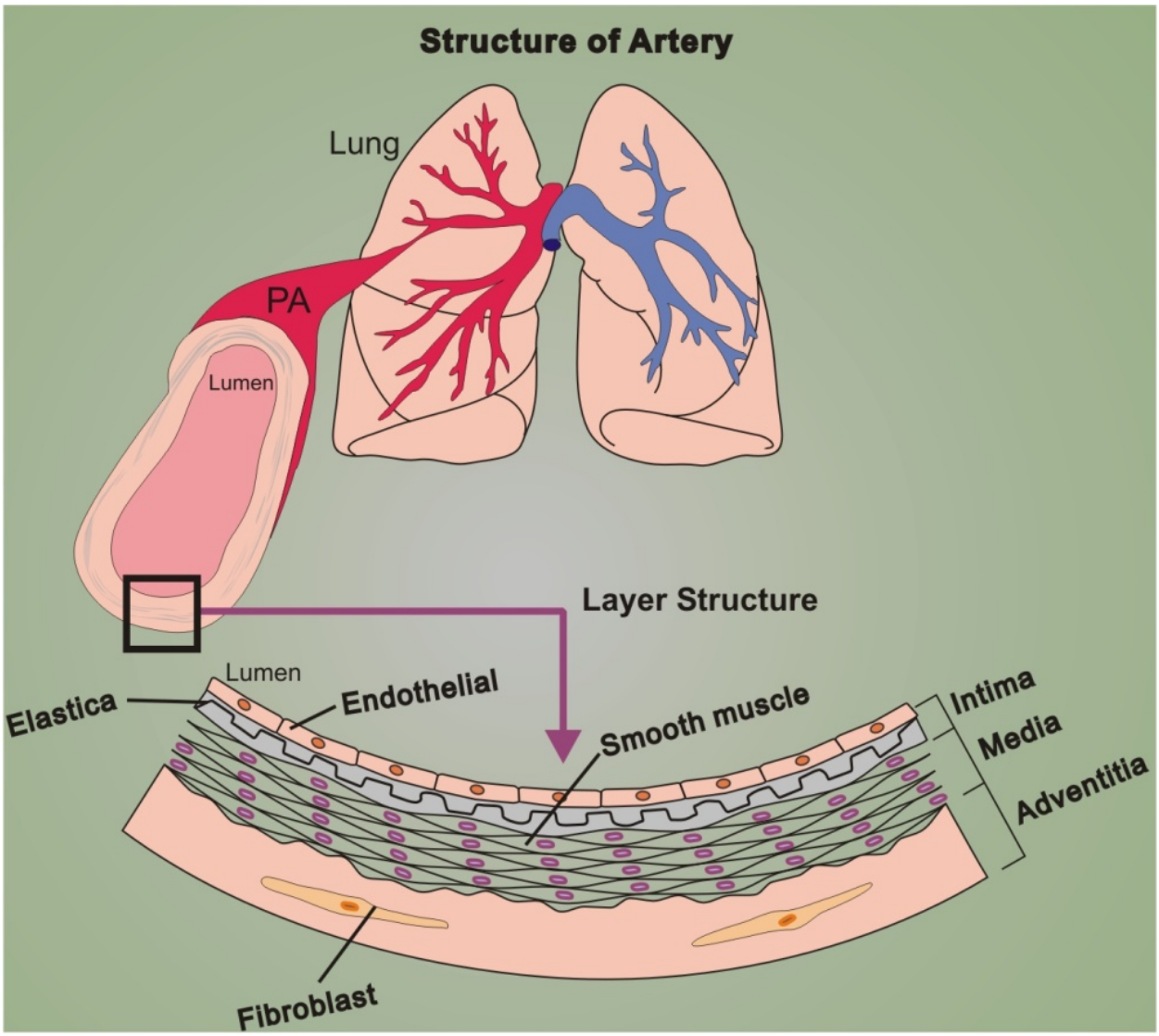

Figure 1: Structure of the pulmonary vasculature. Schematic demonstration of the lung and pulmonary artery, along with the zoomed in diagram of 3-layered structure is depicted above. Image was taken from and modified with permission from Fernandez RA, Sundivakkam P, Smith KA, Zeifman AS, Drennan AR, and Yuan JX (2012). Pathogenic role of store-operated and receptor-operated Ca^{2+} channels in pulmonary arterial hypertension. *J Sign Transduction*. 2012:951497.

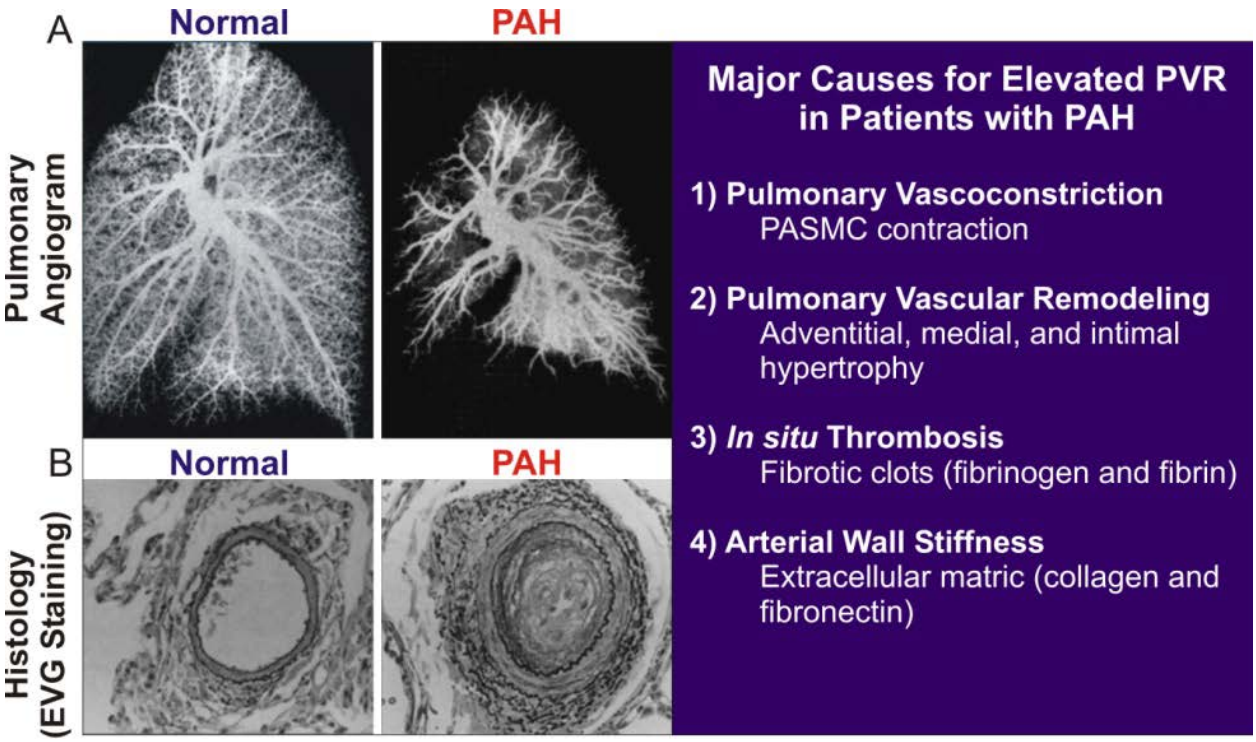

Figure 2: Major causes of elevated PVR in patients with PAH. (A) Pulmonary angiograms from a healthy (normal) patient compared to a PAH patient, depicting the occlusion of the pulmonary arteries. (B) Pulmonary EVG histology staining of cross-sections of a healthy patient compared to a PAH patient, depicting vascular remodeling and medial and intimal layer thickening. (C) Listed are the four major causes of elevated PVR in PAH patients. Image was taken from and modified with permission from Fernandez RA, Sundivakkam P, Smith KA, Zeifman AS, Drennan AR, and Yuan JX (2012). Pathogenic role of store-operated and receptor-operated Ca^{2+} channels in pulmonary arterial hypertension. *J Sign Transduction*. 2012:951497.

C. Major Genes Associated with Hereditary Pulmonary Arterial Hypertension

Over 300 independent bone morphogenic protein receptor type II (BMPR2) gene mutations, which are part of the transforming growth factor (TGF-β) superfamily, have been identified and account for approximately 75% of patients with a known history of familial pulmonary arterial hypertension (FPAH), and up to 25% of apparently sporadic or idiopathic PAH (IPAH). These mutations have not been well-established as a major genetic determinant essential for the development PAH, however, this information provides support for a prominent role of TGF-β superfamily members in the development of PAH.

Furthermore, Caveolin-1 (CAV1) has been identified as a novel gene responsible for hereditary PH (HPAH). CAV1 are membrane-scaffolding proteins, which form the main components of caveolae. Caveolae are found in the endothelium of the lung. Caveolae are consist of cell membrane receptors necessary for the initiation of a signaling cascade which include the TGF-β superfamily, nitric oxide pathway, and G-protein coupled receptors. Irregular signaling at the plasma membrane might be a mechanism for PAH pathogenesis.

Recently the potassium channel KCNK3 was found to have a heterozygous missense variant (Ma et al., 2013). KCNK3 encodes a pH-sensitive potassium channel in the 2-pore domain superfamily (Bournival et al., 2011). These studies reported that this potassium channel is sensitive to hypoxia and plays a role in the regulation of resting membrane potential and pulmonary vascular tone. Identification of this gene as

a cause of HPAH and IPAH and the possibility of rescuing specific mutations may provide a new vasoconstrictive therapeutic target for PAH.

Another approach for identifying genes affecting PAH is to perform association studies recognizing polymorphic markers, for example, single nucleotide polymorphisms (SNP) distributed throughout the whole genome. This approach requires a large number of patients and control subjects to compare the genotype frequencies in the two groups and look for a significant difference that can indicate association between the disease and the marker. With this approach, SNP have been identified that are associated with IPAH (Germain et al., 2013). An example in which studying SNP has provided more insight on the development of PAH, was a previous study conducted in our lab. We identified three C to G (C-->G) SNP in the 5'-regulatory region of the *TRPC6* gene. The allele and genotype frequency of, specifically, the -254 SNP was significantly higher in IPAH patients, and created a canonical nuclear factor-κB (NF-κB) binding site in the promoter region. Furthermore, we confirmed that nuclear translocation of NF-κB upregulates TRPC6 expression and enhances agonist-induced Ca^{2+} influx in IPAH patients. Lastly, inhibition of nuclear translocation of NF-κB attenuates TRPC6 expression and function in PASMC from IPAH patients who carried the -254G allele. The unique SNP found in the *TRPC6* gene links the inflammatory response to the upregulation of TRPC6, leading to increased cytosolic Ca^{2+}, which thus resulted in increased proliferation of PASMC, causing vascular remodeling of the medial layer.

Functional polymorphisms of TGF-β genes have also been studied to investigate a possible differences between BMP and TGF-β signaling pathways, which may explain the occurrence of mutations of BMPR2 mutations in PAH (Newman et al., 2008). They proposed that the TGF-β1 polymorphism modulates the age of diagnosis and the frequency at which the effects of the BMPR2 mutation are present. Of high interest was the expression difference that was observed in the CYP1B1 gene, nearly 10-fold lower in expression, in only female patients (West et al., 2008). CYP1B1 is involved in the syntheses of 2-OH estradiol metabolites, which have anti-proliferative effects on pulmonary vascular smooth muscle cells and attenuates pulmonary hypertension in experimental PH animal models (Tofovic et al., 2008).

Pathophysiological changes occurring during the development of PAH are extremely complex and involve many genetic and epigenetic mechanisms that lead to changes in gene expression, proliferation rate, and metabolic changes in cells. Until recently, approaches have been incomplete and have not allowed a well-rounded understanding of the disease and its development. Recent high-throughput techniques, including genomics, and proteomics, can be performed simultaneously for any given patient, in different cell types and can be repeated throughout the progression of the disease. Such an approach would be beneficial for fully understanding PAH.

We can expect that next generation sequencing in selected families will identify new important genes for explaining the various form of HPAH. Although the identification of novel PAH genes might not account for a large percentage of patients, recent findings would suggest that these data have potential to explain the pathogenesis of PH and provide novel therapy targets. Similarly, with the analysis of

variations in large, well characterized PAH groups, and with the extension of genome-wide association (GWAS) studies, we can demonstrate and provide important insights. Additionally, using these well-defined experimental animals of PH provides a great way to further study this disease.

D. Animal Models of Pulmonary Hypertension

Severe forms of PAH are characterized by various degrees of remodeling of the PA, which increases PVR and right ventricular afterload, thus contributing to the development of right ventricle dysfunction and failure (Gomez-Arroyo et al., 2012; Meyrick and Perkett, 1989; West and Hemnes, 2011). Elucidating the pathobiology of PAH continues to be important to designing new effective therapeutic strategies, thus appropriate animal models of PAH are necessary to achieve the task. Three widely accepted animal models include hypoxic pulmonary hypertension (HPH), monocrotaline (MCT) lung injury model, and the Sugen 5416 (SU5416)/hypoxia model. Currently, these models do not completely recapitulate human PAH, nonetheless, these models have been able to provide an understanding of the underlying pathological cellular and molecular changes that occur in pulmonary arteries.

1. Hypoxic Pulmonary Hypertension

There are various experimental models available to further understand the importance of remodeling and vasoconstriction in the development of PAH. Hypoxic pulmonary vasoconstriction is an important physiological mechanism that enhances ventilation-perfusion matching and pulmonary gas exchange by diverting blood flow

from poorly ventilated areas of the lung to well-ventilated areas in order to maximize oxygenation. In rats and mice, hypoxic exposure results in rapid structural remodeling in pulmonary arteries indicating an imbalance of apoptosis and proliferation of SMC. After chronic exposure to hypoxia right ventricular systolic pressure (RVSP), the Fulton Index, which is the ratio of right ventricle weight over the weight of the left ventricle plus the septum (RV/[LV+S]), and H&E histology images confirmed the presence of PH.

Furthermore, SMC proliferation was evident, indicated by increasing amounts of cells in mitosis (Meyrick and Reid, 1979). Rodents were placed in normabaric hypoxic chambers, which work by releasing nitrogen from a liquid nitrogen tank into a partially sealed chamber, thus reducing the partial pressure of oxygen (10% oxygen) while leaving the overall pressure unchanged. The rodents are exposed to chronic hypoxia for 2 to 4 weeks, with the majority of pathophysiological changes occurring within 3 to 4 weeks. These changes are much more pronounced in rats. Although experimental PH is better represented in rats than mice, in mice we are able to create gene-specific knockout models. Pathophysiological indicators of the rodents developing HPH include increased right ventricular mass in the heart, increased remodeling of the pulmonary vasculature, increased muscularization and thickening of the vessel walls (Meyrick and Perkett, 1989; West and Hemnes, 2011). Additionally, there are increased perivascular inflammatory cells, mediators, fibrosis, and stiffening of the larger pulmonary arteries (Burke et al., 2009). These changes are reversible, when rodents are removed from the hypoxic environment and returned to normoxic conditions. They will eventually return to a state similar to baseline; however, the increase in collagen is still present (Meyrick and Reid, 1980).

2. **Sugen/Hypoxia Model**

A recent modification of the standard hypoxic model was developed by the Tuder lab, which involved adding an angiogenesis inhibitor, Sugen5416 (SU5416, Sigma-Aldrich). SU5416 is a vascular endothelial growth factor (VEGF) receptor blocker that causes mild PH and mild pulmonary vascular remodeling in normoxic rats. VEGF is an important factor in the differentiation and maintenance of vascular endothelial cells. Injection with SU5416, along with hypoxic treatment, results in severe PH with increased vascular remodeling by inducing proliferation of endothelial cells and the development of complex lesions, resulting in precapillary arterial occlusions, similar to *in situ* thrombosis (Abe et al., 2010; Taraseviciene-Stewart et al., 2001; White et al., 2007). Furthermore, SU5416 treated animals exhibit increased SMC proliferation indicating that endothelial cell VEGF receptors can regulate PASMC phenotypic transition. Taken together, the SU5416 in combination with chronic hypoxia exposure, these animals possess the major characteristics of human PAH. This demonstrates that Su5416 plus hypoxia is a more appropriate preclinical model of occlusive PH than the conventional HPH and MCT-injected models.

3. **Monocrotaline Lung Injury Model**

A third experimental model utilized to study PH is monocrotaline (MCT) lung injury model. The MCT rat model is a frequently investigated model of PAH, since it is technically simple and easily reproducible at a low cost compared with hypoxia models of PH. MCT is an 11-membered macrocyclic, pyrrolizidine alkaloid derived from the seeds of the Crotalaria spectabilis plant. The liver converts the MCT alkaloid to its reactive pyrrole metabolite dehydromonocrotaline (MCTP), through a P-450 dependent

mechanism (Gomez-Arroyo et al., 2012). When MCT is ingested it induces a disorder that similarly mimics PH by producing a variety of inflammation-induced destruction of the pulmonary arteries through necrosis of the endothelial layer, along with right ventricular hypertrophy. The MCT-PH model is characterized predominantly by pulmonary arterial medial hypertrophy (Fig. 3). It remains unclear whether an initial MCT-induced damage of pulmonary endothelial cells stimulates SMC proliferation; or whether endothelial cell dysfunction is a result of non-PAEC damage or systemic inflammation triggered by MCT exposure (Kuang et al., 2010). Interestingly, despite having lower PAP and a similar degree of right ventricular dysfunction compared with the SU5416/hypoxia model of PH, MCT-treated rats exhibit a significantly higher mortality. A detailed molecular and cellular mechanism connecting the initial endothelial cell injury and subsequent medial hypertrophy still needs to be explained; however, for the time being, the MCT rat model is the one favored by most investigators.

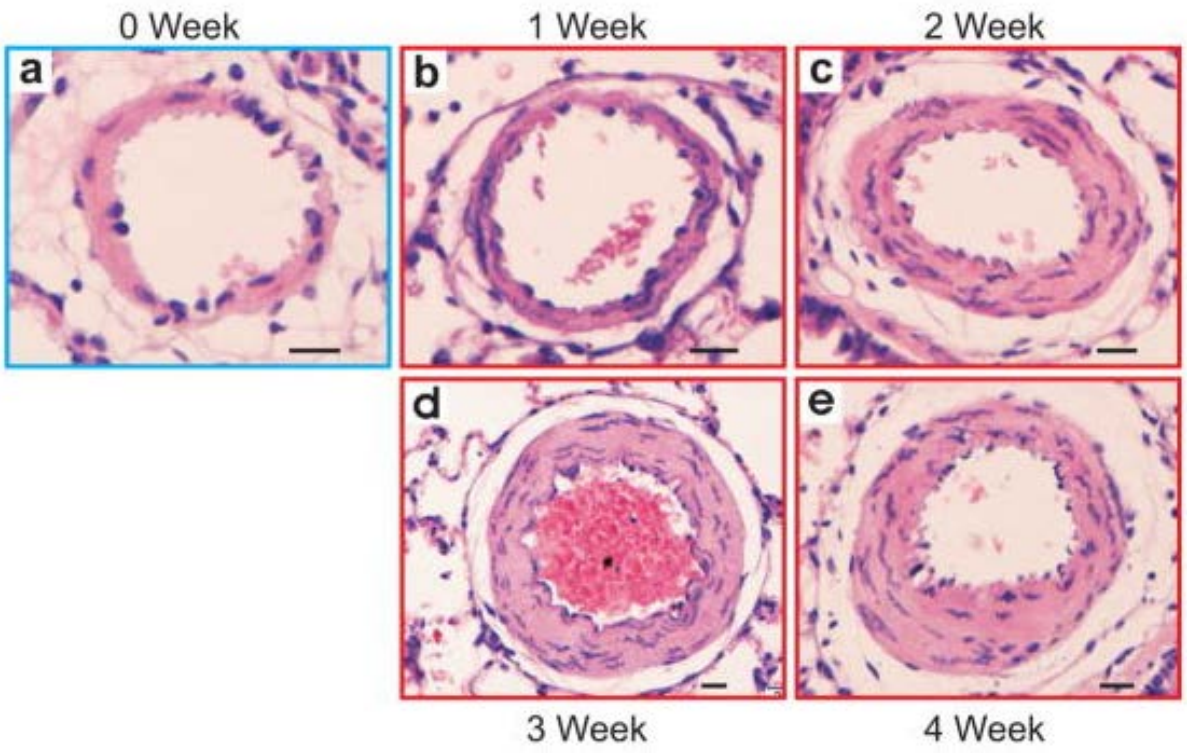

Figure 3: Representative hemotaxylin and eosin staining of pulmonary arterial cross sectional areas images were taken from rats sacrificed at the times indicated after MCT injection. Representative hematoxylin and eosin (H&E) staining of cross-sections of pulmonary arteries. Scale bar=10 μm. Image was taken from and modified with permission from Kuang T, Wang J, Pang B, Huang X, Burg ED, Yuan JX, and Wang C. Combination of sildenafil and simvastatin ameliorates monocrotaline-induced pulmonary hypertension in rats. *Pulm Pharmacol Ther*. 23(5):456-54, 2010.

E. Role of Ca^{2+} in Pulmonary Vasoconstriction and Pulmonary Arterial Smooth Muscle Cell Proliferation

1. Pulmonary Vasoconstriction

Intracellular Ca^{2+} signaling is critical for numerous physiological and pathophysiological processes in PASMC, specifically those associated with pulmonary vasoconstriction, and vascular cell proliferation (Berridge et al., 2003; Somlyo and Somlyo, 1994). The mechanism of contraction in SMC is different than that of striated cardiac or skeletal muscle cells. PASMC undergo a slow tonic contraction, unlike skeletal muscles. Smooth muscle cells contain a single nucleus and lack sarcomeres. Contraction of PASMC involves actin and myosin filaments, which are organized in irregular arrangements, unlike the distinct bands in cardiac and skeletal muscle cells. PASMC Contraction can be initiated either by electromechanical coupling resulting from changes in membrane potential (E_m); or by pharmacomechanical coupling resulting from receptor activation by drugs or hormones, independent of membrane potential change. Regardless of the initial mechanisms, an increase in $[Ca^{2+}]_{cyt}$ is the major trigger for SMC contraction and pulmonary vasoconstriction.

At homeostasis, $[Ca^{2+}]_{cyt}$ is maintained at about 100 nM, creating a gradient greater than 10,000 fold with the extracellular environment, which is at about 1.8 mM. Both Ca^{2+} influx through plasma membrane Ca^{2+} channels and Ca^{2+} release from intracellular stores (e.g., the sarcoplasmic reticulum (SR)) contribute to a rise in $[Ca^{2+}]_{cyt}$. When $[Ca^{2+}]_{cyt}$ rises, Ca^{2+} binds to calmodulin (CaM) and activates myosin light chain kinase (MLCK), which then phosphorylates the myosin light chain (MLC). Phosphorylation of MLC increases myosin ATPase activity, which hydrolyzes ATP to

release energy. The subsequent cycling of the myosin cross-bridges produces a sliding motion of myosin-actin filaments and results in contraction of the smooth muscles (Fig. 3) (Somlyo and Somlyo, 1994). The phosphorylated MLC can be de-phosphorylated by Ca^{2+}-independent myosin light chain phosphatase (MLCP), thus resulting in smooth muscle relaxation. Our previous data show that removal of extracellular Ca^{2+} blocks the high K^{+}-induced (i.e., electromechanical coupling) and phenylephrine (PE)-induced contraction (i.e., pharmacomechanical coupling) in isolated rat pulmonary arterial rings, illustrating that Ca^{2+} influx is necessary for smooth muscle contraction (Fig. 5) (McDaniel et al., 2001). The most common drugs currently used to treat PAH are Ca^{2+} channel blockers, which specifically target L-type voltage dependent Ca^{2+} channels. These channels are responsible for fast increase in Ca^{2+} and results in PASMC contraction.

2. **Pulmonary Arterial Smooth Muscle Cell Proliferation**

In addition to causing PASMC contraction, increased $[Ca^{2+}]_{cyt}$ is also important for cell proliferation and gene expression (Hardingham et al., 1997; Means, 1994). Activation of Ca^{2+}-sensitive signal transduction proteins (e.g., CaM kinase and mitogen-activated protein kinase (MAPK)) and transcription factors (e.g., NFAT, CREB, AP-1, and NF-κB) can stimulate cell proliferation (Fig. 3) (Berridge, 1995; Ginty, 1997; Graef et al., 2001; Sheng et al., 1990). Additionally, the nuclear envelope is highly permeable to Ca^{2+} and an increase in $[Ca^{2+}]_{cyt}$ can rapidly increase nuclear $[Ca^{2+}]$, resulting in activation of Ca^{2+}-sensitive pathways in the nucleus, which leads to activation of mechanism leading to gene transcription. Ca^{2+} influx mechanisms contributing to the maintenance of increased $[Ca^{2+}]_{cyt}$ play a central role during several phases of the cell

cycle. Growth factor-induced increase in $[Ca^{2+}]_{cyt}$ via Ca^{2+} release from the intracellular stores and Ca^{2+} entry from the extracellular space stimulates quiescent cells that are in the G_0 phase to enter the cell cycle (G_1) (Takuwa et al., 1995). In PASMC specifically, maintaining Ca^{2+} in the sarcoplasmic reticulum/endoplasmic reticulum (SR/ER) is vital for cell growth (Short et al., 1993). It has been shown that in the presence of serum and growth factors, the removal of extracellular Ca^{2+} and the depletion of ER-stored Ca^{2+} inhibit proliferation of PASMC, suggesting a crucial role for Ca^{2+} in cell cycle progression and growth (Fig. 4). Thus the maintenance of $[Ca^{2+}]_{cyt}$ is essential for cell proliferation.

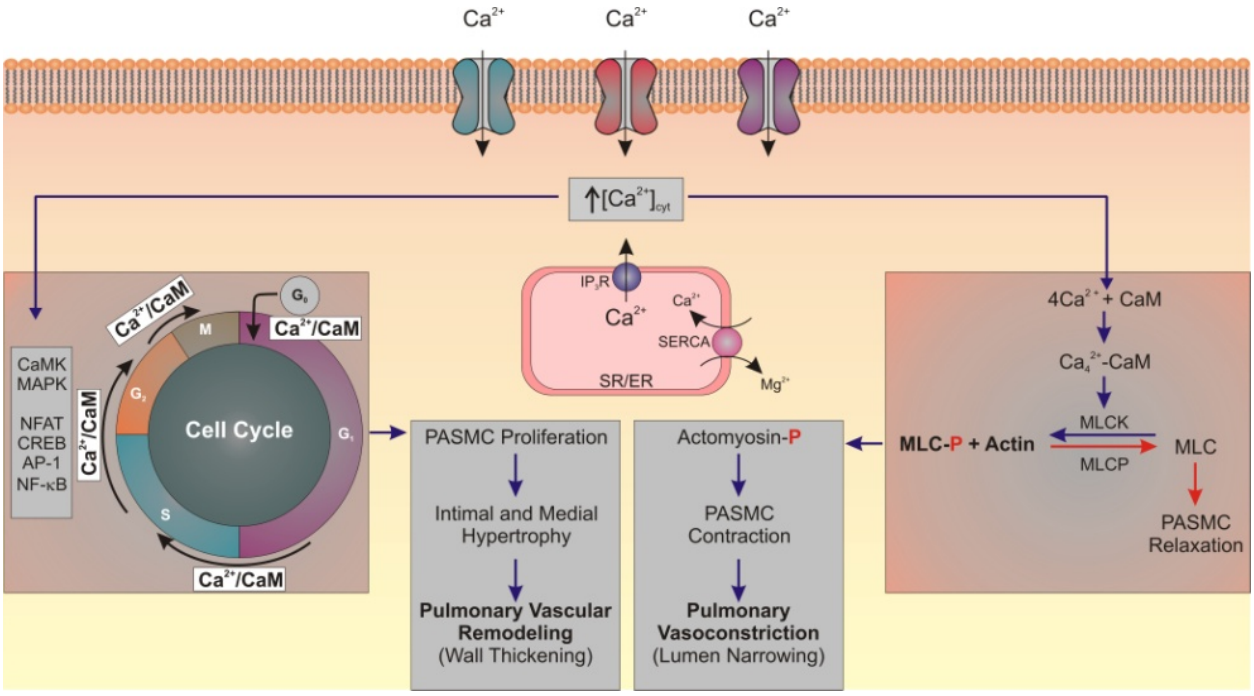

Figure 4: Increased levels of $[Ca^{2+}]_{cyt}$ in PASMC are required for pulmonary vascular remodeling and pulmonary vasoconstriction. When levels of $[Ca^{2+}]_{cyt}$ increase due to influx through various Ca^{2+} channels in the plasma membrane, or by depletion of SR/ER stores, Ca^{2+} can bind to calmodulin (CaM) leading to PASMC contraction by activating myosin light chain kinase (MLCK) causing phosphorylation of MLC, resulting in a sliding motion of the actomysoin complex leading to contraction. Additionally, Ca^{2+} activates intracellular Ca^{2+}-dependent signal transduction proteins such as CaM kinase (CaMK) and mitogen-activated protein kinase (MAPK), as well as activating other transcription factors (e.g. nuclear factor of activate T-cells [NFAT], cAMP response elements binding protein [CREB], activator protein-1 [AP-1], and nuclear factor [NF-κB) that trigger PASMC to enter the cell cycle from a quiescent differentiated state leading to proliferation. Image was taken from and modified with permission from Fernandez RA, Sundivakkam P, Smith KA, Zeifman AS, Drennan AR, and Yuan JX (2012). Pathogenic role of store-operated and receptor-operated Ca^{2+} channels in pulmonary arterial hypertension. *J Sign Transduction*. 2012:951497.

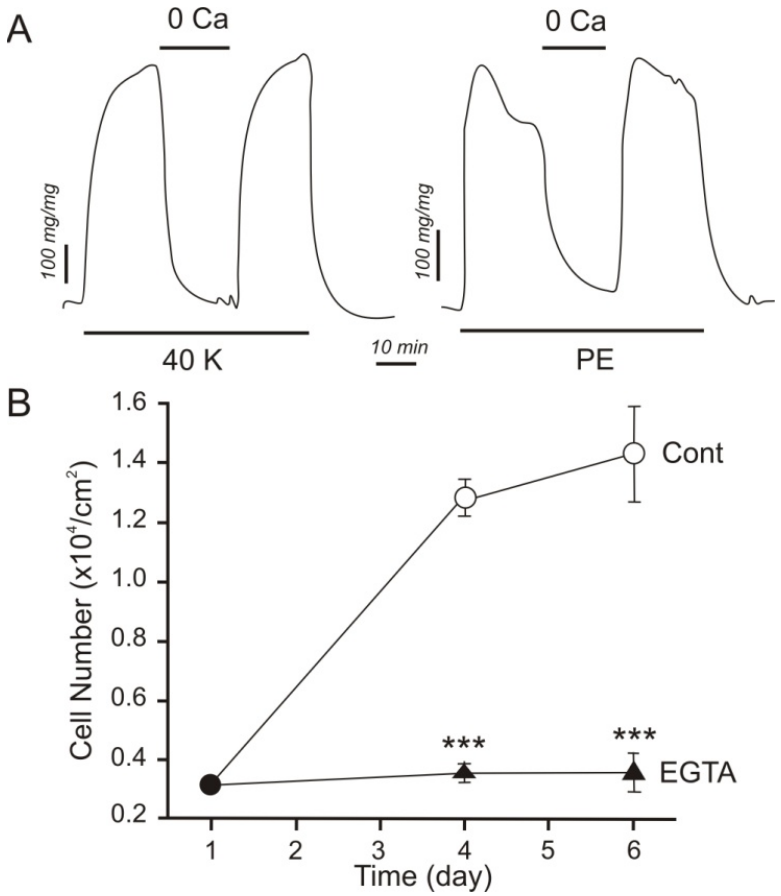

Figure 5: Ca^{2+} is required for PASMC contraction and PASMC proliferation. (A) Extracellular Ca^{2+} is required for pulmonary vasoconstriction induced by 40- mM (40 K) and phenylephrine (PE). (B) Inhibition of rat PASMC growth by chelating extracellular Ca^{2+}. Image was taken from and modified with permission from Fernandez RA, Sundivakkam P, Smith KA, Zeifman AS, Drennan AR, and Yuan JX (2012). Pathogenic role of store-operated and receptor-operated Ca^{2+} channels in pulmonary arterial hypertension. *J Sign Transduction*. 2012:951497.

E. Regulation of Cytosolic [Ca^{2+}] in Pulmonary Arterial Smooth Muscle Cells

Intracellular Ca^{2+}-dependent pathways play an important role in the regulation of numerous physiological and pathophysiological processes in PASMC such as contraction, proliferation, and migration (Remillard and Yuan, 2006). Studies indicate significant changes in the expression levels of Ca^{2+} channels result in alterations in Ca^{2+} homeostasis, due to membrane depolarization, increases in Ca^{2+} entry, elevation of resting $[Ca^{2+}]_{cyt}$, and contractile Ca^{2+} sensitivity, altogether contributing to vasoconstriction and vascular remodeling. Ca^{2+} influx in PASMC is mainly regulated by voltage-dependent Ca^{2+} channels and voltage-independent nonselective cation channels. PASMC have a low resting $[Ca^{2+}]_{cyt}$ (50-100 nM) that increases greatly upon agonist activation and/or membrane depolarization. Furthermore, low resting $[Ca^{2+}]_{cyt}$ is maintained by Ca^{2+} efflux pathways. Ca^{2+} is extruded into the extracellular space by plasma membrane Na^{+}/Ca^{2+}-exchangers and Ca^{2+} is resequestered into the SR/ER by the serca/endoplasmic Ca^{2+} pumps (SERCA).

1. Voltage-Dependent Ca^{2+} Channels in Pulmonary Arterial Smooth Muscle Cells

The voltage-dependent Ca^{2+} channels (VDCC), especially the dihydropyridine-sensitive L-type channels, have established roles in regulation of blood pressure; as a consequence, dihydropyridine Ca^{2+} channel blockers (nifedipine) have been clinically used for the treatment of PH. A functional channel is comprised of pore-forming subunits and regulatory subunits. VDCC are formed by one pore-forming α-subunit associated with several different regulatory subunits (e.g., β-, γ-, and δ-subunits). There are four repeats or units of the six-transmembrane domain structure in the α-subunit,

and each of the units contains a pore region. The Ca^{2+} influx mechanisms in PASMC via L-type VDCC are influenced by changes in the membrane potential, which result in an electrochemical driving force for Ca^{2+} entry. These channels are characterized by high-voltage activation, large single-channel conductance, long current duration, and slow voltage-dependent inactivation. PASMC maintain a resting E_M at about -40 to -60 mV, which is significantly less negative than the equilibrium potential for K^+ (E_K is about -85 mV), suggesting that resting E_M is regulated not only by K^+ currents but also background cation (e.g., Na^+ and Ca^{2+}) currents. Inhibition of K^+ channels causes membrane depolarization, whereas activation of K^+ channels causes membrane repolarization and hyperpolarization. When K_V channels close, the membrane depolarizes. Following depolarization, VDCC opens and permeates the cytosol with Ca^{2+}, leading to a rise in $[Ca^{2+}]_{cyt}$, thus causing PASMC contraction (Fig. 6).

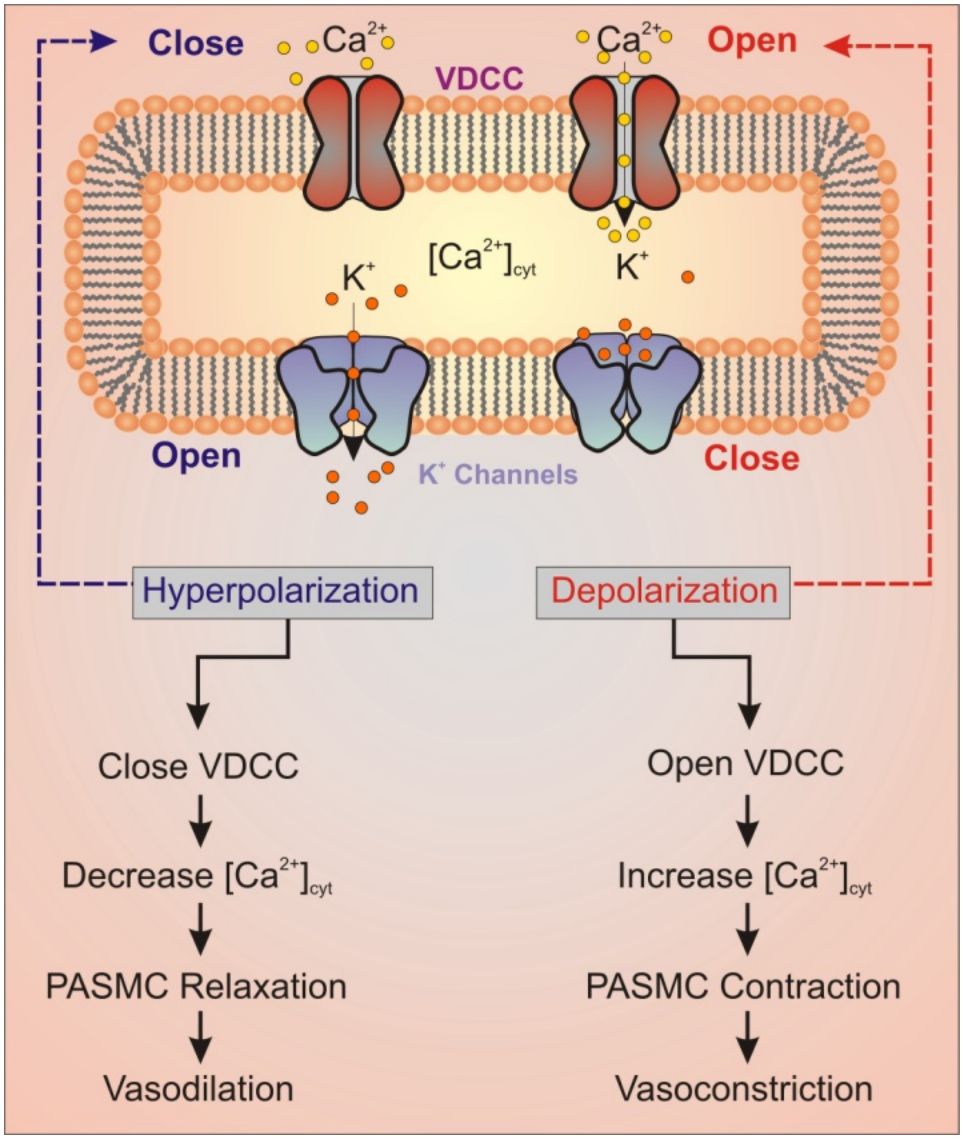

Figure 6: Role of Voltage Dependent Ca^{2+} Channels (VDCC) in membrane depolarization and vasoconstriction. Channels remain closed until stimulated to open. After stimulation, potassium channels open causing membrane hyperpolarization and leading to the closing of VDCC and contributing to decreased levels of $[Ca^{2+}]_{cyt}$, causing PASMC relaxation and thus vasodilation. However, when potassium channels close, this causes membrane depolarization, which opens VDCC, and contributes to increased levels of $[Ca^{2+}]_{cyt}$, leading to PASMC contraction, and causing vasoconstriction. Image was taken from and modified with permission from Fernandez RA, Sundivakkam P, Smith KA, Zeifman AS, Drennan AR, and Yuan JX (2012). Pathogenic role of store-operated and receptor-operated Ca^{2+} channels in pulmonary arterial hypertension. *J Sign Transduction*. 2012:951497.

2. Store-operated and Receptor-operated Ca^{2+} Entry Channels in Pulmonary Arterial Smooth Muscle Cells

Store-operated Ca^{2+} entry (SOCE), formally known as capacitive Ca^{2+} entry (CCE), is identified as one of the significant mechanisms that regulate $[Ca^{2+}]_{cyt}$. The group of channels that mediate SOCE are termed as store-operated Ca^{2+} channels (SOC) (Putney, 1986). SOC channels are activated by a select group of agonists via cell surface receptors such as G-protein-coupled receptors (GPCR) or receptor tyrosine kinases (RTK) (Fig. 6). Various studies have identified that stimulation of receptors, which induces hydrolysis of membrane phosphoinositides by phospholipase C (PLC), yields diffusible Ca^{2+}-mobilizing messenger inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 then binds to IP_3-receptor to cause extrusion of Ca^{2+} from the internal stores, mainly the endoplasmic reticulum (ER) or through sarcoplasmic reticulum (SR). Depletion of Ca^{2+} from the internal stores is followed by a stimulated Ca^{2+} entry through SOC channels, which is termed as SOCE. The ultimate reason for activation of SOC channels is to maintain long-term cytosolic Ca^{2+} signals and to replenish the depleted SR/ER stores (Cahalan, 2009; Roos et al., 2005). SOC channels are comprised of Orai (Orai1-3) proteins and transient receptor potential proteins (TRP). The characteristics and the components of these channels are discussed below in detail.

Receptor-operated channels (ROC) are activated via ligand-mediated activation of RTK and GPCR. ROC is loosely defined as voltage-independent Ca^{2+} channels that require binding of extracellular ligands to their membrane receptors for activation. Receptor-mediated activation followed by hydrolysis of PLC releases DAG along with IP_3 (Fig. 7). DAG opens a selective group of plasma membrane-localized Ca^{2+} channels

leading to Ca^{2+} influx and ultimately a rise in $[Ca^{2+}]_{cyt}$, a process referred to as receptor-operated Ca^{2+} entry (ROCE). Ca^{2+} influx mechanisms either via SOCE or ROCE play an important role in the regulation of vascular tone and arterial wall structure (Remillard and Yuan, 2006). Enhanced SOCE and ROCE in PASMC is evident in patients with PAH and animals with experimental PH (Wang et al., 2006). Studies have shown that inhibition of SOC expression levels or activity attenuates PASMC proliferation, indicating the essential role of SOC channels in vascular remodeling and lesions (Wang et al., 2009).

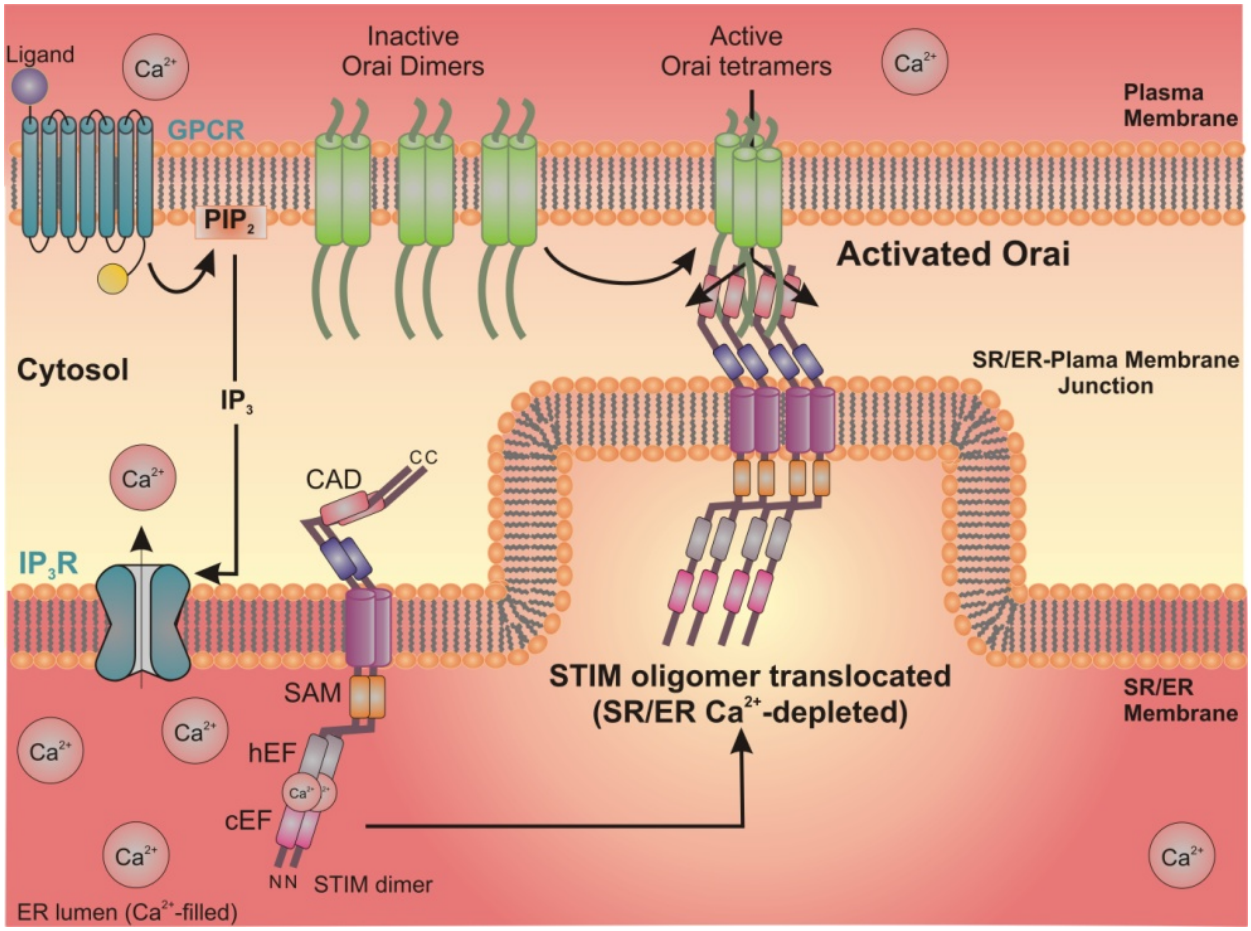

Figure 7: STIM activates store-operated Ca^{2+} channels (SOC) on the plasma membrane, resulting in influx into the cell (SOCE). When the SE is not depleted with Ca^{2+}, STIM is evenly dispersed along the SR/ER membrane and Ca^{2+} is bound to the low-affinity EF hand domain near the N-terminal. Following decreases in Ca^{2+} levels in the SR/ER store, Ca^{2+} will dissociate from STIM causing a conformational change that allows for the dimerization and formation of oligomers through interaction of their sterile-alpha-motifs (SAM). Oligomers then translocate to the SR/ER-plasma membrane junction where they induce the clustering of SOC channels (e.g., Orai) and stimulate opening of the channels by interaction of the STIM-Orai activating region (SOAR) near the C-terminus on STIM, resulting in an influx of $[Ca^{2+}]_{cyt}$. Image was taken from and modified with permission from Fernandez RA, Sundivakkam P, Smith KA, Zeifman AS, Drennan AR, and Yuan JX (2012). Pathogenic role of store-operated and receptor-operated Ca^{2+} channels in pulmonary arterial hypertension. *J Sign Transduction*. 2012:951497.

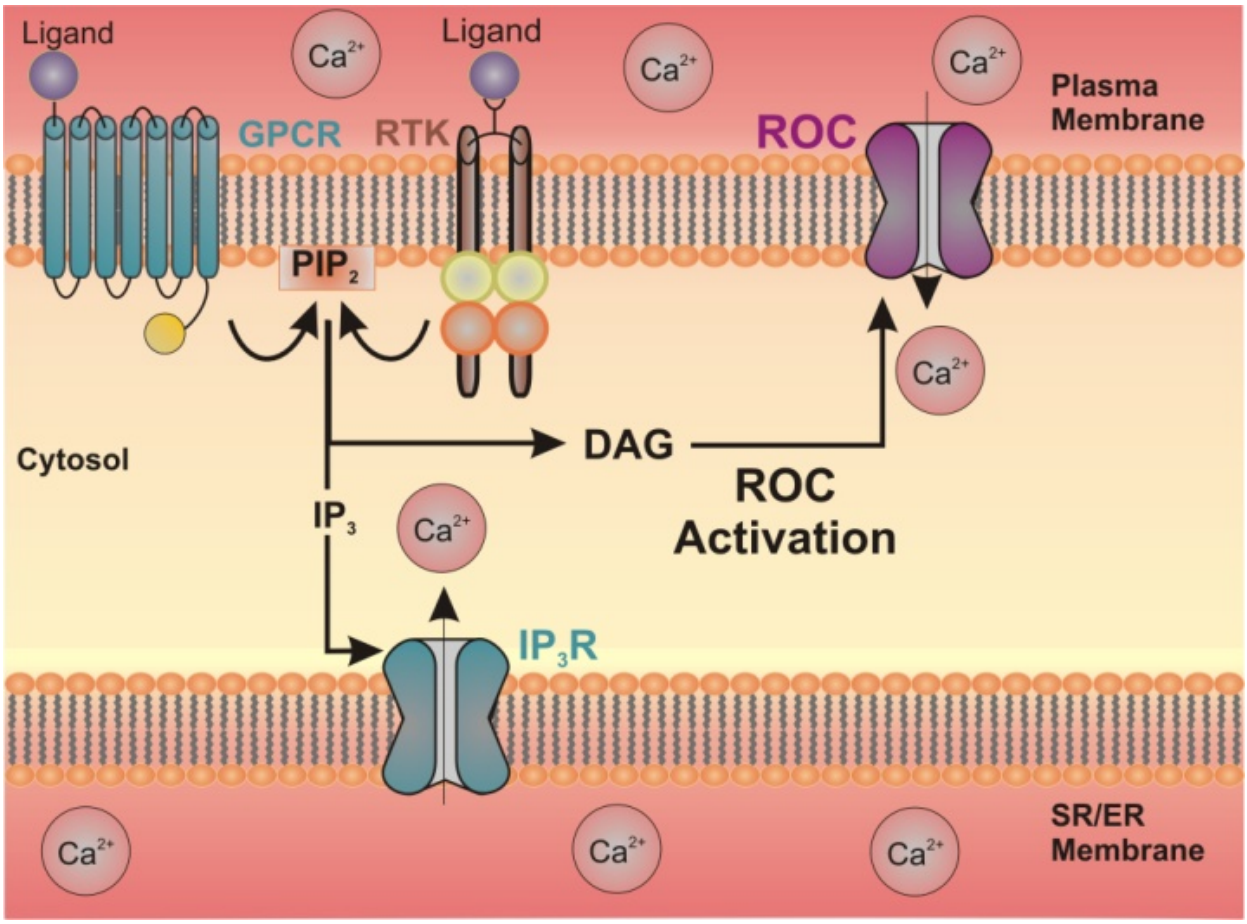

Figure 8: Diacylglycerol (DAG) activates receptor-operated Ca^{2+} channels (ROC) on the plasma membrane, resulting in Ca^{2+} influx into the cell (ROCE). Plasma membrane receptors, such as G-protein-coupled receptors (GPCR) and receptor tyrosine kinases (RTK) can be activated by various ligands, which then leads to activation of phospholipase C (PLC). PLC can then hydrolyze phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate and DAG. DAG remains in the plasma membrane and activates ROC channels resulting in Ca^{2+} influx. Image was taken from and modified with permission from Fernandez RA, Sundivakkam P, Smith KA, Zeifman AS, Drennan AR, and Yuan JX (2012). Pathogenic role of store-operated and receptor-operated Ca^{2+} channels in pulmonary arterial hypertension. *J Sign Transduction*. 2012:951497.

3. Stromal Interaction Molecule Proteins and Store-operated Ca^{2+} Entry

Following the identification of SOC channels, studies were then geared toward searching for the exact molecular mechanisms by which SR/ER Ca^{2+} store depletion is linked with SOC activation in the plasma membrane. These studies resulted in the identification of stromal interaction molecule (STIM) proteins as the fundamental molecular activator of SOCE. Two isoforms of STIM have been identified, STIM1 and its homologue, STIM2. Both STIM1 and STIM2 are expressed in VSMC and endothelial cells, however STIM1 is more highly expressed than STIM2 (Williams et al., 2001).

In recent years, significant progress has been achieved in addressing the role of STIM1 in regulating the plasma membrane (PM)-Ca^{2+} permeable channels, Orai, in various cell types, whereas the contribution of STIM2 in mediating SOCE remains controversial and varies in cell types. STIM senses the SR/ER Ca^{2+} concentration via its EF-hand domain at the N-terminal region (Zhang et al., 2005). The STIM proteins have signal peptides at the N-terminus that contain two cysteine residues which are 8 aa apart, the generate a single helix-loop-helix area that matches to the consensus motif of EF-hand calcium binding domains (Williams et al., 2001). Depletion of SR/ER Ca^{2+}, either actively by IP_3 or passively by inhibiting sarcoma/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), leads to an unfolding of the EF-hand resulting in a rapid oligomerization with similar domains of neighboring STIM molecules (Deng et al., 2009). Confirmed findings prove that a mutation within the EF-hand in STIM1 results in a constitutively active STIM1, which mediates a constant SOCE (Liou et al., 2005; Luik et al., 2008). Following an unfolding of the EF-hand, STIM migrates within the SR/ER membrane to regions that are proximal to the plasma membrane and reorganizes into

punctae that associate with SOC channels to gate or activate these channels (Liou et al., 2005). STIM interacts with PM components, specifically SOC channels through their cytoplasmic C-terminal, which mediates Ca^{2+} influx.

Lately, studies in neurons, skeletal muscle cells, and SMC demonstrated that STIM1 also has the ability to suppress VDCC (Cahalan, 2010; Park et al., 2010; Wang et al., 2010), and led to the term “store-inhibited channels (SIC)” (Moreno and Vaca, 2011). This interaction, in contrast to STIM conventional activation of Ca^{2+} through SOCE, results in inhibition of voltage-dependent Ca^{2+} entry (Fig. 8). As of now, the only channels that STIM negatively regulates are L-type VDCC (e.g., $Ca_V1.2$). The $Ca_V1.2$ L-type channel is involved in specific cellular functions and is an important target for therapeutic agents such as antihypertensive drugs. Using excitable cortical neurons and VSMC, Park et al. and Wang et al. assessed VDCC and SOC channel functions by monitoring $[Ca^{2+}]_{cyt}$. Furthermore, co-immunoprecipitation analysis confirmed the STIM1-$Ca_V1.2$ interaction and functional studies revealed that the SOAR domain was necessary and sufficient to suppress $Ca_V1.2$ current (Park et al., 2010; Wang et al., 2010).

"The second STIM family member, STIM2, is very similar to STIM1 in domain architecture but the mature protein is longer by 69 amino acids than STIM1", [12 aa] of which are located N-terminal to the EF hand domain, and the remainder C-terminal to the distal coiled coil" (Zheng et al., 2011). The EF hand is a Ca^{2+} sensitive helix-loop-helix structural domain found in a many Ca^{2+}-dependent families (e.g., calmodulin, muscle protein troponin-C and STIM). STIM2 is a significantly weaker activator of Orai channels than STIM1 (Bird et al., 2009), but is a more sensitive sensor of SR/ER

luminal Ca^{2+} (Brandman et al., 2007). This possibly results from the 12 aa difference in the EF hand domain. "Unlike STIM1, biotinylation and FAC analysis suggest that STIM2 does not traffic to the plasma membrane, even when overexpressed at a similar level as STIM1" (Dziadek and Johnstone, 2007). Although STIM2 is expressed in a wide variety of cell lines and tissues, conversely it is present at lower levels than STIM1 (Williams et al., 2001).

The protein expression pattern of STIM2 is often different than that of STIM1. As shown in Figure 9, studies from our laboratory demonstrated an important role for STIM2 in mediating SOCE in PASMC. Interestingly, an increase in the expression levels of STIM2, and not STIM1, in PASMC were observed in IPAH patients, which contributes to an augmentation of SOCE and enhancement of PASMC proliferation (Song et al., 2011). More investigation is needed to delineate the potential pathogenic role of STIM2 in the development and progression of pulmonary arterial hypertension, which will be further discussed in this thesis.

The two major Ca^{2+} channels regulated by STIM are SOC channels Orai (Cahalan, 2009) and TRPC (Golovina et al., 2001; Sweeney et al., 1994). Orai channels mediate a highly Ca^{2+}-selective, inward rectifying Ca^{2+} release-activated Ca^{2+} current (Penna et al., 2008), whereas TRPC channels mediate a non-selective, Ca^{2+} permeable current (Wang et al., 2008). Significant progress has been made in recent years in addressing the molecular composition of SOC channels and the fundamental mechanisms by which these channels are activated in VSMC.

The complex role of STIM2 is still under much investigation. Although STIM1 and STIM2 were discovered at the same time in the early dsRNAi studies conducted in

HeLA cells, which solidified its role as a positive regulator of SOCE (Liou et al., 2005), in other cell types a reduction in STIM2 expression has no effect on SOCE (Peel et al., 2006; Roos et al., 2005). Interestingly, in situations where a low ratio of STIM1:Orai1 exist, overexpression of STIM2 can reconstitute SOCE (Parvez et al., 2008). Nevertheless, both the amplitude of Ca^{2+} entry and the kinetics of Orai activation are greatly reduced compared to reintroduction of STIM1. It has been demonstrated that "STIM2 has a slightly lower affinity for calcium than STIM1" does, and "STIM2 EF-SAM domain monomers are more stable than the equivalent STIM1 monomers in the absence of calcium" (Zheng et al., 2008). The differences in the EF-hand would explain the differences in STIM1 and STIM2 oligermization, kinetic, and function differences. It has been observed that cells with increased expression of STIM2 also have increased basal resting $[Ca^{2+}]_{cyt}$. Furthermore, it is important to note that this increase can be blocked by inhibitors of the SOC channels. (Parvez et al., 2008). Both constitutive Ca^{2+} entry and SOCE mediated by STIM2 appear to occur through Orai channels since it is known that STIM2 interacts with Orai1, 2 and 3 (Parvez et al., 2008). Together, these studies suggest a model in which STIM2 has a role in basal Ca^{2+} regulation.

STIM1 has also been implicated in activating Ca^{2+}-permeable TRPC1–6 and in regulating their heteromultimerization directly via its C-terminus (Cahalan, 2009). STIM binds to TRPC channels and contributes to gating of TRPC1. Knockdown of STIM1 by siRNA and prevention of its translocation to the plasma membrane inhibits the activity of native TRPC1 (Worley et al., 2007). Furthermore, it was discovered that all TRPC channels, except TRPC7, function as SOC channels, some activated directly through STIM interactions (e.g., TRPC1, TRPC4 and TRPC5) and others indirectly (Yuan et al.,

2007). It is important to note that STIM1 heteromultimerizes TRPC channels to mediate their function as SOC channels. The specificity of this interaction has been of interest to many. The large range of STIM interaction with SOC channels can result in SOCE provides a greater mechanism of potentiation of downstream effects of Ca^{2+} signaling and also provides the cells with another mechanism to regulate $[Ca^{2+}]_{cyt}$.

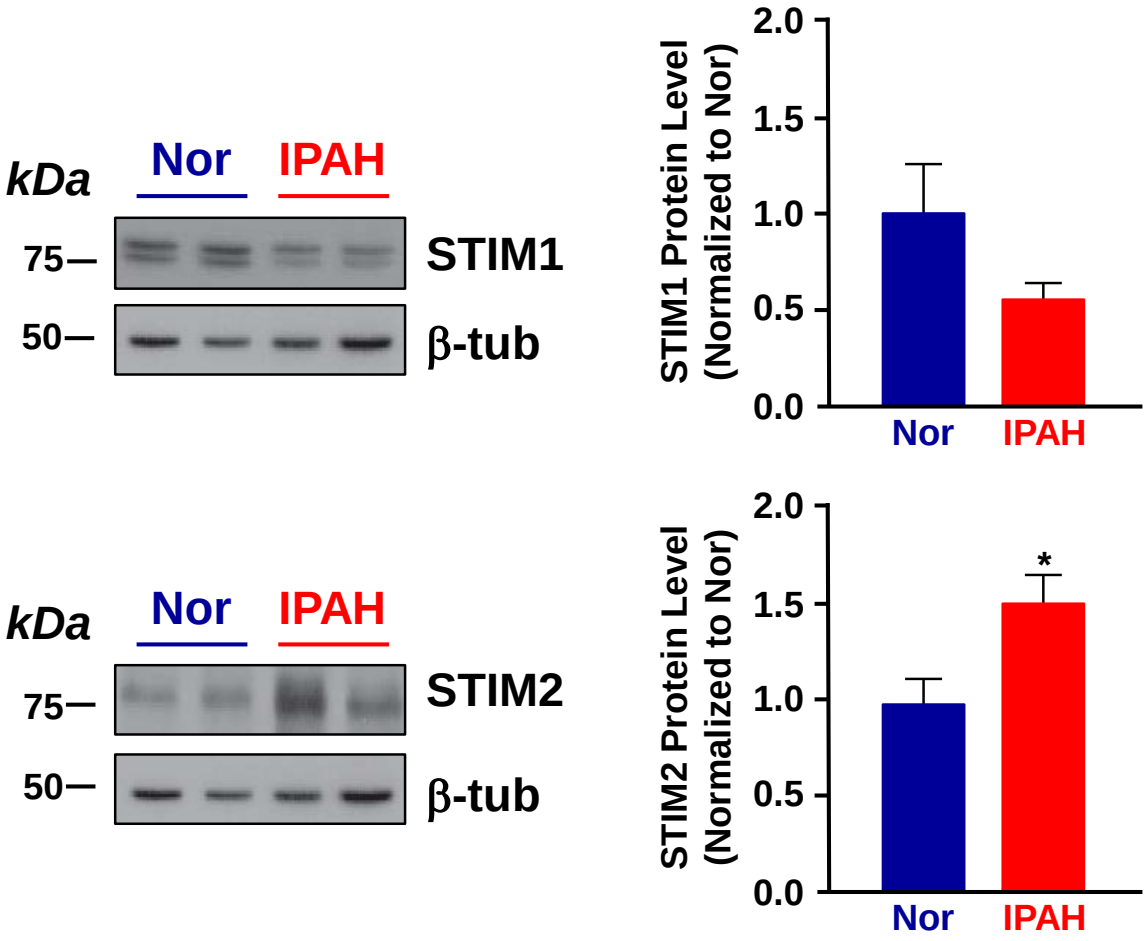

Figure 9: Protein expression of STIM2, but not STIM1, is increased in PASMC from IPAH patients. Representative Western blot images and summarized data for STIM1 and STIM2 protein expression levels in PASMC isolated from IPAH patient or from normal control patients (NPH). B-tubulin was used as a loading control. Summarized data of STIM1 or STIM2 protein expression level (mean±SE) in IPAH-PASMC (*n*=6) and NPH-PASMC (*n*=6). Graph shows protein expression of STIM1 and STIM2 normalized to an average level in NPH-PASMC. **P*<0.05 vs. NPH. Image was taken from and modified with permission from Song MY, Makino A, and Yuan JX (2011). STIM2 Contributes to Enhanced Store-operated Ca^{2+} Entry in Pulmonary Artery Smooth Muscle Cells from Patients with Idiopathic Pulmonary Arterial Hypertension. *Pulm Circ*. 1(1):84-94.

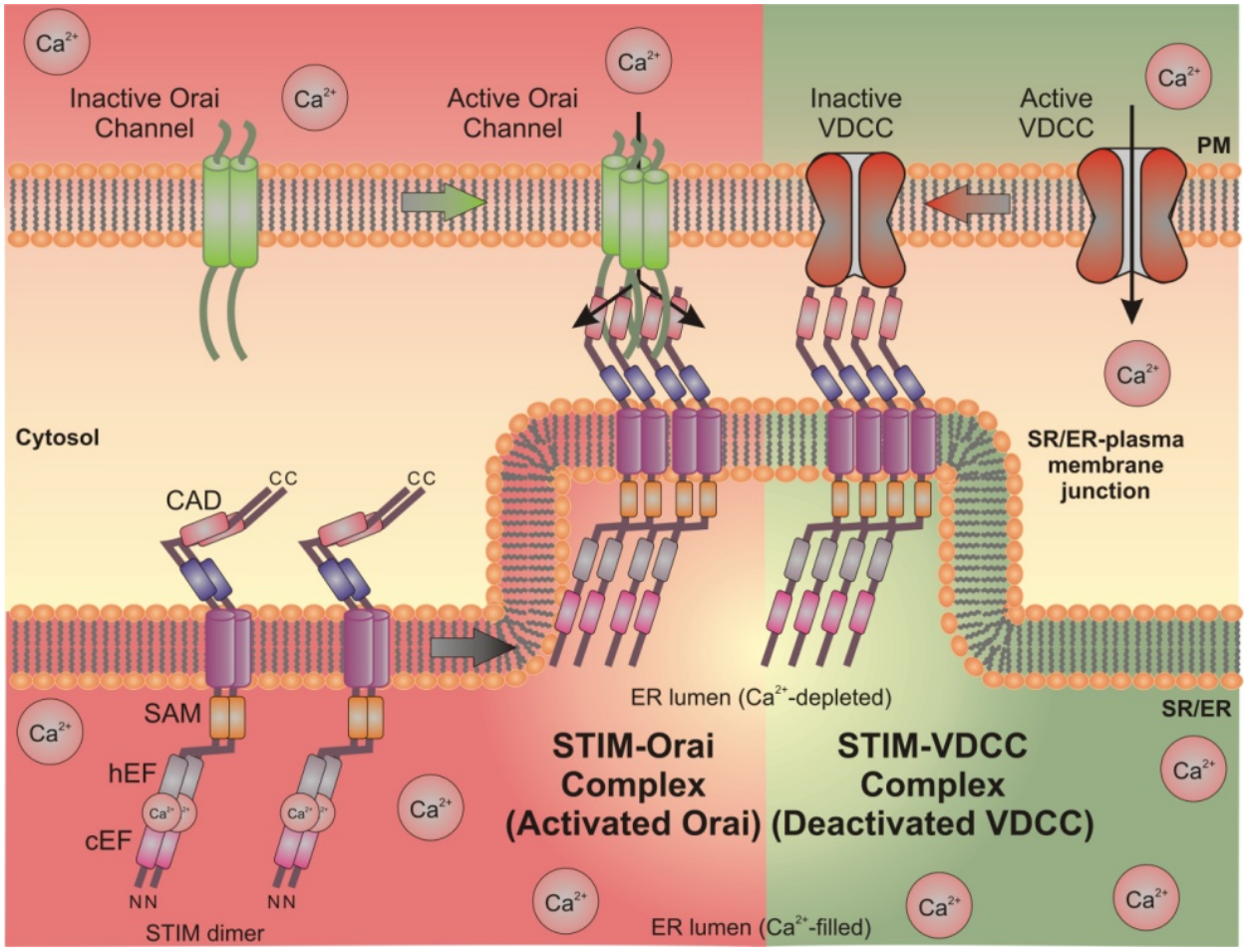

Figure 10: STIM activates SOC channels and inhibits VDCC. STIM not only interacts with SOC channels, but recent data show its ability to interact with VDCC in various cell types such as neurons, skeletal muscles, and SMC. STIM binds to VDCC to inhibit VDCC. Image was taken from and modified with permission from Fernandez RA, Sundivakkam P, Smith KA, Zeifman AS, Drennan AR, and Yuan JX (2012). Pathogenic role of store-operated and receptor-operated Ca^{2+} channels in pulmonary arterial hypertension. *J Sign Transduction*. 2012:951497.

a. **Orai-STIM Signaling in the Pulmonary Vasculature**

Studies in recent years have identified the existence of SOC channels in non-excitable cells such as vascular smooth muscle and endothelial cells (Cioffi et al., 2010). Orai are tetraspanning membrane proteins that play a significant role in cell morphology and motility. Three isoforms of the Orai family (Orai1-3) have been identified, which display notable differences in their features despite a high degree of sequence similarity. Among the three isoforms, Orai1 is the most potent ion pore forming subunit, and its depletion has the highest impact on SOCE in SMC (Baryshnikov et al., 2009; Berra-Romani et al., 2008; Bisaillon et al., 2010). Findings from our laboratory also demonstrated an increase in the expression of STIM1 and Orai1 in SMC induced by platelet-derived growth factor (PDGF), through the mammalian rapamycin (mTOR) pathway (Ogawa et al., 2012). Additionally, the inhibition of the Akt/mTOR pathway significantly suppressed the proliferation rate of PASMC. These observations substantiate the above findings that the expression of Orai proteins may play an important role in maintaining a proliferative phenotype *in vitro* or *in vivo*.

STIM proteins play a central role in activating SOC current mediated by Orai. Studies have also identified the binding of STIM1, STIM2 and/or other potential proteins with the intracellular N- and C-termini of Orai1. Upon store Ca^{2+} depletion, STIM1 co-clusters with Orai and forms punctae, representing a fine stoichiometry between Orai and STIM. A functional channel is comprised of the four pore-forming subunits of the Orai channels with four sets of STIM dimmers (stoichiometry is 4 Orai:8 STIM). It was identified that a minimal domain of STIM1 is sufficient to activate SOC channels (named SOAR for STIM1-Orai activating region). Cross-linking studies indicate that Orai1

proteins form dimers in resting state (Cahalan, 2009). However, once activated, four molecules of Orai1 form an active pore and mediate a sustained influx of Ca^{2+} into the cytosol. In addition to Orai1, Orai2 was identified to yield Ca^{2+}-selective I_{SOCE} currents while Orai3 resulted in a small and slowly developing I_{SOCE} (DeHaven et al., 2007; Lis et al., 2007). Although the Orai-mediated SOC currents are quite well understood in lymphocytes and certain non-excitable cells, a definitive role of Orai in VSMC and endothelial cells is still under investigation.

b. **TRPC-STIM Signaling in the Pulmonary Vasculature**

Transient receptor potential (TRP) channels have been demonstrated to form Ca^{2+}-permeable cation channels that are activated by store depletion (Kunichika et al., 2004). They are generally considered to be important signal transducers for agonist-mediated vascular contractility. The role of TRP channels in Ca^{2+} influx mechanisms was first discovered in *Drosophila(Hardie and Minke, 1995)*. Most TRP channels are nonselective for monovalent and divalent cations with Ca^{2+}: Na^{+} permeability ratio <10 (Salido et al., 2011). The first mammalian TRP protein identified in humans and mice was TRPC1. Since its identification, a number of TRP proteins have been found. Among these TRP families of proteins are; TRPC, TRPV, TRPA, TRPM, which are closely related to each other, while TRPP and TRPML are distantly related subfamilies.

The canonical TRP (TRPC) subfamily is comprised of seven members (TRPC1-7). Initially, TRPC channels were thought to be mainly involved in mediating ROCE mechanisms. However, in recent years, substantial evidence supports a significant role for TRPC proteins in the conduction of Ca^{2+} entry during SOCE. Using approaches such as overexpression and knockdown, several members of the TRPC family are activated

by Ca^{2+} store depletion. Several studies have confirmed the existence of TRPC channels in various vascular preparations (Albert and Large, 2003; Sundivakkam et al., 2012; Sundivakkam et al., 2009). Among the members of TRPC family of proteins, TRPC1, TRPC3, TRPC4 and TRPC6 have been studied extensively showing their abundant expression levels in PASMC (Lu et al., 2010; Peng et al., 2010; Wang et al., 2004; Wang et al., 2006). Most significantly, it was shown that the expression levels of TRPC1 and TRPC6 are upregulated in PASMC of HPH, which produces an increase in SOCE and ROCE (Kumar et al., 2006; Lin et al., 2004). Furthermore, the resulting elevated resting intracellular Ca^{2+} levels in PASMC were shown to augment the resting tension of pulmonary arteries of chronic hypoxic rats (Lin et al., 2004). STIM1 binds to, and directly regulates TRPC1, TRPC4 and TRPC5, as they are sensitive to store depletion. On the other hand, TRPC3 and TRPC6 may be activated indirectly and involve STIM1-dependent heteromultimerization of TRPC1-TRPC3 and TRPC4-TRPC6, in which TRPC1 and TRPC4 present STIM1 to TRPC3 and TRPC6, respectively (Yuan et al., 2007). STIM1-TRPC1/4 interaction is shown to mediate the STIM1 ERM domain. The ERM domain includes the STIM Orai activation region (SOAR) domain. Association of STIM1 and TRPC1 following store depletion was shown in VSMC (Li et al., 2008). Recent results indicate that BMP2 participates in regulating Ca^{2+} signaling in PASMC by inhibiting TRPC1, TRPC4, and TRPC6 expression, and thus leading to reduced SOCE and basal $[Ca^{2+}]_{cyt}$, and inhibition of cell proliferation and migration (Zhang et al., 2013). The STIM, Orai, and TRPC proteins represent highly promising, new pharmacological targets for which antagonist targeting these proteins may aid in the treatment of PH.

F. Pulmonary Arterial Remodeling and Smooth Muscle Cell Phenotypic Transition

Among the variety of underlying conditions that cause PH include increased PVR due to increased vascular remodeling. Pulmonary arterial remodeling is currently irreversible, and involves pathogenic dysregulation of the various cell types including endothelial cells, fibroblasts, and SMC. Vascular remodeling in part involves PASMC growth, which leads to increased concentric thickening of the smooth muscle medial layer of the vessel wall and abnormal muscularization of the normally nonmuscularized PA. PASMC proliferation is regulated by many growth factors and cytokines (Rakesh and Agrawal, 2005), which are also Ca^{2+}-dependent (Kudryavtseva et al., 2013). These factors influence the phenotype of SMC, which results in induced proliferation and migration.

Our hypothesis addresses the differentiation of SMC, and the potential targeting of a molecular pathway that may prove useful in patients with PH. Our approach is important because it can increase the clinical utility of upcoming therapies by targeting the proliferative phenotype of PASMC that leads to a sustained increased proliferation and vascular remodeling. There are several types of Ca^{2+} channels, and a number of classes of Ca^{2+} channel blockers (CCB), but almost all of them specifically or solely block the L-type voltage-dependent Ca^{2+} channel (VDCC). As described earlier, L-type VDCC are responsible for excitation-contraction coupling for smooth muscles. CCB inhibit Ca^{2+} influx into the vascular cells leading to relaxation of SMC and vasodilatation by inhibition of the L-type VDCC. Only about 10-15% of patients with IPAH test positively in potentially having a response to CCB, and only about half of these patients

actually have a long-term CCB response (Tonelli et al., 2010). More recently, studies have been focused on therapies with PAH-approved drugs for patients who are not vasoreactive or are vasoreactive but are not responding appropriately to CCB. Further advances were observed in different areas of PAH treatment in the past 5 years and were updated at the 5th WSPH meeting held in Nice, France. These treatments included drugs interfering with the endothelin (ET) pathway, the nitric oxide (NO) pathway, the platelet-derived growth factor (PDGF) pathway, and the prostacyclin pathway (Galie et al., 2013).

The endothelin pathway is activated when the endothelin peptide is increased in the plasma levels in the lung tissue of patients with PH; it exerts a vasoconstriction affect by binding to endothelin-A or -B receptors. In SMC, endothelin receptors exert vasoconstrive and mitogenic effects, however when present in endothelial cells they exert vasodilative and antiproliferative effects. Specific endothelin receptor antagonists include Ambrisentan and Bosentan. Ambrisentan functions as an endothelin-A receptor antagonist. Bosentan functions as a competitive antagonist of endothelin-1 by inhibiting endothelin-A and endothelin-B receptors.

The NO pathway is activated by synthesis of NO and signaling through NO-soluble guanylate cyclase (sGC). Guanosine monophosphate (cGMP) pathway is decreased in PAH. Specific drugs used to stimulate production of sGC or slow down the degradation of cGMP with Rocigaut, Sildenafil, Tadalafil, and Verdenafil. Rocigaut is a dual mode activator of sGC independent of NO availability. Sildenafil, Tadalafil, and Verdenafil are PDE5 inhibitors that inhibit degradation of cGMP.

The PDGF pathway activates proliferation of endothelial cells and SMC, which results in narrowing or occlusion of the vessel lumen. As of now, the only treatment that targets smooth muscle proliferation is the receptor tyrosine kinase (RTK) inhibitor, imatinib, which interferes with the PDGF pathway. As a potent mitogen, PDGF exerts its effects through two RTK (e.g., PDGF-α and PDGF-β) (Barst et al., 2010). Upon activation by PDGF, the PDGFR dimerize and are able to auto-phosphorylate their domains, which allow for further downstream activation of multiple transduction pathways, some of which include ras-dependent activation of extracellular signal regulated kinase (ERK) and mitogen-activated protein kinases (MAPK), and activation of phosphoinositol 3 kinase (PI_3K). It is well understood that sustained activation of signaling transduction pathways, (e.g., MAPK and PI_3K), are required for progression through G_1 to S phase of the cell cycle. Furthermore, PDGF activation of PDGFR can also activate STAT proteins, which when activated dimerize via sulfhydryl-phosphotyrosine interactions, and translocate to the nucleus, where they mediate gene transcription of early growth response genes (e.g., *c-fos* and *c-myc*). Imatinib, a PDGF-R inhibitor, works by modulation of vascular smooth muscle phenotype and inhibiting vascular smooth muscle cell proliferation and migration, and thus suppresses PDGF-induced vascular remodeling. Although the effects of imatinib in PAH are promising, there are still a number of issues, such as safety and tolerability profile, that need to be addressed. Further understanding of PASMC phenotypic modulation and its prevention or reversal may represent promising future treatments of PAH.

PASMC, unlike other muscle cells, do not terminally differentiate. In response to vascular injury, SMC exhibit a phenotypic change characterized by a loss of contractility

and abnormal proliferation, migration, and matrix secretion. This “synthetic” phenotype plays an active role in repair of vascular damage. SMC phenotype modulation contributes to the pathogenesis of numerous vascular disorders, including PAH. There is a spectrum of different subtypes of SMC that are present in the medial layer, which ranges from a contractile phenotype and a proliferative phenotype. Healthy PASMC are contractile and have a low proliferation rate. On the other hand, PASMC can undertake a phenotypic transition which results in an increase in proliferation rate.

The specific molecular mechanisms responsible for SMC phenotypic switching are many. One of the most significant advances for the field of SMC differentiation was the discovery of myocardin and myocardin-related transcription factors (MRTF), which belong to a family of SAF-A/B, Acinus, Pias (SAP) domain transcription factors. The two family members of MRTF include MRTF-A (also called MAL or MKL-1) and MRTF-B (also called MKL-2). Myocardin is localized in the nucleus, while the MRTF are also found in the cytosol. Myocardin and MRTF do not directly bind to DNA, unlike many other transcription factors which do bind to a conserved DNA sequence. They do, however, form a stable ternary complex with serum-response factor (SRF), which binds to the DNA consensus sequence $CC(A/T)_6GG$ (CArG box). SRF levels appear to be higher in SMC compared with most other tissues (Belaguli et al., 1997), nevertheless there is a lack of compelling evidence that depicts SRF as a critical determinant of SMC phenotype. Myocardin does not have transcriptional activity in $SRF^{-/-}$ cells (Wang et al., 2002). Notably, myocardin may also exert its effects impartially on SRF resulting from its binding to other signaling proteins. Furthermore, selective knockout of myocardin in

neural crest-derived SMC results in the lack of the contractile phenotype of SMC, and thus the cells were in a synthetic phenotype (Huang et al., 2008).

Recruitment of myocardin and MRTF results in the transcriptional activation domains of myocardin and MRTF forming a complex with SRF. Myocardin has been shown to selectively induce the expression of all CArG-dependent SMC marker genes. It has been also demonstrated that myocardin is both necessary and sufficient to activate these various SMC market genes. SMC express a set of contractile proteins that are different from those expressed in skeletal and cardiac muscles (Owens et al., 2004). These marker genes include: smooth muscle alpha actin (SM α-actin), smooth muscle 22-α (SM22α), smooth muscle myosin heavy chain (MYH), smooth muscle myosin light chain kinase (MLCK) and calponin, which all contain CArG elements in their promoter regions.

Previous studies have shown that mutations of the conserved CArG elements in these genes promoter-enhancer's results in abolished expression of SM22α and MYH (Kim et al., 1997; Manabe and Owens, 2001). Furthermore, these SMC markers are useful for assessing SMC differentiation with a particular importance on identifying and assessing the degree of phenotypic switching in SMC. In response to arterial injury, SMC express downregulated contractile genes and also upregulated proliferative genes required for synthetic, migratory, and proliferative functions. As a result, SMC fall within a large spectrum of a synthetic phenotype and contractile phenotype (Fig. 11).

Many efforts have been spent studying the role of the phenotypic switch of SMC in the pathophysiology of numerous vascular diseases (atherosclerosis), yet very little is known concerning the specific environmental cues that regulate SMC differentiation *in*

vivo. Some signaling pathways and epigenetic mechanisms that have been of interest include the regulatory roles upstream of the myocardin-SRF-complex (Owens et al., 2004). Originally, phenotypic switching was largely based on morphological changes. However, studies over the years have expanded to include a full range of possible factors resulting in alterations in the functional and structural properties of SMC *in vitro*. Some of these factors include mechanical forces, contractile agonists, extracellular matrix components, reactive oxygen species, endothelial-SMC interactions, SMC-SMC interactions, and growth factors, all of which have shown to promote expression of at least some SMC markers.

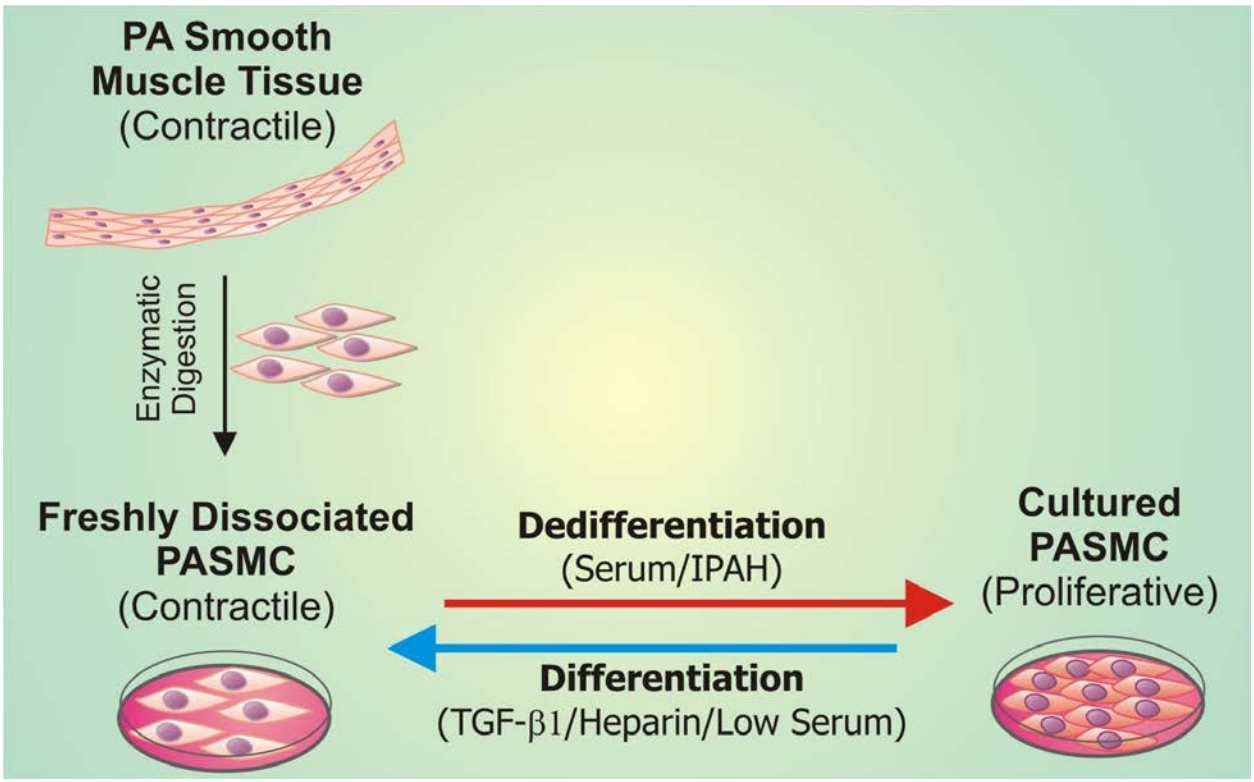

Figure 11: Modulation of Smooth Muscle Cell Phenotype. In response to a variety of stimuli, PASMC transition between contractile phenotype and proliferative phenotype. Contractile phenotype is characterized by high expression of contractile genes and low proliferation rates and migration. Vice versa, the proliferative phenotype of PASMC expresses low levels of contractile genes and has increased proliferation rates and migration

1. Platelet-derived Growth Factor Signaling in Pulmonary Arterial Smooth Muscle Cells

There are at least two studied factors that lead to PASMC differentiation in diseased models: 1) PDGF, which is an effective negative regulator of gene expression of cultured PASMC, and is important for PASMC proliferation, and 2) TGF-β, plays an important role in smooth muscle differentiation.

PDGF is a chemoattractant which is produced by platelets after activation and induces downregulation of specific smooth muscle cell markers in culture, and stimulate SMC dedifferentiation, and promotes proliferation and migration in arterial injury models as previously described above (Blank and Owens, 1990; Corjay et al., 1989; Ogawa et al., 2012; Yu et al., 2003b). It is well established that increased levels of PDGF in lung tissues, as well as increased expression of PDGF receptors in the pulmonary artery is present in patients with IPAH. (Barst, 2005; Ogawa et al., 2012; Ogawa et al., 2008; Yu et al., 2003b). It has been previously demonstrated that in rat PASMC, hypoxia increased PDGF production and consequently results in decreased gene expression of myocardin (Jie et al., 2010). As stated before, decreased myocardin expression may result in a more dedifferentiated SMC phenotype, and thus contribute to increased proliferation.Many different cell types synthesize PDGF. PDGF isoforms are dimeric molecules; they bind to two receptors simultaneously and result in PDGF receptor dimers.

These three dimeric PDGF receptor combinations transduce overlapping, but not identical, cellular signals. There is a difference between the receptors in regards to their effects on the actin filament system. Both the α-receptor and the β-receptor mediate an

increase in $[Ca^{2+}]_{cyt}$, although the β-receptor has a higher affinity than the α-receptor. PDGF induced autophosphorylation ad serves two important functions. First it results in receptor conformational change of the intracellular domains for the receptor for activation of kinases. Secondly, autophosphorylation of tyrosine residue creates docking sites for signal transduction molecules, which contain Src homology 2 (SH2) domains.

There are about 10 different SH2-domain families, which have molecules that are shown to selectively bind to different phosphorylated residues in the PDGF receptors. These include signaling molecules with intrinsic enzymatic activities, such as tyrosine kinases of the Src family, the SHP-2 tyrosine phosphatase, and phospholipase C-γ (PLC- γ). The SH2 domain is a conserved motif of about 100 aa residues that can bind to a phosphorylated tyrosine. A large number of SH2 domain proteins have been shown to bind to PDGF α- and β-receptors. Some of these molecules are themselves enzymes, such as phosphatidylinositol 3'-kinase (PI_3K), phospholipase C (PLC)-γ, the Src family of tyrosine kinases, the tyrosine phosphatase SHP-2, and a GTPase activating protein (GAP) for Ras. Other molecules, such as Grb2, lack enzymatic activity and have adaptor functions, linking the receptor with downstream catalytic molecules.

Secondly, after the PDFG receptors bind and activate signal transducers and activators of transcription (STAT). STATs can translocate to the nuclease which leads activation and regulate transcription by acting as transcription factors. These transcription factors become phosphorylated, then dimerize, followed by nuclear translocatation, which results in transcription of p21 and myc (specific growth and

differentiation genes). Each molecule that binds to the PDGF receptors initiates a signal transduction pathway.

Members of the PI_3K family that bind to and are activated by RTK consist of a regulatory subunit, p85, and a catalytic subunit, p110. Their preferred substrate is phosphatidylinositol 4,5-bisphosphate (PIP2), which is phosphorylated to phosphatidylinositol 3,4,5-trisphosphate (PIP3). PI_3K has a central role in intracellular signal transduction; it can be activated by several different signals, it has a number of downstream effecter molecules, and it mediates many different cellular responses, including of high interest, the serine/threonine kinase Akt/PKB for its proliferative effect (Dudek et al., 1997; Kauffmann-Zeh et al., 1997). Binding of PDGF to its receptor activates Akt/mTOR signaling, through phosphorylation of PIP2 to PIP3 leading to the phosphorylation of AKT then mTOR, which leads to the activation of p70S6K, and eIf4E consequently activating the transcription factors STAT. Furthermore, Forkhead transcription factor (Foxo), a known downstream target of Akt, has been shown to repress SMC differentiation by interacting with and inhibiting the activity of myocardin and resulting in a more proliferative phenotype (Liu et al., 2005).

PLC-γ acts on the same substrate as PI_3K (i.e., PIP2, and the products, IP_3 and DAG), which results in the mobilization of Ca^{2+} SR/ER internal stores. The binding of PLC-γ to the PDGF receptor leads to its phosphorylation on specific tyrosine residues, and thus its activation. Interestingly, full activation of PLC-γ is dependent on PI_3K; the PIP_3 formed by PI_3K binds the Pleckstrin homology domain (PH domain) of PLC-γ and anchors the enzyme at the plasma membrane. PLC-γ does not appear necessary in all

cells; however, in PASMC it plays an important role in Ca^{2+} mobilization (Yadav et al., 2013).

a. Studies of Platelet-derived Growth Factor Signaling in Pulmonary Hypertension

It was recently reported that there is a large amount of PDGF in "distal arteries of patients with chronic [thromboembolic] pulmonary hypertension (CTEPH) and cells isolated from endarterectomized tissues of patients with CTEPH have high expression of PDGFR" (Ogawa et al., 2009). These data demonstrate that no matter which type of pulmonary hypertension a patient has, the contribution of PDGF in the progression of PASMC proliferation is important.

AKT also play a crucial role in the progression of PAH. The downstream signals of AKT include mammalian target of rapamycin (mTOR), p70 ribosomal S6 kinase (p70S6K), both of which control cell proliferation. Rapamycin (mTOR inhibitor) has been shown to demonstrate therapeutic effect on PH by inhibiting proliferation (Krymskaya et al., 2011; Ogawa et al., 2009). Furthermore, it has previously been reported that PDGF significantly increases SOCE, and subsequently promotes cell proliferation of PASMC, contributing to pulmonary vascular remodeling through activation of Akt (Yu et al., 2003b).

STIM1 and Orai1 have been implicated as important components in PDGF-regulated Ca^{2+} entry in VSMC migration, and a role for STIM1 and Orai1 has been suggested in neointimal formation in pulmonary hypertension (Bisaillon et al., 2010). PDGF and its receptor are known to be substantially elevated in lung tissues and

PASMC isolated from patients and animals with pulmonary arterial hypertension (Ogawa et al., 2012). PDGF has been shown to phosphorylate and activate Akt and mammalian target of rapamycin (mTOR) in PASMC. Our lab previously investigated the role of PDGF-mediated activation of Akt signaling and the regulation of $[Ca^{2+}]_{cyt}$ and cell proliferation. We confirmed PDGF activation in the Akt/mTOR pathway and measured the subsequently enhanced SOCE and cell proliferation in human PASMC (Krymskaya et al., 2011; Ogawa et al., 2009; Stenmark and Rabinovitch, 2010; Wessler et al., 2010). Inhibition of Akt attenuated the increase in $[Ca^{2+}]_{cyt}$ due to both SOCE and PASMC proliferation. This effect correlated with a significant downregulation of STIM1 and Orai1, which are known activators of SOCE.

2. Transforming Growth Factor-β Signaling in Pulmonary Arterial Smooth Muscle Cells

Secondly, TGF-β has been shown to induce SMC differentiation in cell culture, corresponding with upregulated SMC markers resulting in downstream activation of the SMAD pathway. TGF-β receptors are constitutively active serine/threonine kinases. Upon activation, TGF-β dimers bind to TGF-β receptors, which results in auto-phosphorylation of the receptors. Specific BMPR (TGF-β superfamily receptors) result in activation of specific SMAD. Heterodimeric SMAD complexes can translocate to the nucleus where they act as a transcription factor for genes that regulate the mitogen-activated protein kinase (MAPK) pathway, which triggers apoptosis.

a. Studies of Transforming Growth Factor-β Signaling in Pulmonary Hypertension

Approximately 70% of known FPAH families have BMPR2 mutations (Machado et al., 2006). Additionally, about 25% of patients with IPAH also have the mutation (Thomson et al., 2000). Reduced mRNA and protein expression of BMPRII have been reported in the lungs of animals with experimental PH (Takahashi et al., 2006). Targeted gene delivery of BMPRII to the pulmonary endothelium significantly reduced PH in chronically hypoxic rats (Reynolds et al., 2007). Studies in knockout mice reveal the critical role of the BMP pathway in early embryogenesis and vascular development (Beppu et al., 2000). Disruption of BMP signaling leads to absence of necessary mechanisms needed to regulate proliferation and differentiation (Austin and Loyd, 2007). In the lung, BMPRII is highly expressed in the vascular endothelium of the PA and is expressed at a lower level in PASMC and fibroblasts (Atkinson et al., 2002). In PAH patients, mutations in the BMPRII results in reduced expression of the protein in the pulmonary vasculature (Atkinson et al., 2002), thus, a reduction in the expression of BMPRII has been identified to be important in the pathogenesis of PAH. Furthermore, it was confirmed that proximal PASMC harboring kinase domain mutations in BMPR2 are deficient in SMAD signaling (Kawabata et al., 1998). Moreover, they showed that the patients with FPAH and IPAH were deficient in the activated form of SMAD1. It was thus determined that SMAD signaling and activation of p38(MAPK)/ERK signaling leads to mutations in BMPR2 and thus the underlying cause for abnormal vascular cell proliferation (Yang et al., 2005).

3. Pulmonary Arterial Smooth Muscle Cells Phenotype is Regulated by Ca^{2+}-dependent Transcription Factors

The changes in $[Ca^{2+}]_{cyt}$ in PASMC, as stated earlier, are mediated either by extra cellular Ca^{2+} influx or release of Ca^{2+} from intracellular SR/ER stores. The release of Ca^{2+} from SR/ER is mediated by IP_3R. As previously mentioned, Ca^{2+} influx in PASMC is mediated by various plasma membrane channels, which are voltage-dependent or voltage-independent Ca^{2+} channels. An increase in $[Ca^{2+}]_{cyt}$ initiates PASMC contraction by a process termed excitation-contraction coupling.

Notably, increased $[Ca^{2+}]_{cyt}$ can also affect gene expression in a process termed excitation-transcription coupling. cAMP response element-binding protein (CREB), nuclear factor of activated T lymphocytes (NFAT), and serum response factor (SRF), are all transcription that are calcium regulated. These Ca^{2+}-dependent transcription factors in PASMC that regulate contractile marker genes code for contractile proteins, and stimulate DNA synthesis (Miano et al., 1993; Ren et al., 2010). Selective activation of genes is essential for defining VSMC functional and morphological properties and, consequently, VSMC phenotype.

a. cAMP Response Element-Binding Protein Function in Pulmonary Arterial Smooth Muscle Cells

CREB, a transcription factor, controls gene expression by binding to SRF and c-jun at their promoter regions of target genes. PASMC gene regulation by CREB requires phosphorylation of Ser133 of CREB, which results in an active transcription complex with CREB-binding proteins and RNA polymerase II (Janknecht and Hunter,

1996). CREB can be phosphorylated by many kinases (e.g., protein kinase A (PKA), protein kinase G (PKG), Ca^{2+}/calmodulin-dependent protein kinase (CaMK)).

In PASMC, CREB phosphorylation is initiated by an increase in $[Ca^{2+}]_{cyt}$. Of high interest to us is the data showing that Ca^{2+} depletion of the SR store by thapsigargin (an irreversible SERCA pump inhibitor), and subsequently activates SOCE, increased phosphorylated CREB in the nuclear compartment and also resulted in the transcription of CREB-dependent genes in cultured VSMC (Pulver et al., 2004). Along with these studies, small interfering (siRNA) for STIM1 decreased the thapsigargin-induced CREP phosphorylation in coronary artery SMC (Takahashi et al., 2007). This links STIM protein to modulation of PASMC proliferative phenotype. It has been demonstrated that short, rapid increases of $[Ca^{2+}]_{cyt}$ are not enough to result in CREB phosphorylation. Although the exact mechanism describing the temporal differences of Ca^{2+}-dependent CREB activation has not been explained, this difference suggests that a sustained rise in $[Ca^{2+}]_{cyt}$ is necessary for CREB activation.

b. Nuclear Factor of Activated T-lymphocytes-dependent Regulation of Transcription in Pulmonary Arterial Smooth Muscle Cells

NFAT, a transcription factor in T cells, was shown to be in charge of transcription of genes responsible for immune responses (Rao et al., 1997). However, members of the NFAT family are also present in various tissues, including cardiac, skeletal, and vascular smooth muscle (Schulz and Yutzey, 2004). There are 5 NFAT family members (NFATc1-5). NFATc5 is the only member that is constitutively found in the nucleus.

NFAT is a cytosolic protein that can be phosphorylated at multiple sites. NFAT can be activated by its dephosphorylationby Ca^{2+}/calmodulin-dependent serine phosphate, calcineurin, which allows for nuclear translocation. Once in the nucleus, activated NFAT can associate with transcription coactivators (e.g., activator protein 1 (AP1) and myocyte enhancer factor 2 (MEF2)), which promote gene expression (e.g., c-jun) (Hill-Eubanks et al., 2003). NFAT activity is highly dependent on temporal and spatial qualities of Ca^{2+} and Ca^{2+} signaling, since increased $[Ca^{2+}]_{cyt}$ results in increased activity of calcineurin. Of significant interest, Ca^{2+} release from the SR/ER, by IP_3R, can induce NFATc3 activation (Gomez et al., 2002). Additionally, NFATc2 has been discovered to be active in patients with PAH, however, not control patients (Bonnet et al., 2007). Furthermore, evidence showing that when SOCE is inhibited NFAT activation is decreased after hypoxia-induced nuclear translocation (Bierer et al., 2011; Hou et al., 2013). It has also been demonstrated that NFAT nuclear translocation is inhibited after knockdown of STIM1 and/or Orai1 with siRNA in proliferative SMC (Zhang et al., 2011). Since both Orai1 and STIM1 knockdown inhibited NFAT nuclear translocation and transcriptional activity, this suggests there is a transcriptional pathway downstream of SOCE.

In summary, increased and sustained $[Ca^{2+}]_{cyt}$ in PASMC results in activation of Ca^{2+}-dependent transcription factors that fundamentally control properties responsible for proliferation and maintenance of a proliferative phenotype in PASMC. Ca^{2+} signaling patterns activate distinct transcription factors that result in initiation of proliferation via different methods. Both voltage-dependent and voltage-independent Ca^{2+} influx can activate transcription factors; however, this activation is through different pathways.

Data suggest that localization of Ca^{2+} signals can offer information about the type of stimulus, while conversely, total increase in $[Ca^{2+}]_{cyt}$ informs us about the degree and intensity of the stimulus (Kudryavtseva et al., 2013). As of yet, the exact co-localization of Ca^{2+} and specific transcriptional targets is uncertain. Understanding the relationship between different Ca^{2+} sources, transcription factors, signaling pathways, and activation of selective genes and how they regulate PASMC phenotypic switch is also important. This knowledge will provide a foundation for the adaptive and pathological transition of PASMC from contractile to a proliferative phenotype during the development of PAH and other vascular diseases.

Future experiments detailing the specific downstream effects of enhanced SOCE and ROCE will shed light on important therapeutic strategies for PAH. More specifically, understanding the role of other proteins, such as transcription factors, signal transduction proteins, and nuclear transporters, as well as their effect on vasoconstriction and proliferation, could provide future pharmacological blockades of SOC/ROC channels. Additionally, downregulation of these proteins could reveal therapeutic effects for PH. Application of new experimental methods, along with further use of well-established methods, is crucial to the advancement of the field of pulmonary hypertension.

The role of STIM2 as a mediator of sustained PASMC proliferation may be of some major importance. Although the role of STIM2 in PASMC differentiation has not been directly examined, our studies have confirmed that enhanced SOCE is apparent in the proliferative phenotype of PASMC when compared to quiescent PASMC. These data suggest that downregulation of SOCE is associated with decreased proliferation

and potentially affects the transition into a more differentiated/quiescent phenotype. Furthermore, in this thesis, there is strong evidence suggesting that in a dedifferentiated (proliferative) phenotypic state, PASMC express upregulated expression of STIM2, in combination with enhanced SOCE. Therefore, we suggest a function for STIM2 in the dedifferentiated and proliferative of PASMC. It has been previously shown that decreased expression of STIM2 can reduce PASMC proliferation (Song et al., 2011). Cells with decreased STIM1 resulted in the inability to progress into the S phase of the cell cycle and was associated with the upregulation of p21, reduction in CREB phosphorylation (Takahashi et al., 2007), and NFAT transcription activity (Aubart et al., 2009). These data suggest that multiple signaling pathways may be regulated by STIM in PASMC.

In summary, based on the above literature, we tested the hypothesis that upregulation of STIM2 contributes to the phenotypic transition of PASMC from the contractile phenotype to the proliferative phenotype.

III. MATERIALS AND METHODS

A. Isolation of Pulmonary Artery

Protocols involving the use of experimental animals for all experiments were reviewed and approved by the Ethics/Animal Care Committee of the University of Illinois at Chicago. Sprague-Dawley male rats (125-150 g) were decapitated via guillotine, followed by the whole lung and heart being removed *en bloc* and placed in warm Hank's balanced salt solution (HBSS, Life Technologies; Carlsbad, CA) supplemented with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Sigma Aldrich; St. Louis, MI). The right and left branches of the main pulmonary, as well as the intrapulmonary arteries, were isolated from the whole-lung tissues with fine forceps under a dissecting microscope.

1. Isolation of Pulmonary Arterial Smooth Muscle Tissue

The fat and connective tissues were removed gently from the isolated PA under sterile conditions. The isolated PA was incubated in HBSS containing 1.7 mg/mL collagenase type II (Worthington Biochemical; Lakewood Township, NJ) for 20 min at 37°C. Then, the shortly digested PA ring was rinsed with HBSS to remove residual collagenase; fine forceps carefully stripped off the adventitia of the PA ring and the endothelium was denuded with sterile cotton swab. The remaining PA smooth muscle tissue was then used to prepare pure PASMC.

2. Isolation and Preparation of Rat Pulmonary Arterial Smooth Muscle Cells

Following removal of the adventitia and endothelium, the rat PA was further digested in HBSS 1.7 mg/mL collagenase type II, 0.5 mg/mL elastase (Sigma) and 1 mg/mL bovine serum albumin (BSA, Sigma) at 37°C for 50 min. The PA tissue was agitated every 15-20 min to speed digestion. The digested PA tissue was then triturated approximately 8-10 times with a fire-polished Pasteur pipette to further dissociate the cells. 10 mL of Dulbecco's Modified Eagle Medium (DMEM; Corning; Herndon, VA), supplemented with 7 mM NaH_2CO_3, 10 mM HEPES (pH 7.2), 20% fetal bovine serum (FBS, Corning), and 1% penicillin and streptomycin (Pen/Strep, Corning) was then added to the enzymatic solution to stop the digestion. The suspension was centrifuged for 5 min at 1,500 rpm at RT (22-24°C). Next we aspirated the supernatant and the resulting pellet was resuspended in 2 mL of fresh 10% FBS-DMEM and triturated to separate the cells.

For the experiments using freshly dissociated PASMC, aliquots of the cell suspension were plated directly onto glass cover slips coated with 5% gelatin (procine, Sigma Aldrich) or in 6-well Petri dishes (Corning) with 2.5 mL of 10% FBS-DMEM. These freshly dissociated rat PASMC were allowed to attach to the cover slips for 3-4 hours before loading with Fura 2-AM for measurement of $[Ca^{2+}]_{cyt}$. The freshly dissociated cells plated directly onto the 6-well Petri dishes were then used for immunocytochemistry and immunoblotting experiments to detect protein expression levels of ion channels of interest.

To prepare primary cultured rat PASMC, aliquots of the cell suspension were plated onto gelatin-coated cover slips in 25-mm Petri dishes or directly onto 10-cm Petri dishes with 10% FBS-DMEM, and incubated in a humidified atmosphere of 5% CO_2 at 37°C. 24 hours later, the culture media was changed to Medium 199 (M199), supplemented with 10% FBS, 100 μg/mL GS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 50 mg/L D-valine (SMC medium). The medium was changed 24 hrs after initial seeding and every 48 hours subsequently. When the cells reached 80-90% confluency, cells were gently washed with phosphate buffered saline (PBS), incubated briefly with 1 mL of 0.025% trypsin/EDTA solution until detachment (3-5 min), and then 9 mL 10% FBS-M199 was added to the plate. The cell suspension was transferred to a sterile 15 mL round bottom tube, centrifuged at room temperature for 5 min at 1,500 rpm, and resuspended in the appropriate growth media before being seeded onto cover slips or Petri dishes. They were then used for Western blot and $[Ca^{2+}]_{cyt}$ measurement experiments.

B. *in vitro* Differentiation of Pulmonary Arterial Smooth Muscle Cells

PASMC differentiation was induced by serum-starvation (3% or 1% FBS-M199), TGF-β treatment (1 or 2ng/mL TGF-β, Sigma), or by treatment with Heparin in low serum (30 μg Heparin + 1% FBS). Primary PASMC at 80-90% confluency were serum starved (1% FBS) overnight before being plated for experiments. After being serum starved, cells were washed 3 times, and either vehicle-control media (M199) or dedifferentiating media (M199) was added for the duration of the experiment. After 48 hours or 72 hours, cells were collected and PASMC phenotype was determined by relative expression of smooth muscle contractile proteins (SM22α, MYH, and calponin).

The proliferative phenotype was evaluated by the relative expression of proliferation markers (proliferating cell nuclear antigen (PCNA)). Both contractile and proliferative phenotypes were analyzed by Western blot.

C. Measurement of $[Ca^{2+}]_{cyt}$ in Pulmonary Arterial Smooth Muscle Cells

$[Ca^{2+}]_{cyt}$ was measured using fura-2 acetoxymethyl ester (Fura-2/AM, Invitrogen-Molecular Probes, Eugene, OR), a membrane-permeable Ca^{2+}-sensitive fluorescent indicator, and a Nikon digital imaging fluorescent microscopy system. Cells on 25-mm cover slips were loaded with 4 μM Fura-2/AM in normal physiological salt solution (PSS) for 60 minutes at room temperature (22-25°C) in the dark. The PSS solution contained (in mM): 137 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, and 10 glucose (pH was adjusted to 7.4 with 1 N NaOH). The Fura-2/AM-loaded cells were next placed on the stage with an inverted fluorescent microscope (Eclips Ti-E; Nikon, Japan) with an objective lens (S. Plan Flour 20x/0.45 ELWD; Nikon) and Em-CCD camera (Evolve; Photometrics, Tucson, AZ). The continuous perfusion of the recording chamber with PSS was maintained at a rate of 2 ml/min controlled with a mini pump (Model 3385; Control, Friendswood, TX). The fura-2/AM-loaded cells were then washed by perfusion of normal PSS for 20 min to remove excess extracellular Fura-2/AM and allowed sufficient time for intracellular esterase to cleave Fura-2/AM to the active Fura-2. The cells were excited at 340- and 380-nm wavelengths (D340xv2 and D380xv2 filters, respectively; Chroma Technology, Bellows Falls VT) by a xenon arc lamp (Lambda LS; Sutter Instrument, Novato, CA) and an optical filter changer (Lambda 10-B). Emission of

fura-2 fluorescence was composed through a dichroic mirror (400DCLP) and a wide band emission filter (D510/80m). $[Ca^{2+}]_{cyt}$ within the ROI (4×4 μm), which was specifically located at the edge of each individual cell. The ratio of fluorescence intensities (F_{340}/F_{380}) was measured every 2 seconds. $[Ca^{2+}]_{cyt}$ measurements were conducted at 32°. The temperature was controlled by using an automatic temperature controller (TC-344B, Warner Instruments, Hamden, CT). The setting was not set to physiological temperature, 37°C, because this results in increased Fura-2 compartmentalization in intracellular organelles (Roe et al., 1990).

D. Tension Measurements of Pulmonary Artery

The isolated rat PA ring was cut into 2-mm long segment and two tungsten hooks (0.1 mm in diameter) were carefully inserted into the lumen of the ring under a dissecting microscope. The first hook then was linked to the base of a perfusion chamber and the second hook was linked to the isometric force transducer (Harvard Apparatus). Passive resting tension was set to and maintained at 300 mg. This was followed by 1 hour stabilization of the rings at resting tension before experimentation. Isometric tension was recorded continuously. Followed by data collection using DATAQ software (DATAQ Instruments). 40 mM K^+-containing solution (40K) was applied to the PA ring 3 times for a steady contractile response. Isolated PA rings were perfused with modified Krebs solution (MKS: at 37C) which consisted of the following chemicals: 138 mM NaCl, 1.8 mM $CaCl_2$, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 5 mM HEPES, and 10 mM glucose (pH 7.4). In the calcium-free solution (0Ca MKS), $CaCl_2$

was substituted by equimolar $MgCl_2$, and to chelate residual Ca^{2+}, 1 mM EGTA was added. High-K^+ (40 mM K) solution, NaCl was substituted by equimolar KCl, this allowed for maintenance of osmolarity. Cyclopiazonic acid (CPA, Sigma Chemical) was dissolved in DMSO and then diluted to 1:1,000–2,000 in MKS or 0Ca MKS on the day of use in order to obtain their final concentration. The pH values of the solutions were determined after addition of drugs and adjusted to a pH of 7.4. The rings were weighed using a fine balance, and the active tension was normalized wet tissue weight after induction by 40 mM K^+ solutions was added. The active tension, in relation to the basal tension was determined and expressed as the net increase in tension (mg).

E. **Western Blot Analysis**

Smooth muscle tissue were placed in ependorf tubes over ice and lysed with cold RIPA lysis buffer (Millipore, Billerica, MA) supplemented with protease inhibitor cocktail (Roche; Basel, Switzerland) by sonification 3 times for 30 seconds each time. Cultured rat PASMC and HEK cells were washed with ice cold PBS, scraped, placed into the ependorf tubes, and centrifuged. The pelleted cells were resuspended in 20-50 μl of RIPA buffer supplemented with protease inhibitor cocktail. Lysed tissues and cells were incubated in lysis buffer for 15 minutes on ice. The lysates were then centrifuged at 13,300 rpm for 15 minutes at 4°C. The pellet was discarded and from the supernatant, protein concentration was determined by Bradford Protein Assay (BioRad; Hercules, CA) with BSA as a standard. Proteins (10-20 μg) were mixed and boiled in Laemmli sample buffer supplemented with 2-mercaptoethanol (BME, Sigma) reducing agent. Protein lysates were resolved on sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE) and transferred onto 0.45 μm nitrocellulose membranes (BioRad). Membranes were incubated for 1 hour at 22–24°C in a blocking buffer (0.1% Tween 20 in TBS, (TBST)) containing 5% nonfat dry milk powder. The membranes were then incubated with primary antibodies diluted in TBST containing 5% BSA, shaking overnight at 4°C. Membranes were washed three times in TBST for 5 minutes each, followed by incubation in secondary antibody conjugated to horseradish peroxidase for 2 hours at room temperature in TBST containing 5% milk. Membranes were washed 3 times for 5 minutes each and peroxidase activity was visualized with enhanced chemiluminescence substrate (Pierce; Rockford, IL). Primary antibodies included: anti-calponin (1:1000, Santa Cruz Biotechnology, Inc.; Santa Cruz, CA), myosin heavy chain (MYH, 1:1200, Santa Cruz), SM22α (1:1000, Santa Cruz), proliferating cellular nuclear antigen (PCNA, 1:1000, Santa Cruz), STIM1 (1:1000, Pro-Sci), STIM2 (1:1000, Sigma), Orai1 (1:500, Pro-Sci), Orai2 (1:500, Alomone; Israel), Orai3 (1:500, Alomone), TRPC6 (1:500, Sigma), and β-actin (1:2000, Santa Cruz). Band intensity was quantified with ImageJ, normalized to β-actin control, and expressed as arbitrary units.

F. Transient Transfection of Pulmonary Arterial Smooth Muscle Cells

Twenty-four hours after seeding, HEK293 cells at 60-80% confluency were transiently transfected with STIM2 and Orai2 expression constructs using Lipofectamine 2000 transfection reagent, based on manufacturer's protocol. Transfection was performed at 37°C in serum-free Opti-MEM medium (Gibco) with .5ug/ml DNA and Transfection reagent (μl). After 5-6 hours of incubation at 37°C with transfection media,

cells were refed with were serum-containing DMEM and incubated 24-72 hours before experiments. Rat PASMC were transiently transfected with STIM2 and Orai2 expression constructs and siRNA targeting STIm2 and Orai2 using Amaxa Basic Nucleofector kit (Lonza; Walkersville, MD) via electroporation according to manufacturer's instructions. Serum-free Opti-MEM medium (Gibco) was used to dilute cells, transfection reagent, DNA, and siRNA. After 5-6 hours of incubation at 37°C with transfection medium, cells were refed with serum-containing M199 and incubated 24-72 hrs. $[Ca^{2+}]_{cyt}$ measurements and Western blots using DNA- and siRNA-transfected cells was preformed 48-72 hours after transient transfection.

1. **cDNA Vectors Used for Upregulation**

STIM2 (Plasmid 18868: pEX-CMV-SP-STIM2 (15-746))

Orai2 (Plasmid 16369: pcDNA3.1 Orai2)

2. **small interfering RNA Agents Used for Downregulation**

STIM2 (Ambion Life Technology, Carlsbad CA)

Orai2 (siGENOME Thermo scientific)

scramble si-RNA (siGENOME Thermo scientific)

G. Immunocytochemistry

Purity of the PASMC population in cells isolated from PA was tested by anti-α-smooth muscle actin (SMA, a marker for SMC). Cells were seeded on a glass coverslip in a six-well chamber with M199 supplemented with 10% FCS, 100 μg/mL GS, 100

IU/mL penicillin, 100 μg/mL streptomycin, and 50 mg/L D-valine (SMC medium). Cells were fixed with 100% methanol at −20°C and blocked with 5% BSA in PBS and incubated SMA (1:400 dilution) for 1 hour at room temperature. Cells were washed and incubated with secondary antibody, in the dark for 1 hour. Cells were stained with membrane-permeable nucleic acid stain, 4, 6-diamidino-2-phenylinodole (DAPI) and mounted with mounting medium (VECTASHIELD Mounting Medium with DAPI, Vector Laboratories; Burlingame, CA). Blue fluorescence emitted at 461 nm was used to estimate total cell numbers in the culture and a secondary antibody conjugated with indocarbocyanine (Cy3) was used to display the fluorescent image (emitted at 570 nm). Fluorescence was captured by a charge-coupled device camera connected to a fluorescence microscope with a 20x objective lens. The Cy3 fluorescence was colored green and DAPI fluorescence was colored blue to display images with green-blue overlay.

H. **Pulmonary Arterial Smooth Muscle Cells Proliferation Assays**

1. **Bromodeoxyuridine Incorporation Assay**

To determine rat PASMC proliferation rate *in vitro* Bromodeoxyuridine (BrdU) incorporation assay (Millipore) was used according to manufacturer's protocol. Primary confluent Cells were detached with 0.05% trypsin/EDTA, seeded into 96-well plates ($2.5x10^3$ cells/well) in M199 containing 10% FBS, and allowed to attaché overnight. PASMC were starved in serum-free M199 for 24 hours and then incubated with vehicle control, or 1, 2, or 3 ng/mL of TFG-β for 24, 72, or 96 hours. In addition, cells were

incubated with 0.3%, 1.0% or 10% FBS/M199 for 24, 72, or 96 hours. Cells were also incubated with 500 nM Ca^{2+} of 1.2 mM Ca^{2+} in 10% FBS/M199 for 24 or 72 hours. During the final 4 hours of incubation BrdU was incorporated into actively dividing cells. Cells were fixed/denatured, in room temperature fixative solution. Anti-BrdU antibody was added, followed by incubation with secondary anti-mouse IgG peroxidase-conjugate. Fluorescence was measured using a spectrophotometer micorplate reader at 450/540 nm.

2. **Cell Counting**

PASMC growth curves were examined by cell counting with the Bio-Rad TC10 automated cell counter. Subculture cells were growth arrested overnight, after which they were collected counted and equally seed into 8-well multidishes (Nunclon Δ, 10.5 cm2 of culture area per well; Thermo Scientific), with 1×105 cells/well (0.95×104 cells/cm2, 3 wells/sample) this number was used as baseline (0 hours). Treatment cells were counted 24, 72, and 96 hours after start of experiment. Each count was an average of three repeats, and each data point was the average of 4 experiments.

I. Preparation of Monocrotaline-induced Pulmonary Hypertensive Rats

Male Sprague-Dawley rats were injected with monocrotaline (MCT), which is a poisonous pyrolizidine alkaloid found in the leguminous plant *Crotalaria spectabilis*, and results in the progressive development of pulmonary hypertension. Rats were injected with a single subcutaneous (sc) injection of vehicle (dimethyl sulfoxide, DMSO, Sigma) or 60 mg/kg MCT. 14 days after injection, rats were anesthetized with ketamine/xylazine and then right ventricular systolic pressure (RVSP) was measured using an MPVS Ultra

system (Millar Instruments) to determine severity of development of pulmonary hypertension. Additionally, Fulton Index measurements and H&E staining were obtained to further confirm the development of PH. The Fulton Index The Fulton's index is the ratio of right ventricle weight over the weight of left ventricle plus the septum (RV/[LV+S]).

J. Chemicals

Human TGF-β (R&D Systems) was prepared as a stock solution in sterile HCl (4 mM) that contained 0.1% BSA. Cyclopiazonic acid (CPA, Sigma) was dissolved in DMSO resulting in a stock solution of 100 mM. The stock solution was aliquoted. Then the stock solution were then diluted 1:1,000 in MKS or 0Ca MKS. Fura 2/AM was reconstituted in 50 μL DMSO to make a stock solution 10 mM and kept away from light to avoid degradation. Aliquots of the stock solution were then diluted to make a working solution of 4 μM.

K. Statistical Analysis

Data were expressed as mean±S.E. and analyzed for statistical significance by the unpaired Student's *t*-test or One Way ANOVA for many groups using Sigma Plot software. Differences were considered to be significant at $P < 0.05$. Significant difference is expressed the figures as $*P<0.05$, $**P<0.01$, and $***P<0.001$.

IV. **RESULTS**

A. **Overview**

Patients with early stages of PH present with increased vasoconstriction and are more likely to be responsive to calcium channel blockers (CCB). Severe PH patients do not respond to the traditional CCB treatments, which target L-type VDDC, thus a new method of studying PH needs to be considered. Hence, examining the mechanism of SMC phenotypic switching which leads to a sustained increased proliferation and progression of PH has provided an alternative approach in finding a cure for PH.

Our laboratory has documented the upregulation of STIM2 and SOC channels (Orai2 and TRPC6) in PASMC from patients with PAH (Song et al., 2011; Yu et al., 2009). However, the role of upregulated STIM2 and SOC channels in a proliferative phenotype has not been investigated. Whether STIM2 upregulation is a necessary regulator of PASMC phenotypic modulation and sustained proliferation will be discussed in this thesis.

B. **Upregulated Expression of STIM2, TRPC6 and Orai2 in the Proliferative Phenotype of Pulmonary Arterial Smooth Muscle Cells in Cultured Compared to the Contractile Phenotype of Pulmonary Arterial Smooth Muscle Cells in Pulmonary Arteries**

As previously described, PASMC from IPAH patients show enhanced SOCE compared to normal PASMC, along with increased levels of STIM2, Orai2, and TRPC6

(Song et al., 2011; Yu et al., 2004). However, which role these proteins play in PASMC phenotypic transition is unknown. STIM2 may play an important role in the early phenotypic transition of PASMC from a contractile phenotype to a proliferative phenotype. To examine whether STIM2 and SOC channels correlates with this transition, we first measured and compared protein expression levels of STIM2, Orai2, and TRPC6 in isolated PA (contractile phenotype) and cultured PASMC (proliferative phenotype). First we wanted to determine the purity of our PASMC isolation method. Nearly all cells stained by nucleic acid dye, DAPI, cross-reacted with the smooth muscle α-actin antibody (Fig. 12), indicating that the cultured PASMC were pure SMC without fibroblast and endothelial cell contamination. Furthermore, Figure 12a shows that cultured PASMC were spindle shaped, which is a typical characteristic of SMC. Additionally, Figure 12b represents the whole rat PA smooth muscle tissue, used as a representation of healthy contractile smooth muscle.

As shown in Figure 13, the protein expression levels of STIM2, Orai2, and TRPC6 in rat PA and PASMC were significantly upregulated in the proliferative PASMC compared to contractile PA (n=4; Fig. 13). The STIM2 and SOC channel upregulation in proliferative PASMC suggests that these proteins may play a role in maintaining increased PASMC proliferation as seen in IPAH patients. Furthermore, a rise in $[Ca^{2+}]_{cyt}$ due to Ca^{2+} influx through the plasma membrane Ca^{2+} channels can activate MAPK (Chao et al., 1992), which is part of the phosphorylation cascade that leads to activation of DNA synthesis-promoting factor, and can rapidly increase nuclear $[Ca^{2+}]$ and promote cell proliferation (Allbritton et al., 1994).

The expression levels of contractile SMC marker and proliferative marker genes were used to characterize PASMC phenotype. Since smooth muscle specific MYH, SM22α, and calponin are gold-standard marker proteins for the SMC contractile phenotype, we measured expression levels of these proteins in contractile PA tissues and proliferative PASMC. As shown in Figure 14, protein expression levels of MYH, SM22α, and calponin were significantly downregulated in proliferative PASMC in comparison to contractile PA (n=4; Fig. 14). These results confirm that PASMC dedifferentiate to a more proliferative phenotype when cultured in smooth muscle basal medium plus 10% FBS and growth factors for 72-96 hours.

To link the role of Ca^{2+} in proliferation, we measured BrdU incorporation in growth arrested cells and cells with 1.8 mM or 0.5 mM Ca^{2+} for 24 or 72 hours. The addition of 2 mM EGTA, a Ca^{2+} chelator, to culture media decreased the free [Ca^{2+}] from 1.8 mM to about 525 nM and almost abolished rat PASMC growth in the presence of serum and growth factors (n=4, Fig. 15). These data are consistent with our previous observations (Golovina et al., 2001) in human PASMC showing that chelation of extracellular Ca^{2+} with 2 mM EGTA significantly inhibited cell growth in media containing 10% FBS.

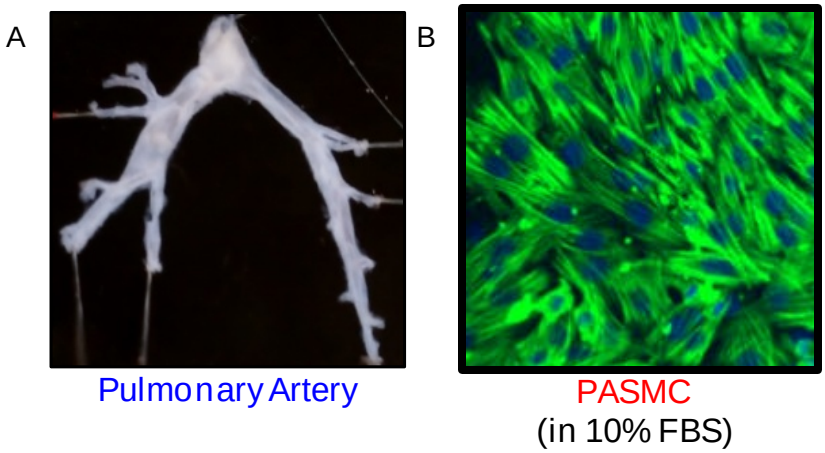

Figure 12: PA and primary culture PASMC isolated from normal male rat PA. A) PA was isolated from 5 lobes of the lung and fibroblasts were removed. B) Primary cultured cells were stained with specific antibody against smooth muscle α-actin (green fluorescence) and the nucleic acid dye DAPI (blue fluorescence). Scale line is 10x.

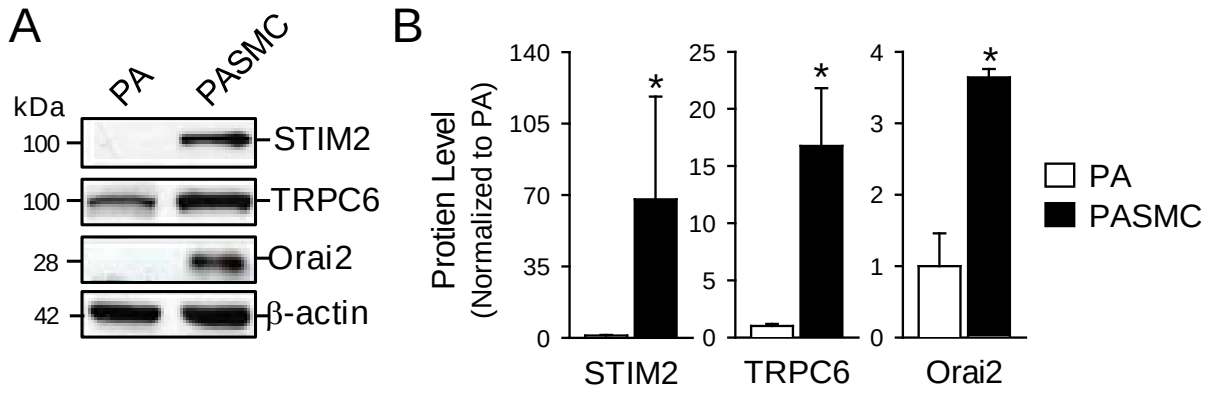

Figure 13: STIM2, TRPC6, and Orai2 expression is upregulated in proliferative pulmonary arterial smooth muscle cells (PASMC) compared to smooth muscle pulmonary artery tissue (PA). A. Representative Western blot images (left panels) and summarized data (right panels) for STIM2, TRPC6 and Orai2 proteins in cultured PASMC isolated and contractile PA tissue. β-actin was used as a loading control. Summarized data of STIM2, TRPC6 and Orai2 (B, right panel) protein expression level (mean±SE) in rat-PASMC (n=6). Graph shows protein expression of STIM2, TRPC6, Orai2 normalized to an average level in PA. *P<0.05 versus PA. STIM2, TRPC6 and Orai2 protein expression levels were increased in proliferating PASMC.

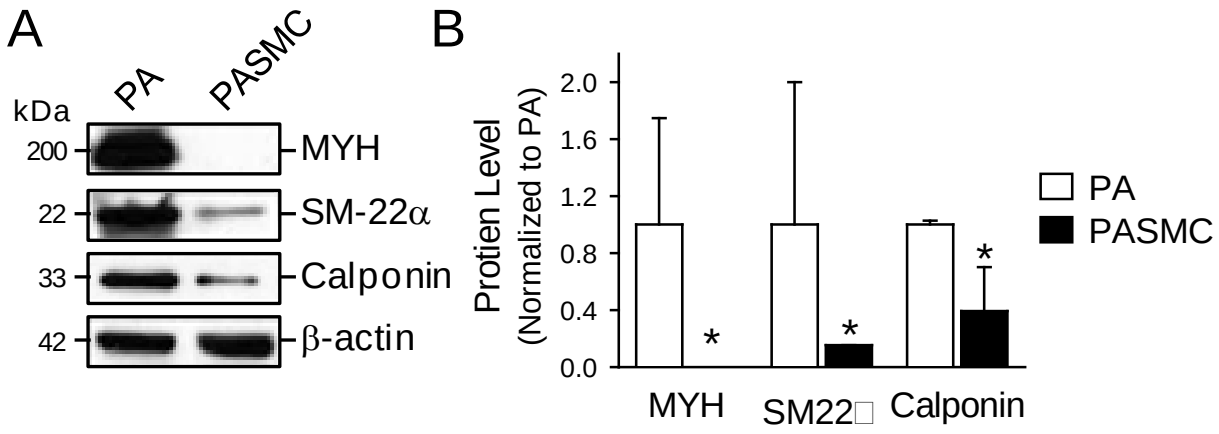

Figure 14: Contractile marker expression is downregulated in proliferative pulmonary arterial smooth muscle cells (PASMC) compared to smooth muscle pulmonary artery tissue (PA). A. Representative Western blot images (A) and summarized data (B) for MYH, SM22α and calponin proteins in cultured PASMC isolated and contractile PA tissue. β-actin was used as a loading control. Summarized data of MYH, SM22α, and calponin (B, right panel) protein expression level (mean±SE) in rat-PASMC (n=6). Graph shows protein expression of MYH, SM22α and calponin normalized to an average level in PA. *P<0.05 versus PA. MYH, SM22α, and calponin protein expression levels were decreased in Proliferating PASMC.

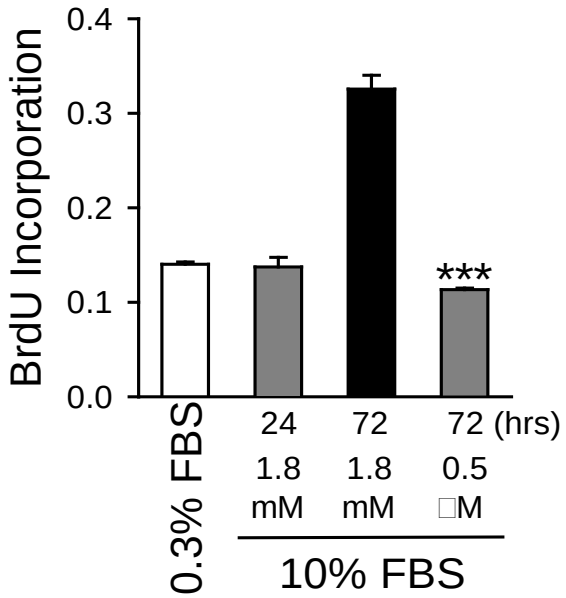

Figure 15: Inhibition of rat pulmonary arterial smooth muscle cell growth by chelating extracellular Ca^{2+}. Cells were cultured in 10% FBS-DMEM in the absence (Cont.) and presence of 2 mM EGTA. BrdU incorporation was measured after either 24 or 72 hours after cells were plated. Data are means ± SE (n=4 wells of cells/group). *** $P< 0.001$. Decreased [Ca^{2+}] inhibits PASMC proliferation.

C. Upregulated Expression STIM2, TRPC6, and Orai2 and Enhanced Store-Operated Ca^{2+} Entry in Proliferative Pulmonary Arterial Smooth Muscle Cells Compared to Contractile Pulmonary Arterial Smooth Muscle Cells

A rise in $[Ca^{2+}]_{cyt}$ due to Ca^{2+} release from intracellular Ca^{2+} stores and Ca^{2+} influx through SOC channels or VDCC is an important mechanism for increased $[Ca^{2+}]_{cyt}$. Fast, short-lasting increases in $[Ca^{2+}]_{cyt}$ through VDCC are important mechanisms for pulmonary vasoconstriction. However, sustained long lasting increases in $[Ca^{2+}]_{cyt}$, specifically through SOC channels, are important mechanisms for stimulation of PASMC proliferation. To determine whether plasma membrane Ca^{2+} channel activity was specific to PASMC phenotype, we measured Ca^{2+} influx through VDCC and SOC channels in both contractile and proliferative PASMC.

Raising the extracellular K^+ concentration, in the presence of extracellular Ca^{2+}, causes membrane depolarization, which subsequently increases the open probability of VDCC and promotes Ca^{2+} influx (Xu et al., 2008). In freshly dissociated contractile rat PASMC, application of high-K^+ (60 mM) solution resulted in a larger influx of Ca^{2+} through VDCC than in cultured proliferative rat PASMC (Fig. 16B, bottom panel). On the contrary, in the absence of extracellular Ca^{2+} (0Ca), extracellular application of SERCA inhibitor, cyclopiazonic acid (CPA, 10 μM), induces a transient increase in $[Ca^{2+}]_{cyt}$ due to leakage from the SR (first peak-release, Figure16B-top panel). Restoration of extracellular Ca^{2+} to 1.8 mM causes a second rise of $[Ca^{2+}]_{cyt}$ via SOCE. In cultured proliferative rat PASMC, application of CPA resulted in a larger influx of Ca^{2+} through SOC channels than freshly dissociated contractile PASMC (second peak-SOCE, Fig. 16B-top panel). These data suggest that the activity and/or expression levels of VDCC

and SOC channels correlate with PASMC phenotype. We can infer that a proliferative phenotype relies on SOC channels for an increase in $[Ca^{2+}]_{cyt}$, while a contractile phenotype relies on VDCC for an increase in $[Ca^{2+}]_{cyt}$.

The different gating mechanisms between SOC channels and VDCC channels could account for the cells' need to utilize one Ca^{2+} channel over the other, and could account for their increased function in each particular phenotype. A contractile phenotype may require fast activating Ca^{2+} channels (i.e., VDCC) to mediate contraction, whereas a proliferative phenotype may require a longer sustained activating Ca^{2+} channel (i.e., SOC channels) to mediate sustained proliferation. Additionally, this could suggest that the significantly increased $[Ca^{2+}]_{cyt}$ seen in proliferating PASMC is specifically due to increased expression levels or activation of SOC channels or SOC activator (i.e. STIM). Furthermore, the passive release of Ca^{2+} from SR stores was significantly increased under these conditions as seen in the first peak in Figure 16B (top panel). These results suggest there is an increase in the amount of Ca^{2+} leaking from the SR stores from proliferative PASMC; this could suggest that there is an increase of $[Ca^{2+}]$ in the SR.

Once it was apparent that SOC channels were important for increased $[Ca^{2+}]_{cyt}$ in proliferating PASMC, we went on to determine the protein expression levels of STIM2, Orai2, Orai3, and TRPC6 in contractile and proliferative PASMC. In proliferative PASMC, expression levels of STIM2, Orai2, Orai3, and TRPC6 were increased compared to contractile PASMC (n=4, Figure 17, left panel). Additionally, we compared relative expression of contractile marker, MYH, and proliferation marker, PCNA, to analyze the phenotypic state of these PASMC. As seen in Figure 18, freshly dissociated

PASMC have increased expression levels of MYH and decreased expression levels of PCNA, indicating that they are in a less proliferating and more contractile phenotypic state. Overall, these results reveal that dedifferentiated, proliferative PASMC mediated Ca^{2+} entry through SOCE and that STIM and SOC channels are a significant contributors for PASMC proliferation.

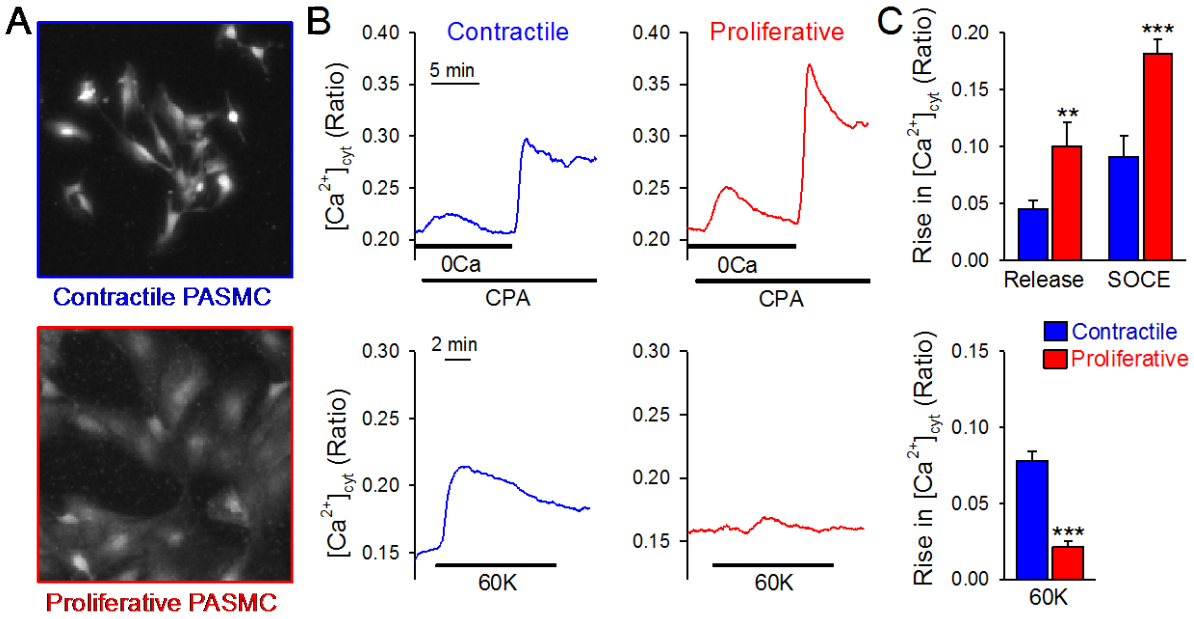

Figure 16: Enhanced store-operated Ca2+ entry (SOCE) in the proliferative phenotype of PASMC compared to the contractile PAMSC (freshly-dissociated PASMC). A: fura 2 fluorescence (F360) images (top) showing freshly dissociated contractile PASMC and (bottom) showing primary cultured PASMC from which $[Ca^{2+}]_{cyt}$ were measured. B: time courses of the CPA-induced $[Ca^{2+}]_{cyt}$ changes in the presence (1.8 mM) or absence (0 Ca) of extracellular Ca^{2+} in PASMC from contractile and proliferative PASMC (top) and 60K (60 mM K^{+})-Induced $[Ca^{2+}]_{cyt}$ changes in the presence of extracellular Ca^{2+} (bottom). C: summarized data (means ± SE) showing the release of Ca^{2+} and amplitudes of the CPA-induced transient increases in $[Ca^{2+}]_{cyt}$ ration (top) in the presence of extracellular Ca^{2+}, and summarized data showing the influx of Ca^{2+} after 60K-induced VDCC activation. ***P*< 0.01, ** *P*<0.01, *** *P*<0.001 vs. contractile phenotype.

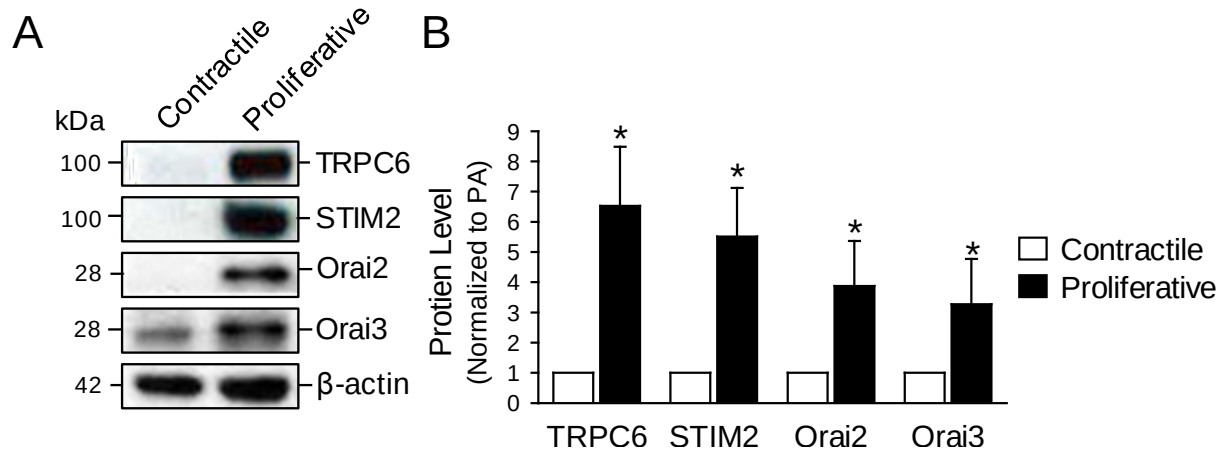

Figure 17: TRPC6, STIM2, Orai2 and Orai3 expression is upregulated in proliferative PASMC compared to freshly dissociated PASMC. A. Representative Western blot images (left panels) and summarized data (right panels) for TRPC6, STIM2, Orai2, and Orai3 proteins in PASMC isolated from contractile and proliferative phenotypes. β-actin was used as a loading control. Summarized data of STIM2, TRPC6, Orai2 and Orai3 (B, right panel) protein expression level (mean±SE) in rat-PASMC (n=6). Graph shows protein expression of STIM2, TRPC6, Orai2 and Orai3 normalized to an average level in Contractile PASMC. *P<0.05 versus Contractile PASMC. STIM2, TRPC6, Orai2 and Orai3 protein expression levels were increased in proliferating PASMC.

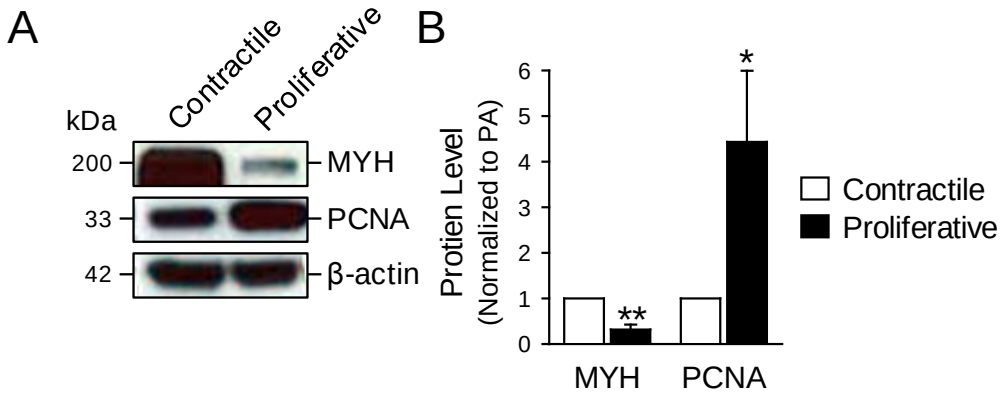

Figure 18: Contractile marker expression is downregulated while proliferative marker expression is upregulated in proliferative PASMC compared to freshly dissociated PASMC. A. Representative Western blot images (left panels) and summarized data (right panels) for MYH and PCNA proteins in rat PASMC isolated from contractile and proliferative PASMC. β-actin was used as a loading control. Summarized data of MYH (n=4) and PCNA (n=4) (b, right panel) protein expression level (mean±SE) in proliferative PASMC and contractile PASMC. Graph shows protein expression of MYH and PCNA normalized to an average level in contractile PASMC. **P*<0.05 vs. contractile PASMC. MYH protein expression level was decreased in proliferative PASMC, but PCNA protein expression level was increased in proliferative PASMC.

D. Low Serum-induced Pulmonary Arterial Smooth Muscle Cell Differentiation is Associated with Downregulation of STIM2, Orai2 and TRPC6

Removal of serum and growth factors from cultured medium abolished cell growth (n=6, Fig. 19 and Fig. 20). These data indicate that the cells cultured in smooth muscle basal medium with low serum (1.0% or 3% FBS) are growth-arrested cells or quiescent, while the cells cultured in smooth muscle growth medium (smooth muscle basal medium supplemented with 10% FBS and growth factors) are proliferating cells, confirming that serum-starvation can convert PASMC into a more differentiated phenotypic state. Previous studies have also utilized this method of differentiated SMC.

Subsequently, we measured the degree of expression of PCNA, a well-known indicator for the cell proliferation in growth arrested (differentiated) PASMC. As seen in Figure 19 (n=4, left pane), expression levels of PCNA are decreased in growth-arrested cells, further supporting the fact that growth-arrested PASMC are in a less proliferative phenotypic state. However, these data are not significant, but they do indicate a trend to decreased proliferation.

Since we had previously demonstrated that in cultured proliferative PASMC expression levels of STIM2, Orai2, Orai3, and TRPC6 were increased compared to freshly dissociated contractile PASMC (Fig. 21), we then sought to determine the expression levels of these proteins in growth-arrested differentiation PASMC. In addition to the level of PCNA expression in growth-arrested PASMC, we also examined whether dedifferentiated quiescent PASMC had decreased expression levels of STIM2, Orai2, and TRPC6 via both Western blot. The protein expression levels of TRPC6 were

significantly decreased (by 2.5 fold, n=5, left panel), while the expression levels of Orai2 (0.5 fold) and STIM2 (1.5 fold) were only slightly decreased in growth-arrested PASMC compared to proliferative PASMC (Fig. 21). Growth-arrested, differentiated PASMC demonstrated a decrease in STIM2 and SOC channels, thus suggesting these effectors play an active role in the proliferative phenotype PASMC.

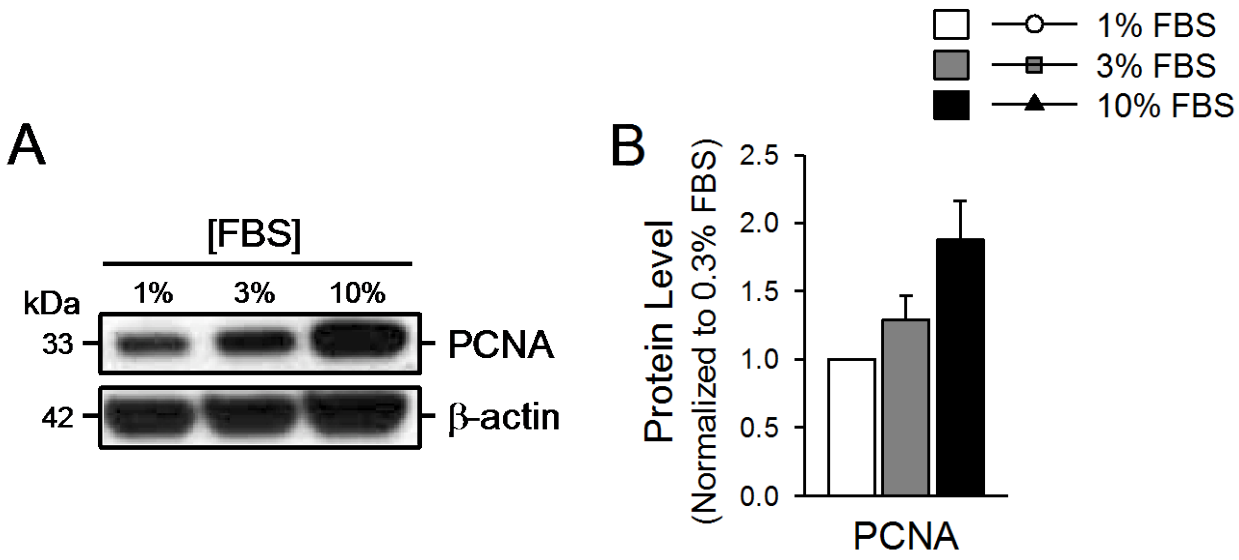

Figure 19: Low serum-induced inhibition of PASMC proliferation. Differential modulation of PASMC by low serum starvation decreases expression of proliferation indicator, PCNA. The protein expression of proliferating cell nuclear antigen (PCNA) is downregulated in growth arrested PASMC. A. Representative Western blot images (left panels) and summarized data (right panels) for PCNA protein (n=4) from growth arrested (1% or 3%) PASMC. β–actin was used as a loading control. Summarized data of PCNA (B, right panel) protein expression level (mean±SE) in growth arrested PASMC with 1% FBS, growth arrested PASMC with 3% FBS, proliferative PASMC with 10% FBS. Graph shows protein expression of PCNA normalized to an average level in 1% FBS growth arrested PASMC.

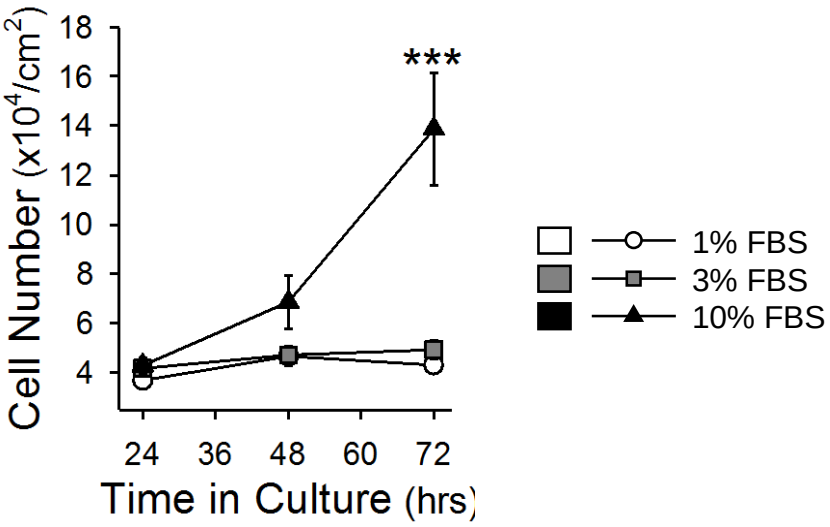

Figure 20: Low-serum-induced inhibition of PASMC Growth. Inhibition of rat PASMC growth by serum starvation. A: cells were cultured in 10%, 3%, or 1% FBS-M199. Viable cell numbers were determined 24, 48, or 72 hours after cells were plated. Data are means ± SE (*n* = 12 wells of cells/group). *** *P*<0.001 vs. 10% FBS-M199

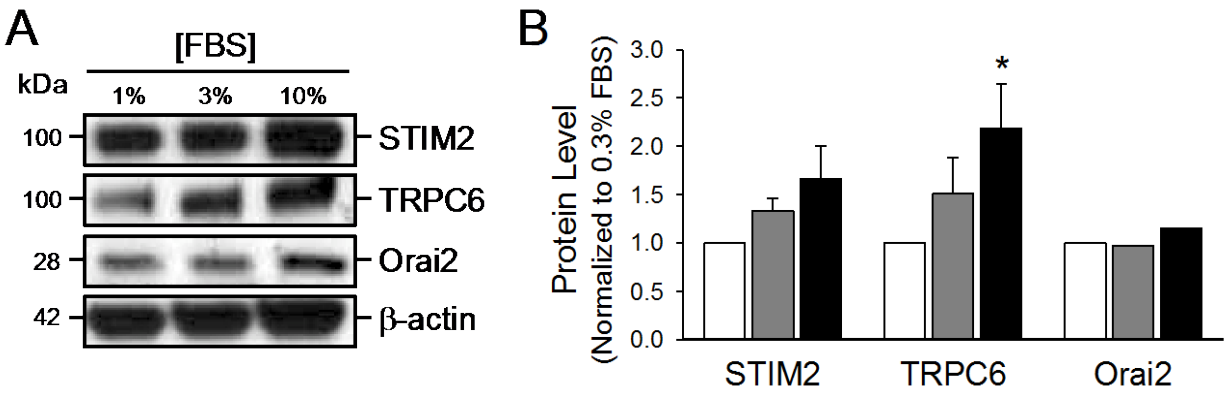

Figure 21: Low serum-induced PASMC differentiation is associated with downregulation of STIM2, TRPC6 and Orai2. The protein expression of STIM2, TRPC6 and Orai2 is downregulated in growth arrested PASMC. A. Representative Western blot images (left panels) and summarized data (right panels) for STIM2 (n=4), Orai2 (n=4) and TRPC6 (n=4) proteins from growth arrested (1% or 3%) PASMC. β-actin was used as a loading control. Summarized data of PCNA (B, right panel) protein expression level (mean±SE) in growth arrested PASMC with 1% FBS (n=6), growth arrested PASMC with 3% FBS (n=6), proliferative PASMC with 10% FBS. Graph shows protein expression of STIM2, Orai2 and TRPC6 normalized to an average level in 1% FBS growth arrested PASMC. *P<0.05 vs. 1% FBS growth arrested PASMC. STIM2, Orai2 and TRPC6 protein expression levels were higher in proliferative PASMC.

E. TGF-β-induced Pulmonary Arterial Smooth Muscle Cell Differentiation is Associated with Downregulation of STIM1, STIM2, TRPC6, Orai1 and Orai2

TGF-β is one of the most potent soluble growth factors that promote SMC differentiation (Deaton et al., 2005; Hu et al., 2003; Sinha et al., 2004; Tang et al., 2010). However, the exact effects of TGF-β on SMC proliferation remain highly controversial. It has been reported that TGF-β exerts a growth-inhibitory effect on VSMC by inducing cell cycle arrest at G_1 phase (Reddy and Howe, 1993; Seay et al., 2005). Conversely, some labs have shown that TGF-β promotes proliferation of cultured VSMC through activation of SMAD2 and the ERK/MAPK pathways (Stouffer and Owens, 1994; Suwanabol et al., 2012; Tsai et al., 2009). In our studies we use TGF-β to differentiate cultured PASMC from a contractile phenotype to a proliferative phenotype. Our studies demonstrated that treatment with 1, 2, or 3 ng/mL of TGF-β in the presence of 10% serum decreased PASMC proliferation compared to vehicle control after 48 and 72 hours (n=6, Fig. 22). These studies suggest that cells cultured in smooth muscle basal medium with serum and TFG-β are growth-arrested and differentiated, while the cells cultured in smooth muscle growth medium without TGF-β are dedifferentiated.

Additionally, we compared the degree of expression of contractile markers, calponin and SM22α, and proliferation indicator, PCNA, to PASMC phenotype. As seen in Figure 23 (n=6, left panel), after treatment with TGF-β (1 or 2 ng/mL) 72 hrs, PASMC expressed increased levels of calponin and SM22α and decreased expression levels of PCNA, which is consistent with decreased proliferation. These studies validate that TGF-β dedifferentiates PASMC into contractile phenotypic state.

We then wanted to determine whether dedifferentiated quiescent PASMC by TGF-β treatment also express decreased levels of STIM2, Orai2, and TRPC6. The protein expression levels of TRPC6 and STIM2 were significantly decreased, while the expression levels of STIM1 were not altered in TGF-β treated PASMC (n=5, Fig. 25). Furthermore, the protein expression levels of Orai1 and Orai2 were slightly decreased in TGF-β treated PASMC (n=5, Fig. 26). These data correlate the increased expression of STIM2 and SOC channels, Orai2 and TRPC6 to the proliferative phenotype of PASMC.

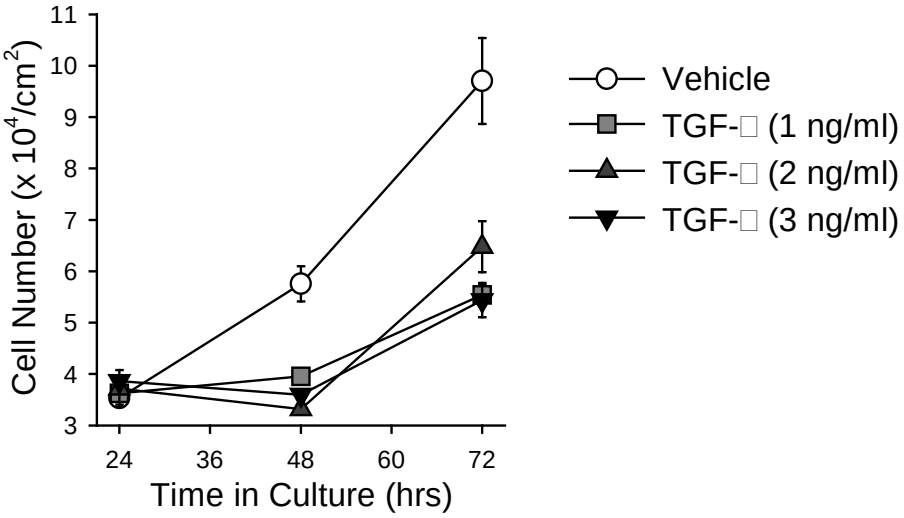

Figure 22: TGF-β inhibits PASMC proliferation. A: cells were cultured in 10% FBS-M199 with vehicle control, 1, 2, or 3 ng/ml. Viable cell numbers were determined 24, 48 or 72 hours after cells were plated. Data are means±SE (*n*=6 wells of cells/group). ****P*<0.001 vs. vehicle control.

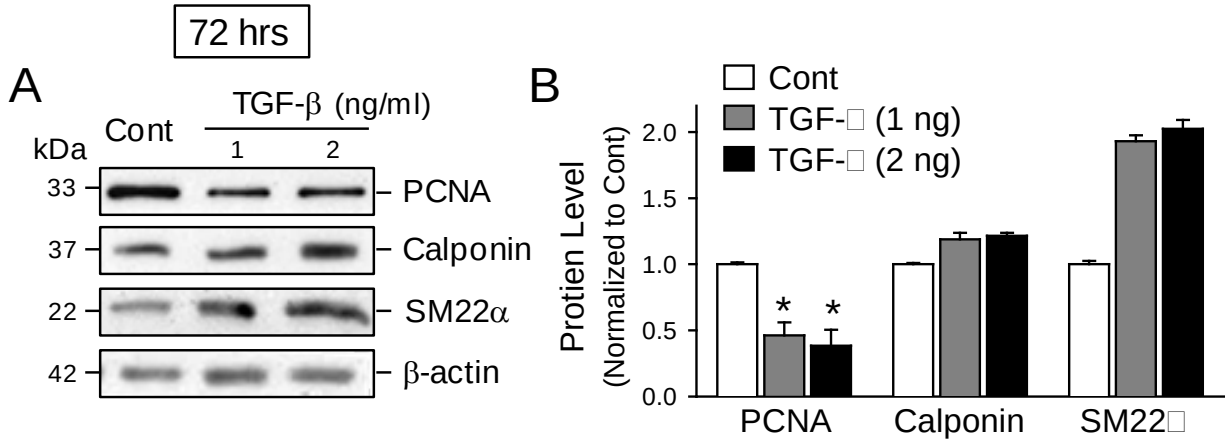

Figure 23: TGF-β inhibits PASMC proliferation and caused PASMC differentiation. Differential modulation of PASMC by TGF-β treatment decreases the protein expression levels of proliferative indicator, PCNA, while increasing the protein expression levels of contractile marker calponin and SM22α in culture PASMC. A. Representative Western blot images (left panels) and summarized data (right panels) for PCNA, calponin, and SM22α proteins from TGF-β treated (vehicle control, 1 or 2 ng/mL) PASMC. β-actin was used as a loading control. Summarized data of PCNA, calponin, and SM22α (B, right panel) protein expression level (mean±SE) in TGF-β treated PASMC with vehicle-control (n=6), 1 ng/mL TGF-β treatment (n=6) and with 2 ng/mL TGF-β treatment (n=6). Graph shows protein expression of PCNA, calponin, and SM22α normalized to an average level in vehicle control PASMC. *P<0.05 vs. vehicle control PASMC. PCNA protein expression levels were decreased in TGF-β treated PASMC, while contractile markers, calponin and SM22α were increased.

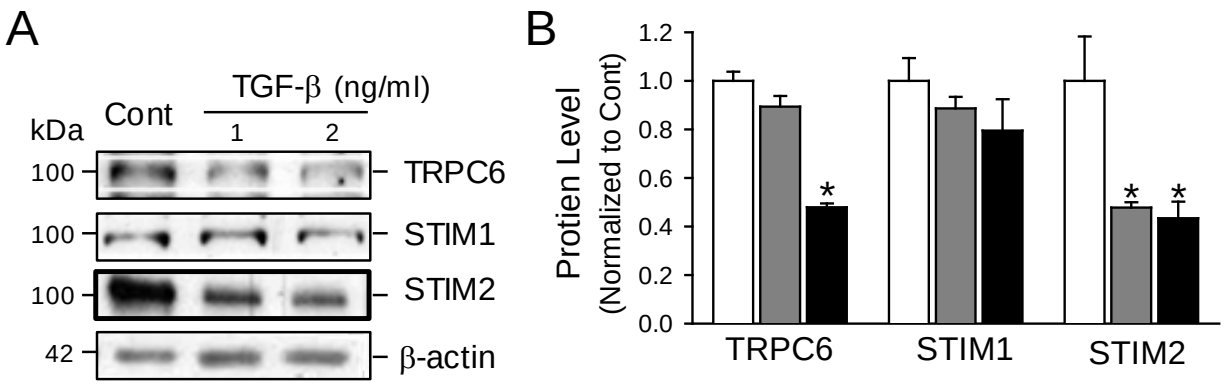

Figure 24: TGF-β-induced PASMC differentiation is associated with downregulation of TRPC6, STIM1 and STIM2. Differential modulation of PASMC by TGF-β treatment decreases the protein expression levels of TRPC6, STIM1, and STIM2 in cultured PASMC. A. Representative Western blot images (left panels) and summarized data (right panels) for TRPC6, STIM1, and STIM2 proteins from TGF-β treated (vehicle control, 1 or 2 ng/mL) PASMC. β-actin was used as a loading control. Summarized data of TRPC6, STIM1, and STIM2 (B, right panel) protein expression level (mean±SE) in TGF-β treated PASMC with vehicle-control (n=6), 1 ng/mL TGF-β treatment (n=6) and with 2 ng/mL TGF-β treatment (n=6). Graph shows protein expression of TRPC6, STIM1 and STIM2 normalized to an average level in vehicle control PASMC. *P<0.05 vs. vehicle control PASMC. TRPC6, STIM1 and STIM2 protein expression levels were decreased in TGF-β treated PASMC.

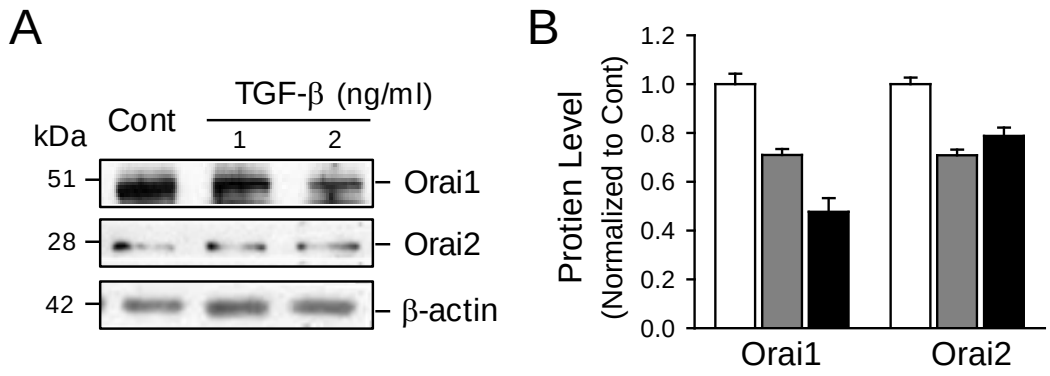

Figure 25: TGF-β-induced PASMC differentiation is associated with downregulation of Orai1 and Orai2. Differential modulation of PASMC by TGF-β treatment decreases the protein expression levels of Orai1 and Orai2 in cultured PASMC. A. Representative Western blot images (left panels) and summarized data (right panels) for Orai1 (n=4) and Orai2 (n=4) proteins from TGF-β treated (vehicle control, 1 or 2 ng/mL) PASMC. β-actin was used as a loading control. Summarized data of Orai1 and 2 (B, right panel) protein expression level (mean±SE) in TGF-β treated PASMC with vehicle-control (n=6), 1 ng/mL TGF-β treatment and with 2 ng/mL TGF-β treatment. Graph shows protein expression of Orai1 and 2 normalized to an average level in vehicle control PASMC. Orai1 and 2 protein expression levels were slightly decreased in TGF-β treated PASMC.

F. Heparin-induced Differentiation is Associated with Downregulation of STIM1, STIM2, TRPC6, Orai1, Orai2 and Orai3

PASMC proliferation has long been considered to be a key point in the remodeling of the vascular wall following vascular injury. It is well established that antiproliferative heparins significantly inhibit pulmonary vascular remodeling induced by PASMC proliferation in culture. Heparin inhibits PASMC growth *in vitro* and *in vivo* (Garg et al., 2003; Joseph et al., 1997), however, the mechanisms leading to antiproliferative effects of heparin are unknown. There are some evidences that heparin inhibits cell proliferation by inhibiting the activation of protein kinase C (PKC), activator protein 1 (AP-1), c-fos, and c-jun (Kazi et al., 2002). Recently, it has been determined that p27 plays a critical role in heparin inhibition of PASMC proliferation *in vitro* and PH *in vivo* (Yu et al., 2006). Consistent with previous studies, we characterized the PASMC phenotype after heparin treatment for 72 hours. As seen in Figure 26, following treatment with heparin for 72 hours, PASMC expressed increased levels of contractile markers, calponin, and SM22α and decreased expression levels of proliferation marker, PCNA, indicating that the PASMC were in a less proliferating and more contractile phenotypic state.

Next, we determined whether differentiated quiescent PASMC, by heparin treatment, had decreased expression levels of STIM2, Orai2, and TRPC6. The protein expression levels of STIM1 were significantly decreased, while the expression levels of STIM2 and TRPC6 were trending towards decreased expression levels in differentiated PASMC via heparin treatment compared to proliferative PASMC (n=4, Fig. 27). Furthermore, the protein expression levels of Orai1, Orai2, and Orai3 were decreased in

growth-arrested PASMC via heparin treatment compared to proliferative PASMC (n=4, Fig. 28A). Considering the fact that proliferative PASMC have increased expression levels of SOC channels responsible for increased $[Ca^{2+}]_{cyt}$, we can infer that STIM2 and SOC channels Oria1 and TRPC6 may mediate a sustained increase of $[Ca^{2+}]_{cyt}$ and thus initiate a PASMC phenotypic switch to a more proliferative phenotype.

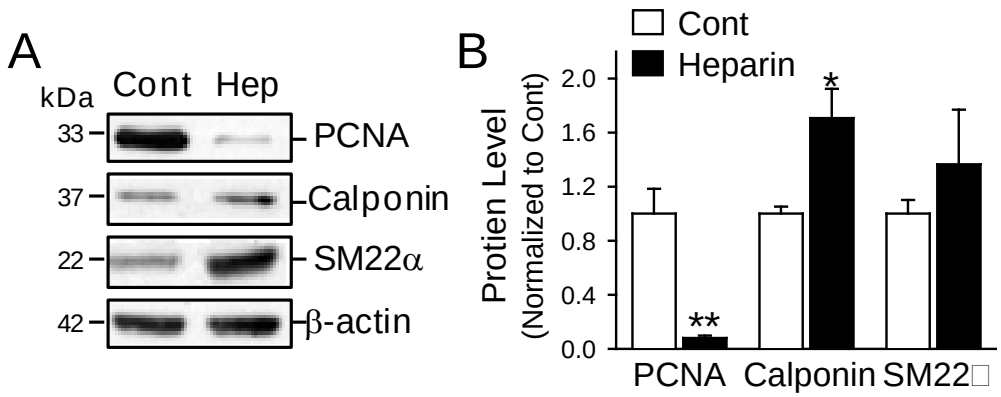

Figure 26: Heparin inhibits PASMC proliferation and causes PASMC differentiation. Differential modulation of PASMC by low-serum and heparin decreases the expression levels of proliferation indicator, PCNA, while increasing the expression levels of calponin and SM22α in cultured rat PASMC. A. Representative Western blot images (A) and summarized data (B) for PCNA (n=4), calponin (n=4), and SM22α (n=4) proteins in PASMC treated with heparin for 72 hrs (vehicle control or Hep). β-actin was used as a loading control. Summarized data of PCNA, calponin, and SM22α (B, right panel) protein expression level (mean±SE) in Heparin-treated PASMC or control. Graph shows protein expression of PCNA, calponin, and SM22α normalized to an average level in control PASMC. **P*<0.05, ***P*<0.01 vs. vehicle control PASMC. PCNA protein expression levels were decreased in Heparin-treated PASMC, while contractile markers, calponin and SM22α were increased.

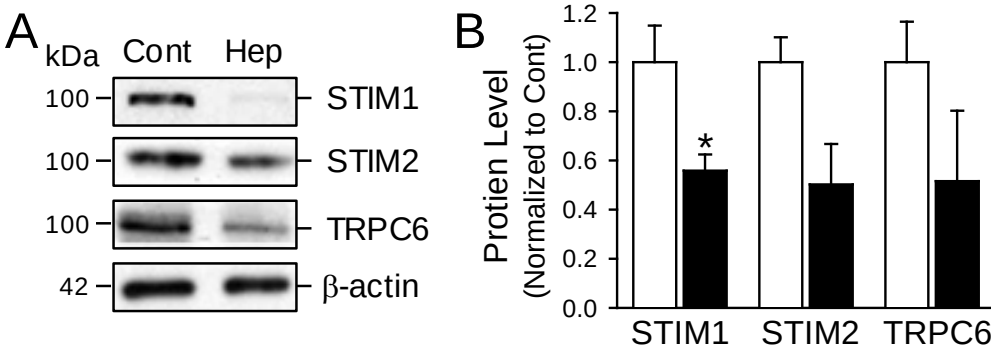

Figure 27: Heparin-induced PASMC differentiation is associated with downregulation of STIM1, STIM2 and TRPC6. Differential modulation of PASMC by low-serum and heparin decreases the expression levels of STIM1, STIM2, and TRPC6 in cultured rat PASMC. A. Representative Western blot images (A) and summarized data (B) for STIM1 (n=4), STIM2 (n=4), and TRPC6 (n=4) protein from PASMC treated with heparin for 72hrs (vehicle control or Hep). β-actin was used as a loading control. Summarized data of STIM1, STIM2 and TRPC6 (B, right panel) protein expression level (mean±SE). Graph shows protein expression of STIM1, STIM2 and TRPC6 normalized to an average level in control PASMC. *P<0.05 vs. vehicle control PASMC. STIM1, STIM2 and TRPC6 protein expression levels were decreased in heparin-induced PASMC differentiation.

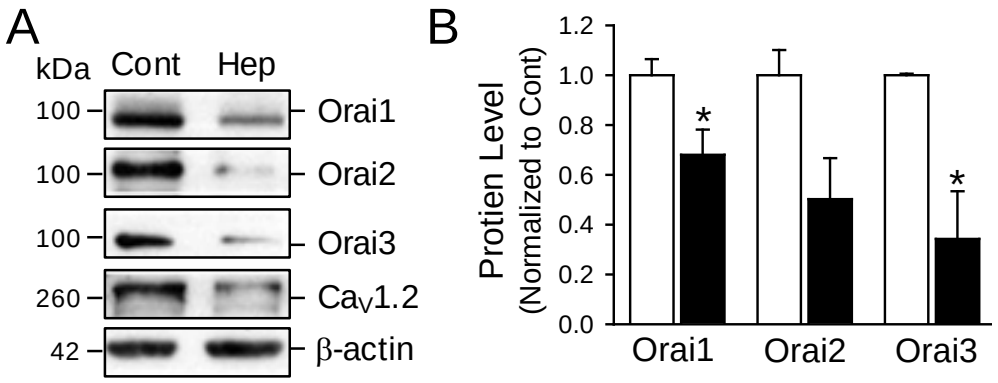

Figure 28: Heparin-induced PASMC differentiation is associated with downregulation of Orai1, Orai2, and Orai3. Differential modulation of PASMC by low-serum and heparin decreases the expression levels of Orai1, Orai2 and Orai3 in cultured rat PASMC. A. Representative Western blot images (A) and summarized data (B) for Orai1, 2 and 3 proteins from PASMC treated with heparin or control (vehicle control or Hep). β-actin was used as a loading control. Summarized data of Orai1 (n=4), Orai2 (n=4) and orai3 (n=4) (B) protein expression level (mean±SE). Graph shows protein expression of Orai1, 2 and 3 normalized to an average level in control PASMC. *P<0.05 vs. vehicle control PASMC. Orai1, 2 and 3 protein expression levels were decreased in Heparin-treated PASMC.

G. Orai2 is Necessary for Enhanced Store-operated Ca^{2+} Entry in Highly Proliferating Pulmonary Arterial Smooth Muscle Cells

Enhanced SOCE contributes to an increase in $[Ca^{2+}]_{cyt}$ by either increasing the numbers of SOC channels or by increasing the open probability of the channels. Orai are membrane-spanning Ca^{2+} channels that form tetrameric SOC channels in the plasma membrane and are demonstrated to be the major channels responsible for SOCE. As previously demonstrated, proliferating rat PASMC expresses increased levels of Orai family members (Orai1, 2, and 3). To determine whether Orai2 proteins were necessary for enhance SOCE in proliferating PASMC, we used si-RNA mediated downregulation of Orai2. To measure SOCE in proliferating PASMC, Ca^{2+} stores were depleted in the absence of extracellular Ca^{2+} (0Ca) with CPA (10 μm), a specific reversible SERCA pump inhibitor (first peak, labeled release). Then, following store depletion, Ca^{2+} (1.8 mM) was added back to extracellular space and a rise in $[Ca^{2+}]_{cyt}$ through SOC channels was measured (second peak, labeled SOCE). In cultured proliferating PASMC, a larger SOCE is induced following SR Ca^{2+} store depletion with CPA (10 μM) in control (scramble siRNA) compared to PASMC treated with siRNA for Orai2 (n=12, Fig. 29, top left panel). These results indicate that Orai2 is essential for activation of SOCE in proliferating PASMC, and the increased Orai2 plays an important role in contributing to an increase of $[Ca^{2+}]_{cyt}$, and thus leads to a phenotypic switch of PASMC. Furthermore, the passive release of Ca^{2+} from SR stores was not significantly different under these conditions. Suggesting there is no difference in the amount of Ca^{2+} leaking from the SR stores (first peak, release, Fig. 30, top panel).

Transient transfection with Orai2 siRNA resulted in a 50% knockdown of Orai2 protein expression levels as seen in Figure 30 (n=12). Similarly, we measured a decrease in expression levels of proliferating-marker, PCNA, in PASMC transfected with siRNA for Orai2, thus validating that increased Orai2 is necessary for increased proliferation of PASMC.

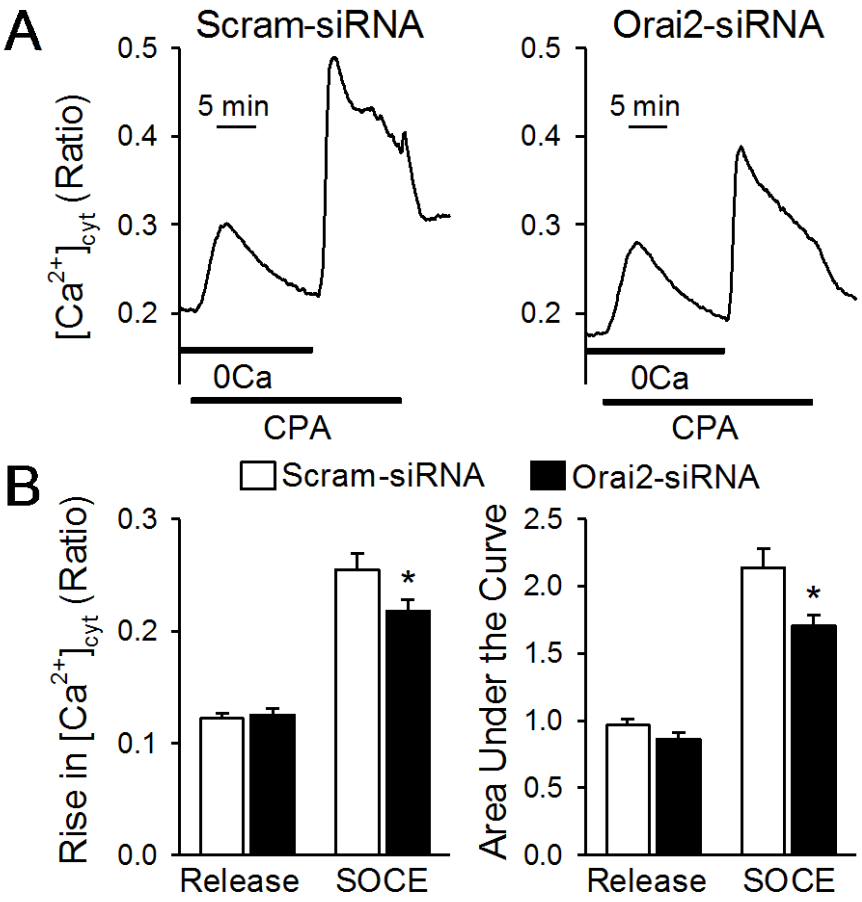

Figure 29: downregulation of Orai2 in PASMC by siRNA inhibits SOCE. A. Representative records show CPA-induced changes in $[Ca^{2+}]_{cyt}$ in the absence or presence of extracellular Ca^{2+} in control rat-PASMC (Scram-siRNA, left panel) and rat-PASMC treated with siRNA targeting Orai2 (Orai2-siRNA, right panel). SOCE (indicated by the CPA-induced increase in $[Ca^{2+}]_{cyt}$ when extracellular Ca^{2+} is restored) is induced by the passive depletion of SR Ca^{2+} using 10 μM CPA. B. Summary data (mean±SE) showing changes in CPA-induced increase in $[Ca^{2+}]_{cyt}$, immediately following the re-addition of Ca^{2+} after store depletion (indicative of SOCE) in scram-PASMC (white bar), Orai2 siRNA-PASMC (black bar). *P>0.05 vs. scram-siRNA.

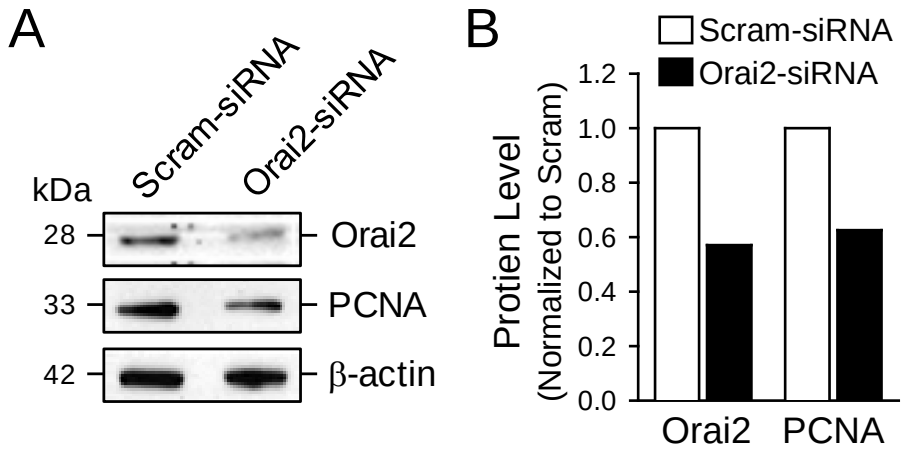

Figure 30: Downregulation of Orai2 in proliferative PASMC by siRNA inhibits proliferation. Knockdown of Orai2 in proliferative rat PASMC with siRNA decreased protein expression of Orai2 (n=1) and proliferation indicator, PCNA. A. Western blot representative image of Orai2 protein expression in control-PASMC (Scram-siRNA), and Orai2 targeted siRNA-PASMC (Orai2-siRNA). Scram-siRNA-PASMC (white bar) and Orai2-siRNA-PASMC (black).

H. Overexpression of STIM2 is Sufficient to Enhance Store-Operated Ca^{2+} Entry in Highly Proliferating Pulmonary Arterial Smooth Muscle Cells

STIM functions as a SR/ER membrane-bound sensor of [Ca^{2+}] in the SR/ER. When the SR is depleted, STIM proteins dimerize then translocate to the plasma membrane-SR/ER junction where they cluster and recruit Orai subunits to form SOC channels and induce SOCE. The role of STIM2, Orai2, and TRPC6 in the proliferative phenotype of PASMC is still not fully understood. We thus determined the involvement of STIM2 in the regulation of SOCE in proliferating PASMC. As previously demonstrated, proliferating rat PASMC express increased levels of STIM2.

To examine enhanced SOCE, Ca^{2+} stores were depleted in the absence of extracellular Ca^{2+} (0CA) with CPA (10 μM). Then, following store depletion, Ca^{2+} (1.8 mM) was added back to extracellular space and a rise in $[Ca^{2+}]_{cyt}$ through SOC channels was measured (second peak, labeled SOCE). In cultured proliferating PASMC, a larger SOCE was induced following SR Ca^{2+} store depletion with CPA in control (empty vector) compared to transiently transfected PASMC with the STIM2 vector (n=30, Fig. 31, top right panel). These results indicate that STIM is sufficient for activation of SOCE in proliferating PASMC, as well as that STIM2 and SOCE play an important role in PASMC proliferation. Furthermore, the passive release of Ca^{2+} from SR stores was significantly different under these conditions, suggesting that increased STIM expression levels are important for maintaining a normal basal level of $[Ca^{2+}]_{SR}$, and may explain for variations in passive release of Ca^{2+} in proliferative PASMC compared to contractile PASMC. To determine whether STIM2 is sufficient to enhance SOCE in proliferating PASMC, we overexpressed STIM2 in cultured PASMC. Transfection with

STIM2 plasmid resulted in a 30-fold increase in the level of expression of STIM2 as seen in Figure 32 (n=1).

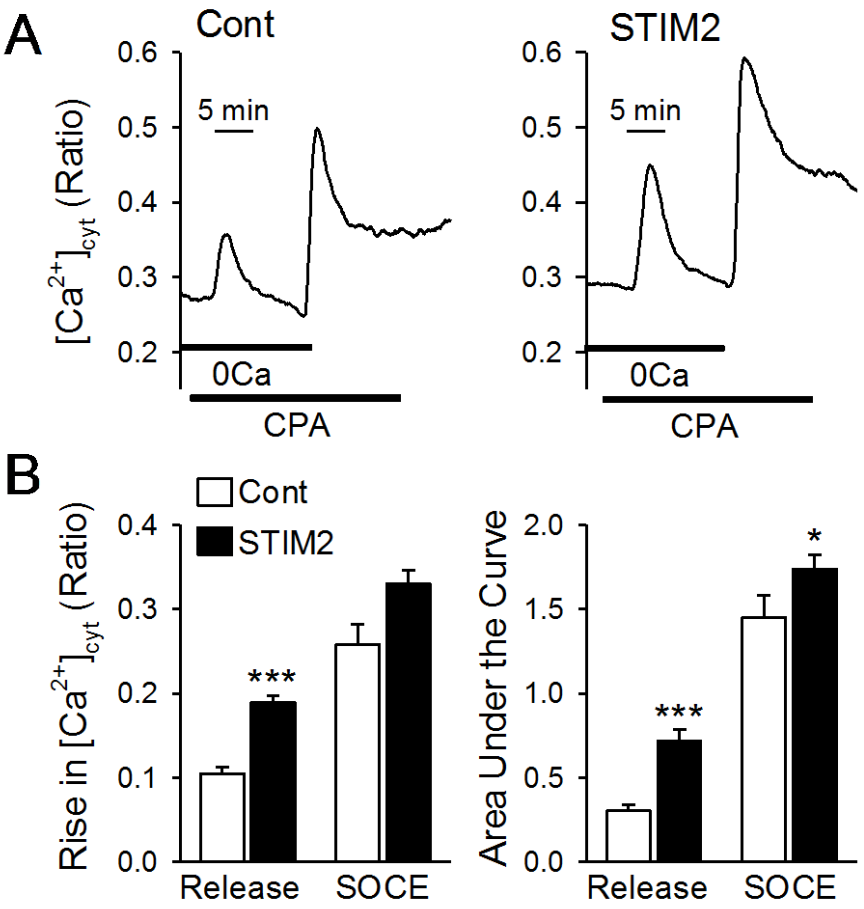

Figure 31: Upregulated protein expression of STIM2 is sufficient to enhance SOCE. Overexpression of A. Representative records showing changes in $[Ca^{2+}]_{cyt}$ before, during, and after the application of CPA (10 μM), in the absence or presence of extracellular Ca^{2+} in the vector control (left panel) and STIM2-transfected (right panel) rat-PASMC. SOCE was induced by the passive depletion of SR Ca^{2+}, using CPA. B. Summary data (mean±SE) showing changes in $[Ca^{2+}]_{cyt}$ ratio immediately following the addition of CPA in the absence of extracellular Ca^{2+}, which reflects SR Ca^{2+} release (SR Ca^{2+} Release, left bars), in control (white) and STIM2-transfected (black) rat-PASMC. There was an increase in magnitude of SR Ca^{2+} release between the two groups. **P*<0.05, ****P*<0.001 vs. empty-vector control.

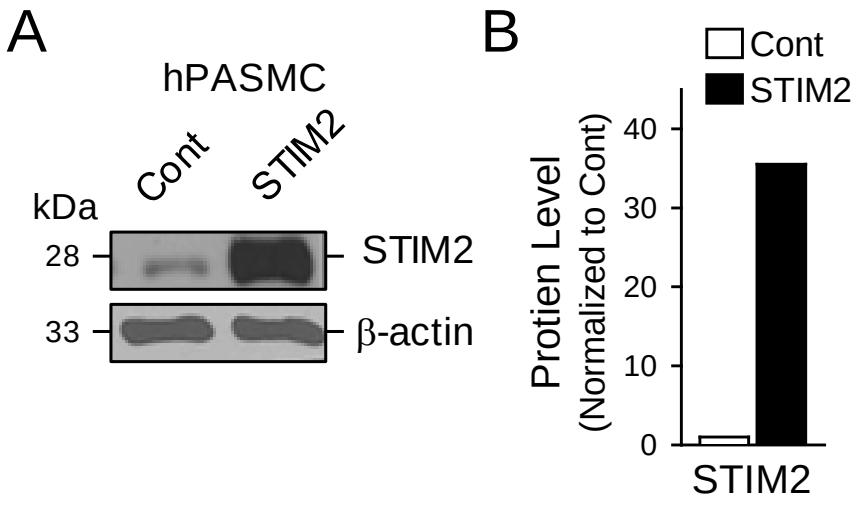

Figure 32: Overexpression of STIM2 increased the protein expression levels of STIM2. A. Western blot representative image of STIM2 protein expression in control-PASMC (Cont), and STIM2-overexpressing PASMC (STIM2). Control-PASMC (white bar) and STIM2-overexpressing PASMC (black).

I. Pulmonary Vasoconstriction due to Ca^{2+} Influx through Voltage-Dependent Ca^{2+} Channels is Greater than Vasoconstriction due to Ca^{2+} Influx through Store-Operated Ca^{2+} Entry

In order to determine which plasma membrane Ca^{2+}-permeable channel, VDCC, specifically L-type ($Ca_V1.2$) or SOC channels, was more important in inducing PA ring contraction, we compared 40K-induced active tension to that of CPA-induced (10 μM) active tension. As stated earlier, perfusion of PA rings with 40K (40 mM K^+) solution induces Ca^{2+} influx through VDCC, while CPA solution induces Ca^{2+} influx through SOC channels. These results show that PA contraction induced by increased $[Ca^{2+}]_{cyt}$ through 40K-mediated membrane depolarization, resulting in activation of VDDC, is greater that PA contraction due to SOCE (n=1, Fig. 33). Consistent with the previous findings in our lab (Kunichika et al., 2004), these observations indicate that the degree of vascular contractility and vasoconstrictive response to specific agonists are dependent on whether which plasma membrane Ca^{2+}-permeable channel, VDCC or SOC channels, are recruited. These data imply that VDCC contribution is far more efficient at inducing PA contraction than SOC channels. This difference is likely attributed to the differences in the gating, open probability, and inactivation kinetics of these channels. Through activation of VDCC via depolarization of the plasma membrane provides a faster influx of Ca^{2+} resulting in larger and faster increases of $[Ca^{2+}]_{cyt}$, resulting in a larger and stronger contractile force. Conversely, CPA activation of SOC channels provides a slow but longer sustained increase in $[Ca^{2+}]_{cyt}$, resulting in a weak generation of contraction of the PA. These data also suggest that an increase in $[Ca^{2+}]_{cyt}$ through SOCE is not only important to weakly induce contraction but may also

be important for maintaining a sustained increase in $[Ca^{2+}]_{cyt}$, which may contribute to activation of Ca^{2+}-dependent signals, which are responsible for maintenance of proliferation.

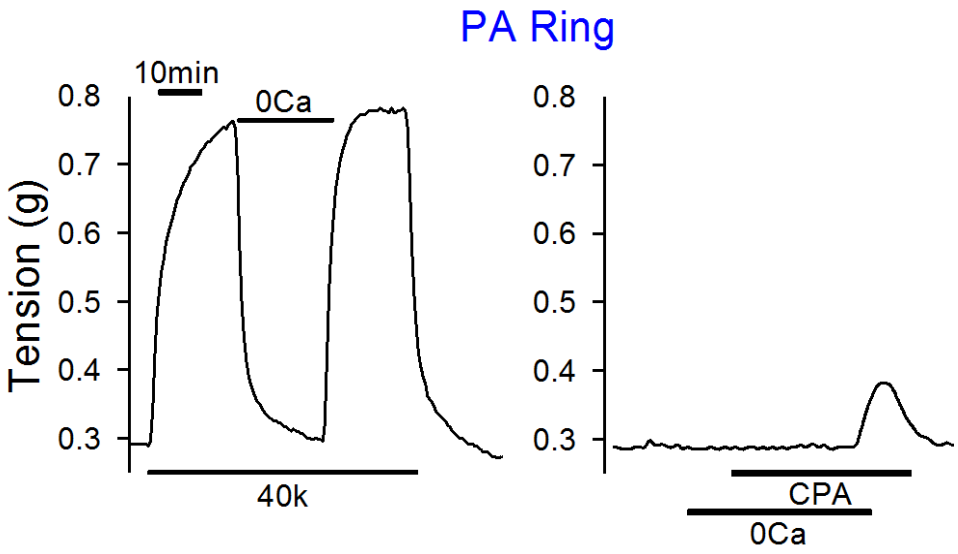

Figure 33: Vasoconstriction due to VDCC Ca^{2+} influx is greater than vasoconstriction due to SOCE. Isometric tension was measured in isolated PA rings from rat. In the absence of Ca^{2+}, (0Ca) causes vasodilatation in a rat pulmonary artery. A. Representative tension record showing 40K-induced active tension before and during the application of Ca^{2+} free solution (0Ca) (left panel, n=1) and CPA-induced active tension before and during the application of Ca^{2+} free solution (0Ca) (right panel, n=1).

J. Expression of STIM2 and Orai2 is Upregulated in Pulmonary Arteries Isolated from Animals with Monocrotaline-Induced Pulmonary Hypertension

Our *in vitro* data demonstrated an upregulation of STIM2 and Orai2 and augmented SOCE in proliferative PASMC. To investigate whether STIM2 can be a target for treatment for PAH, we used the severe monocrotaline (MCT)-induced rat model of pulmonary hypertension (MCT-PH). MCT-PH displays increased PASMC proliferation, migration, and contraction through, at least partially, a Ca^{2+}-dependent mechanism (Kuang et al., 2010). In rats with more severe MCT-PH (4 weeks after initial injection of MCT), we examined and compared protein expression level of STIM2 and Orai2 in PA. As shown in Figure 34, the protein expression levels of STIM2 and Orai2 from rat PA with MCT-PH were significantly greater than in PA isolated from vehicle control (Cont.). Our lab previously established and defined the *in vivo* effects and perimeters of MCT-PH. We examined and compared the right ventricular systolic pressure (RVSP), the Fulton index (i.e. the ratio of right ventricle/left ventricle + septum, RV/(LV+S)), and the muscularization of distal pulmonary arteries in control rats and MCT-injected rats. Injection of MCT (60 mg/kg) in rats significantly increased RVSP and caused right ventricular hypertrophy compared with control rats injected with vehicle (DMSO) (Fig. 35). The *in vivo* animal experiments were consistent with the *in vitro* experiments demonstrating increased protein expression levels of STIM2 and Orai2. Furthermore, our lab previously confirmed that PA isolated of HPH rats also had increased expression levels of STIM2 and Orai2 compared to normoxic control PASMC. These data were also consistent with our previous findings from our lab, demonstrating

that STIM2 and Orai2 protein expression levels were increased in PASMC from IPAH patients. Together our data strongly suggest that increased expression and function of STIM2 and Orai play a crucial pathogenic role in the development of pulmonary vascular remodeling by initiating and/or maintaining the proliferative phenotype of PASMC.

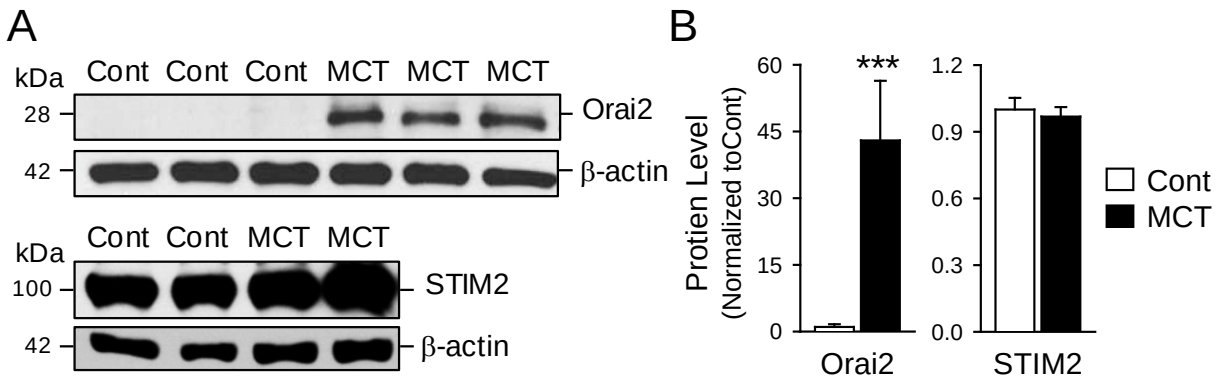

Figure 34: STIM2 and Orai2 are upregulated in PA smooth muscle from rats with MCT-induced PH for 4 weeks. A. Representative Western blot analyses for STIM2 (n=3) and Orai2 (n=2) in Control and MCT-induced PH PASMC. β-actin was used as loading control. B. Summarized data showing protein expression levels of STIM2 and Orai2 in Control and MCT-induced PH PASMC. Graph shows protein expression of Orai2 and STIM2 normalized to average level in control PASMC. ****P*<0.001 vs. Con.

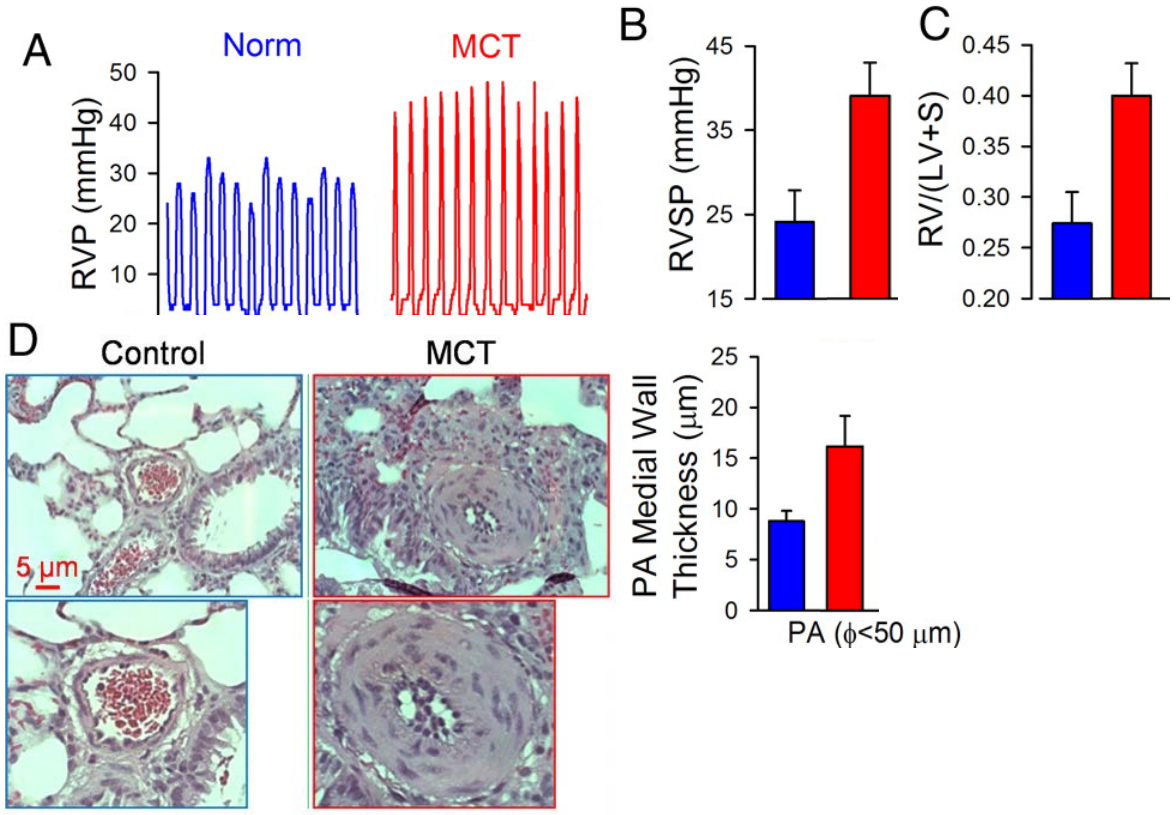

Figure 35: Injection of MCT in rats significantly increased RVSP and caused right ventricular hypertrophy compared with Norm rats injected with vehicle. MCT treatment promotes the development of pulmonary vascular remodeling and pulmonary hypertension in rats. A and B, Representative record of right ventricular pressure (RVP, A) and summarized data (mean±SE) showing the peak value of right ventricular systolic pressure (RVSP, B) in normal control rats (norm, n=6) and MCT-injected rats (MCT, n=6). C, Averaged Fulton index [RV/(LV+S) ratio, mean±SE] showing that RV hypertrophy is significantly inhibited in MCT-rats. D, representative hematoxylin and eosin images of small pulmonary arteries (D) and summarized data of the medial thickness of pulmonary arteries with a diameter (Ø) less than 50 μm, between 50 in normal control rats (norm, n=6) and MCT-injected rats (MCT, n=6). *P<0.05 versus MCT-treated rats (red bars). Image was taken from and modified with permission from Yamamura A, Guo Q, Yamamura H, Zimnicka AM, Pohl NM, Smith KA, Fernandez RA, Zeifman A, Makino A, Dong H, and Yuan JX. (2012). Enhanced Ca^{2+}-sensing receptor function in idiopathic pulmonary arterial hypertension. *Circ Res.* 3;111(4):469-81.

K. Knockout of Akt1 and Akt2 Inhibits Pulmonary Arterial Smooth Muscle Proliferation and Downregulates STIM2 and Orai2 Expression in Whole Lung Tissues of Mice

To further understand the role of STIM2 and Orai2 in proliferating PASMC, and its contribution to enhanced SOCE, we examined the expression of STIM2 and Orai2 in whole lung tissues from Akt1 (*Akt1*$^{-/-}$) and Akt2 (*Akt2*$^{-/-}$) knockout mice. Akt is part of the serine/threonine-specific kinase family known for promoting cell survival through inhibition of apoptotic pathways and promotion of gene expression. It also contains a pleckstrin homology (PH) domain that binds to PIP_3 and PIP_2 after conversion of PIP_2 by PI3K. Akt promotes cell survival directly by phosphorylation of proapoptotic proteins such as the Forkhead transcription factor family FOXO1a and 3a and by phosphorylation of proteins such as STAT transcription factors (Moylan et al., 2008). We examined the involvement of the Akt pathway in the regulation of STIM2 and Orai2 proteins in whole lung tissues from Akt1 and Akt2 knockout mice. We also evaluated PASMC proliferation from cultured cells isolated from wild type, *Akt1*$^{-/-}$ and *Akt2*$^{-/-}$ knockout mice. As demonstrated in Figure 36, knockout of Akt1 significantly inhibits proliferation in isolated PASMC compared to wild type. However, in mice with knocked down Akt2, the decrease in proliferation was not significant. This suggests that Akt1, but not Akt2, plays a role in regulation of PASMC proliferation. Additionally, 24 hour hypoxia treatment (3% O_2) increased proliferation, as expected, however, PASMC from *Akt1*$^{-/-}$ demonstrated significant inhibition of increased proliferation, while PASMC from *Akt2*$^{-/-}$ only demonstrated a slight inhibition, after hypoxia treatment. Furthermore, we were able to demonstrate a drastic decrease in Orai2 expression levels in *Akt1*$^{-/-}$ mice whole

lung tissue; however the decrease in *Akt2*$^{-/-}$ mice whole lung tissue was not as significant (Fig. 37), suggesting a that Ak1$^{-/-}$ may play a role in regulation of Orai2 expression. Previous studies in our lab also correlated activation of Akt/mTOR pathway with increased expression of TPR6, suggesting that activation of Akt is involved in initiation of transcription of TRPC6. These data showing that with knockout of Akt also results in decreased expression of STIM2 and Orai2, along with inhibition of proliferation further supports the role of STIM2 and Orai2 in proliferation of PASMC. It could also be inferred that activation of the Akt/mTOR pathway may play a role in the phenotypic transition of PASMC, from a contractile phenotype to a proliferative phenotype. Akt/mTOR activation by growth factors (e.g., PDGF) is well established to induce proliferation of PASMC and is also known to contribute to the development of PAH. Increased expression of STIM2 and SOC channels Orai2 and TRPC6 may be a mode by which cells increase $[Ca^{2+}]_{cyt}$ to fuel PASMC proliferation.

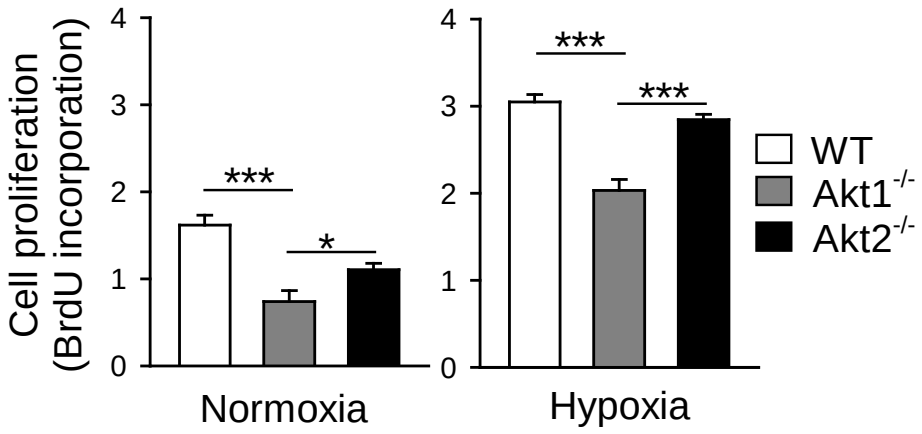

Figure 36: Knockout of Akt1, not Akt2, inhibits PASMC growth. Inhibition of rat PASMC growth in Akt (*Akt1*$^{-/-}$) and Akt2 (*Akt2*$^{-/-}$) knockout mice. Cultured PASMC was isolated from wild type (WT) *Akt1*$^{-/-}$ and *Akt2*$^{-/-}$ mice. Cells were cultured in 10% FBS-M199 in the presence of growth factors. BrdU incorporation was measured after 72 hours after cells were plated. Data are means ± SE (n=10 (WT), n=9 (Akt1$^{-/-}$), and n=11 (Akt2$^{-/-}$) wells of cells/group). * P<0.05, *** P<0.001 vs. WT control.

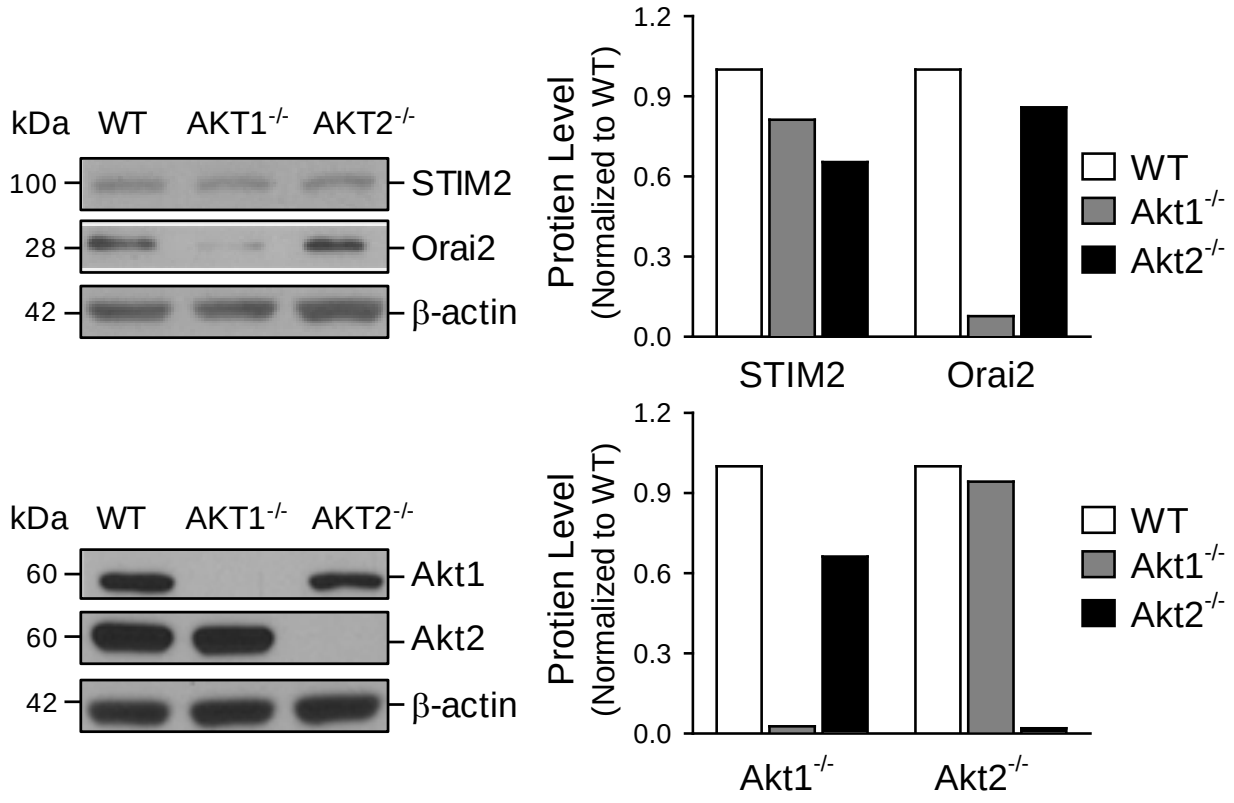

Figure 37: Knockout of Akt1 and Akt2 Inhibits STIM2 and Orai2 Expression in whole lung tissue. A. Representative Western blots showing *Akt1*$^{-/-}$ and *Akt2*$^{-/-}$ mice had downregulation of STIM2 and Orai2 in whole lung tissues. β-actin was used as a loading control. B. Summarized data of STIM2, Orai2, Akt1, and Akt2 (B, right panel) protein expression level (mean) in Ak1 and Ak2 knockout SM22α-cre knockout mice whole lung tissues (n=1). Graph shows protein expression of STIM2, Orai2, Akt1, and Akt2 normalized to an average level in WT control. STIM2 and Orai2 protein expression levels were decreased in whole lung tissue from Akt1 and Akt2 knockouts.

V. **DISCUSSION**

A. **Upregulated Expression of STIM2, TRPC6 and Orai2 in the Proliferative Phenotype of Pulmonary Arterial Smooth Muscle Cells in Culture Compared to Contractile Pulmonary Arterial Smooth Muscle Cells**

The role of STIM and SOC channels as regulators of proliferation in PASMC is only now emerging. Some of the interest has stemmed from recognition of the role of increased $[Ca^{2+}]_{cyt}$ on activating Ca^{2+}-dependent transcription factors that lead to increased proliferation of PASMC. The pathogenesis of PAH is attributed to the effects of both sustained vasoconstriction and increased vascular remodeling (Morrell et al., 2009). However, patients with later, more severe stages of PH do not effectively respond to the common CCB therapy, partially due to the excessive amount of vascular remodeling. Therefore, more studies need to be completed to elucidate the mechanisms of which PASMC transition into a more proliferative phenotype and how PASMC proliferation is maintained in the proliferative phenotype.

To establish the role of STIM2 in the proliferative phenotype, we studied STIM2 expression in both differentiated and dedifferentiated PASMC. In this study, we examined whether STIM2 upregulation may play an important role in the early transition of PAH pathogenesis from the sustained vasoconstrictive stage to highly proliferative stage due to the transition of PASMC from contractile phenotype to highly proliferative phenotype. Furthermore, we investigated the role of SOCE and voltage dependent Ca^{2+} entry (VDCE) in contractile and proliferative phenotypes. We determined that STIM2, Orai2, and TRPC6 protein increased expression levels is associated with specific

PASMC phenotypes. We linked the increased STIM2 to enhanced SOCE, which leads to sustained increase of $[Ca^{2+}]_{cyt}$ and which could possibly lead to PASMC phenotypic transition, through activation of Ca^{2+}-dependent transcription factors which increase PASMC proliferation.

While the inhibition of Ca^{2+} channels alleviates pathogenic effects, such as VDCC induced sustained vasoconstriction, inhibition of SOCE may also have beneficial effects on inhibition of vascular remodeling. The present study identifies beneficial roles for targeting STIM2, by determining whether downregulation or blockade STIM2 will inhibit PASMC phenotypic and transition to a more proliferative phenotype, and highlights the need to selectively target the pathogenic effects of increased SOCE. By understanding the mechanism of enhanced cell proliferation via STIM activation of SOCE, we may be able to specifically inhibit this interaction.

The molecular mechanisms driving SMC differentiation/dedifferentiation in the PA media layer are not completely explained yet, although many studies established the loss of contractile markers (MYH, SM22α, and calponin) and increased proliferation in dedifferentiated/proliferative SMC. Based on our results we have confirmed that PASMC are highly plastic when cultured *in vitro* and culturing induces a phenotypic transition to a more dedifferentiated SMC. With this knowledge, and with the previous discovery that IPAH-PASMC have increased expressions of STIM2, Orai2, and TRPC6, it could be inferred that these proteins may play a role in contributing to vascular remodeling through mediating PASMC proliferation (Song et al., 2011; Yu et al., 2004).

In these studies the phenotypic characterization of rat PASMC led to the determination that proliferative PASMC also have increased STIM2, TRPC6, and Orai2 expression levels compared to healthy contractile PA. We first verified the purity and efficiency of the PASMC isolation by confirming that nearly all cells stained with DAPI cross-reacted with smooth muscle α-actin (Fig. 12). This figure demonstrates the fine meshwork of actin filaments seen at the more superficial layers of the cell Furthermore, the morphology of the isolated PASMC match the characteristics of typical SMC. By using our *in vitro* model of inducing proliferation by culturing freshly dissociated PASMC in smooth muscle basal medium plus 10% FBS and growth factors for 72-96 hours, we determined that this induces a phenotypic transition of PASMC from a contractile phenotype to a proliferative phenotype. As validated in Figure 14 by Western blot analysis, the protein expression levels of contractile markers, MYH, SM22α, and calponin were all downregulated in the cultured proliferating PASMC, compared to contractile PA tissue. Furthermore, the proliferating marker, PCNA, was increased in the proliferating PASMC. These markers are well accepted in the field as indicators of phenotype differentiation in SMC (Chamley-Campbell et al., 1979).

We further demonstrated that the protein expression levels of STIM2, Orai2, and TRPC6 in proliferative PASMC were significantly upregulated in the dedifferentiated phenotype (Fig. 13). Although the function of STIM2 in PASMC differentiation has not been elucidated, the role of enhanced SOCE in proliferative PASMC has been studied (Berra-Romani et al., 2008; Golovina et al., 2001; Potier et al., 2009; Sweeney et al., 2002), suggesting that downregulation of SOCE is associated with the acquisition of a differentiated phenotype. Moreover, in this model of PASMC dedifferentiation, STIM2

expression is upregulated, and associated with enhanced SOCE. This is coinciding with the role of STIM2 in regulating dedifferentiated

To link the role of increased $[Ca^{2+}]_{cyt}$ to proliferation, we measured BrdU incorporation in growth arrested cells and cells with 1.8 mM or 0.5 mM Ca^{2+}. We verified that in human PASMC, chelation of extracellular Ca^{2+} markedly inhibited cell growth in media containing serum and growth factors. Previous studies also verified that resting $[Ca^{2+}]_{cyt}$ was much higher in proliferating cells than in growth-arrested cells (Golovina et al., 2001). These results imply that elevated $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{SR}$, due to a constant Ca^{2+} influx, are necessary for human PASMC proliferation. SOCE is a critical mechanism involved in maintaining sustained Ca^{2+} influx and refilling Ca^{2+} into depleted SR/ER stores. Indeed we determined that SOCE-mediated increase in $[Ca^{2+}]_{cyt}$, and STIM2, Orai2, and TRPC6 protein expression were all significantly enhanced in PASMC during proliferation. Inhibition of SOC channels with siRNA for Orai2 reduced SOCE and significantly attenuated human PASMC growth. These results suggest that SOCE, potentially through the upregulated STIM2, Orai2, and TRPC6 expression and the resultant increase in Ca^{2+} influx, is a critical mechanism required to maintain the elevated $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{SR}$ in PASMC during proliferation.

Cytosolic Ca^{2+} stimulates cell proliferation, whereas $[Ca^{2+}]_{SR}$ may regulate cell proliferation by modulating the amplitude and frequency of Ca^{2+} signals in the cytosol and nucleus. Maintaining a sufficient level of Ca^{2+} in the SR/ER is also critical for cell growth, and depletion of the SR Ca^{2+} stores induces growth arrest. Therefore a rise in $[Ca^{2+}]_{cyt}$ plays a critical role in stimulating PASMC proliferation.

As stated above, rise in $[Ca^{2+}]_{cyt}$ due to Ca^{2+} release from intracellular Ca^{2+} stores and Ca^{2+} influx through SOC channels or VDCC is an important mechanism for increased $[Ca^{2+}]_{cyt}$. Fast-short lasting increases in $[Ca^{2+}]_{cyt}$ through VDCC is an important mechanism for pulmonary vasoconstriction. However, sustained long-lasting increases in $[Ca^{2+}]_{cyt}$, specifically through SOC channels, is an important mechanism for stimulation of PASMC proliferation. To determine whether plasma membrane Ca^{2+} channels were specific to a PASMC phenotype, we measured Ca^{2+} influx through VDCC and SOC channels in both contractile and proliferative PASMC. In freshly dissociated contractile rat PASMC, application of high-K^+ (60 mM) solution resulted in a larger influx of Ca^{2+} through VDCC than in cultured proliferative rat PASMC (Fig. 16). On the contrary, SOCE was enhanced in dedifferentiated/proliferative PASMC. These data suggest that the activity and/or expression levels of VDCC and SOC channels correlate with PASMC phenotype. This could suggest that the significantly increased $[Ca^{2+}]_{cyt}$ seen in proliferating PASMC is specifically due to increased expression levels or activation of SOC channels or SOC activators (i.e. STIM).

After confirming that SOC channels were more important for increased $[Ca^{2+}]_{cyt}$ in proliferative state, we then correlated the protein expression levels of STIM2, Orai2, Orai3, and TRPC6 in the contractile phenotype and the proliferative phenotype of PASMC. In proliferative PASMC, expression levels of STIM2, Orai2, Orai3, and TRPC6 were increased compared to contractile PASMC. Additionally, we confirmed the phenotypic state of these cells and showed the decreased expression of contractile makers (MYH) and increased proliferative marker, PCNA. Overall, these results revealed that dedifferentiated, proliferative PASMC mediated Ca^{2+} entry through SOCE

and that STIM and SOC channels can be a significant component of induced proliferation of PASMC.

It is important to note that both the HPH and MCT-PH experimental model of pulmonary hypertension are characterized by excessive proliferation of SMC in the medial layer of the PA. Both these models demonstrated an increase in STIM2 and Orai2 in isolated PASMC compared to their controls. In the future, we would like to determine whether SOCE is also elevated in freshly isolated PASMC from these animal models, to verify the role of SOCE in the development of PH. Furthermore, we would like to determine whether inhibition of SOCE by knockdown of STIM2, would prevent or reverse the development of PH. The important role of STIM2 in regulating pathological events linked to vascular remodeling and hypertension are depicted in these studies.

B. Effect of Differential Modulation of Pulmonary Arterial Smooth Muscle Cells on STIM2, Orai2, and TRPC6 Expression

As was expected, removal of serum and growth factors from cultured medium inhibited cell growth (Fig. 20) (Owens et al., 1986). These data infer that the cells cultured in smooth muscle basal medium with low serum are more differentiated/quiescent, while the cells cultured in smooth muscle growth medium (smooth muscle basal medium supplemented with 10% FBS and growth factors) are dedifferentiated/proliferated cells, indicating that serum-starvation can convert PASMC into a more differentiated phenotypic state. Subsequently, we confirmed the decreased proliferative state of the differentiated PASMC by verifying the decreased protein

expression levels of PCNA. Since we previously demonstrated that cultured, proliferative PASMC expression levels of STIM2, Orai2, Orai3 and TRPC6 were increased compared to freshly dissociated contractile PASMC, we decided to determine the expression levels of these proteins in growth-arrested/quiescent PASMC. The protein expression levels of TRPC6, Orai2 and STIM2 were decreased in growth-arrested PASMC compared to proliferative PASMC (Fig. 21). Growth-arrested, differentiated PASMC demonstrated a decrease in STIM2 and SOC channels, thus suggesting a role in PASMC proliferative phenotype.

After serum starvations, we decided to use a second mode of differentiation to further evaluate and compare the expression of STIM2, Orai2, and TRPC6 following phenotypic transition from dedifferentiated/proliferative to differentiated/quiescent PASMC phenotype via TGF-β treatment. TGF-β is a potent soluble growth factors which promote SMC differentiation (Deaton et al., 2005; Hu et al., 2003; Sinha et al., 2004; Tang et al., 2010). Our studies demonstrated that treatment with TGF-β in the presence of 10% serum decreased PASMC proliferation compared to vehicle control after 48 and 72 hours (Fig. 24). Taken together with the inhibition of proliferation and increased contractile proteins after TGF-β treatment, we suggest that the upregulated STIM2, Orai2 and TRPC is, important for the enhancement of SOCE in proliferative PASMC. The exact methods involved in the increased expression of these proteins during PASMC proliferation is unclear and needs further study. However, the role of increased $[Ca^{2+}]_{cyt}$ has been well established in proliferating SMC, and will be discussed below. Additionally, we verified a phenotypic transition after TGF-β treatment, by analyzing the contractile markers and proliferative markers, which confirmed the PASMC

differentiation. The contractile markers, calponin and SM22α, were increased, while the proliferation marker, PCNA, was decreased after TGF-β treatment.

Following TGF-β treatment, we decided to use a third mode of differentiation, to further evaluate and compare the expression of STIM2, Orai2 and TRPC6 after a phenotypic transition from dedifferentiated/proliferative to differentiated/quiescent PASMC phenotype via heparin + low serum treatment. Heparin significantly inhibits pulmonary vascular remodeling induced by hypoxia in rodents (Garg et al., 2000) and inhibits PASMC proliferation in culture (Cindhuchao et al., 2003). Heparin may inhibit PASMC growth in culture via the TGF-β pathway (Garg et al., 2003; Joseph et al., 1997), however, the mechanisms responsible for the anti-proliferative effects of heparin are not well understood. Heparin (a structural and biosynthetic relation of the glycosaminoglycan heparin sulphate) can maintain a contractile phenotype (Bingley et al., 1998) and induce differentiation into a more contractile phenotype by upregulating the expression of contractile proteins. Heparin effectively inhibited the mitogenic and proliferative response of a variety of growth factors, suggesting that heparin does not act by blockage of specific membrane receptor, but rather through a more generalized, undefined pathway.

Consistent with previous studies, we characterized the PASMC phenotypic transition from a dedifferentiated/proliferative phenotype to differentiated/quiescent phenotype. Our data demonstrated that after heparin treatment, PASMC increased expression levels of contractile markers, calponin and SM22α and decreased expression levels of proliferation marker, PCNA. We next determined whether

differentiated/quiescent PASMC, by heparin treatment, had decreased expression levels of STIM2, Orai2, and TRPC6. We discovered that the protein expression levels of STIM1 were significantly decreased, while the expression levels of STIM2 and TRPC6 were trending towards decreased expression levels in differentiated PASMC (Fig. 27). Furthermore, the protein expression levels of Orai1, Orai2 and Orai3 were also decreased in these PASMC. These data further indicate that these proteins exert a proliferative effect by enhancing proliferation, although their exact mechanism has yet to be determined.

In summary, regardless of the mode of PASMC differentiation, STIM2, Orai2 and TRPC6 expressions were all downregulated, coinciding with decreased proliferation and, to a certain degree, an increased contractile phenotype. Indeed, it has been previously confirmed that depletion of STIM2 reduces the proliferative capabilities of differentiated PASMC (Song et al., 2011). Additionally, it has been shown that in STIM1 knockout cells there is a failure to progress to the S phase of the cell cycle due to upregulation of p21 (CDK inhibitor) and reduction in Rb phosphorylation (Rb is one of the major modulators of the G1/S transition in mammalian cells) (Guo et al., 2009) as well as a reduction in CREB phosphorylation (Takahashi et al., 2007) and NFAT transcriptional activity (Aubart et al., 2009), suggesting that multiple signaling pathways may be regulated by STIM2 in PASMC. However, these suggestions have yet to be confirmed, and would be necessary to complete the story.

C. STIM2 is Sufficient while Orai2 is Necessary to Enhance Store-operated Ca^{2+} Entry in Proliferative Pulmonary Arterial Smooth Muscle Cells

Enhanced SOCE contributes to increased $[Ca^{2+}]_{cyt}$ by either increasing the numbers of SOC channels or by increasing the probability of these channels being open. Orai are a membrane-spanning Ca^{2+} channel that form tetrameric SOC channels in the plasma membrane, and are demonstrated to be the major channels responsible for SOCE. As previously confirmed, proliferating rat PASMC express increased levels of Orai family members (Orai1, 2 and 3).

In this study we confirmed, with transient transfection of Orai2 siRNA, downregulation of Orai2 protein expression results in a 50% decline in SOCE. Similarly, we measured a decrease in expression levels of proliferating-marker, PCNA, in PASMC transfected with siRNA for Orai2, thus demonstrating that increased Orai2 is necessary for increased proliferation of PASMC. These results indicate that Orai2 is essential for activation of SOCE in proliferating PASMC, and plays an important role in contributing to an increase of $[Ca^{2+}]_{cyt}$, and thus contributes to proliferation in PASMC.

Secondly we wanted to specifically examine the role of STIM2 and SOCE in proliferative PASMC phenotype. STIM functions as a SR/ER membrane-bound sensor of $[Ca^{2+}]$ in the SR. When the SR is depleted, STIM proteins dimerize resulting in movement of the proteins to the plasma membrane-SR junction where they cluster and recruit Orai subunits to form SOC channels and induce SOCE. The role of STIM2, Orai and TRPC in the phenotypic transition of PASMC is still not fully understood. We thus determined the involvement of STIM2 in the regulation of SOCE in proliferating PASMC. As previously demonstrated, proliferating rat PASMC expresses increased levels of

STIM2. We verified that STIM2 was sufficient to enhance SOCE in proliferating PASMC, by overexpressing STIM2 in cultured PASMC. Transient transfection with STIM2 plasmid resulted in an increase in the level of expression of STIM2 (Fig. 31). Furthermore, these cells demonstrated a larger SOCE compared to control (Fig. 31). These results indicate that STIM is sufficient for activation of SOCE in proliferating PASMC, and that STIM2 and SOCE play an important role in PASMC proliferation. These findings may be helpful in finding new potential drug targets which will inhibit the modulation of phenotypic switching which drives PASMC proliferation.

D. Genetic Deletion of Akt1 and Akt2 Leads to Downregulation of STIM2 and Orai2 in Whole Lung Tissues of Mice

We previously demonstrated that the Akt/mTOR pathway may regulate the expression of STIM and Orai2 (Ogawa et al., 2012), which was shown to be a result of increased SOCE. We showed a significant decreased in proliferation in *Akt1*$^{-/-}$ PASMC isolated from mice, however, there was only a slight decrease in proliferation of *Akt2*$^{-/-}$ PASMC (Fig 36). This demonstrates a role of Akt in regulating proliferation. Even more interesting was that knockdown of Akt1, and not so much Akt2, decreased expression of STIM2 and Orai2, demonstrating that Akt/mTOR leads to increased expression of STIM2 and Orai2 (Fig. 37). This suggests that STIM2 is involved in the proliferation of PASMC. Our lab has previous proposed a pathway in which after PDGF binds to PDGFR, Akt/mTOR pathway is turned on. As a result this causes increased expression of STIM1/Orai, and hence enhanced SOCE, and sustained cellular proliferation (Ogawa et al., 2012).

Unfortunately, there is no STIM2 inhibitor currently available for use in the clinic; however, there are a few candidate drugs known to inhibit SOCE (e.g., Synta 66). In conclusion, we highlight the importance of Akt phosphorylation and signaling in the regulation of proliferation and Ca^{2+} regulation. The data suggest a potential for STIM2 inhibitors in the treatment of pulmonary hypertension by their ability to reduce enhancement of SOCE, and decrease the sustained increase in $[Ca^{2+}]_{cyt}$.

In summary, SMC are extremely plastic and can dedifferentiate in response to various environmental stimuli from a contractile/quiescent to a proliferative/synthetic phenotype (Owens et al., 2004; Rensen et al., 2007). Generally, a phenotypic switch of PASMC is inevitable for any pathological and physiological vascular remodeling process to occur. Contractile PASMC represent the majority of PASMC in a healthy vessel; they are responsible for maintenance of pulmonary arterial tone and thus manage blood vessel diameter. Contractile phenotype preserve SMC differentiation marker genes (e.g., MYH, SM22α, and calponin) (Chamley-Campbell et al., 1979). However, under pathological conditions or vascular injury, PASMC undergo a change in phenotype, characterized by an increase in proliferation rate, migration, and vascular repair, due to increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) (Marchand et al., 2012).

Importantly, an increase in PASMC $[Ca^{2+}]_{cyt}$ is a major trigger for pulmonary vasoconstriction and an important stimulus for proliferation (Morrell et al., 2009; Reid, 1990). $[Ca^{2+}]_{cyt}$ is increased by: 1) Ca^{2+} influx through plasma membrane VDCC, SOC and ROC channels (Putney, 1986; Putney, 1990); and 2) Ca^{2+} release from intracellular stores (e.g., SR/ER) through Ca^{2+} release channels (e.g., IP_3R). Nuclear and cytosolic Ca^{2+} pools stimulate proliferation by activating Ca^{2+}-dependent kinases (e.g., CaMK),

which are necessary for cell growth, and other transcription factors (e.g., c-Fos, NFAT, and CREB) (Berridge, 1995; Graef et al., 2001; Sheng et al., 1990).

Increased $[Ca^{2+}]_{cyt}$ can also affect gene expression by interacting with PKC and calmodulin or by activating cyclins and cyclin-dependent kinases (proteins involved in the cell cycle). In PASMC specifically, both increases in $[Ca^{2+}]_{cyt}$ and intracellular stored Ca^{2+} are essential for proliferation (Golovina et al., 2001). In the presence of serum and growth factors, the removal of extracellular Ca^{2+} and the depletion of intracellular stored Ca^{2+} inhibits proliferation of PASMC, further establishing the role of Ca^{2+} for cell cycle progression and cell growth (Golovina et al., 2001).

It is important to note that studies have well characterized the clinical manifestations of IPAH, the cellular and molecular mechanisms are still under investigation. Therefore, additional studies clarifying role of STIM2 and its regulation of SOCE are necessary for PASMC proliferation, and STIM2 regulation may be a potential target in the therapeutic intervention for patients with several forms of pulmonary hypertension.

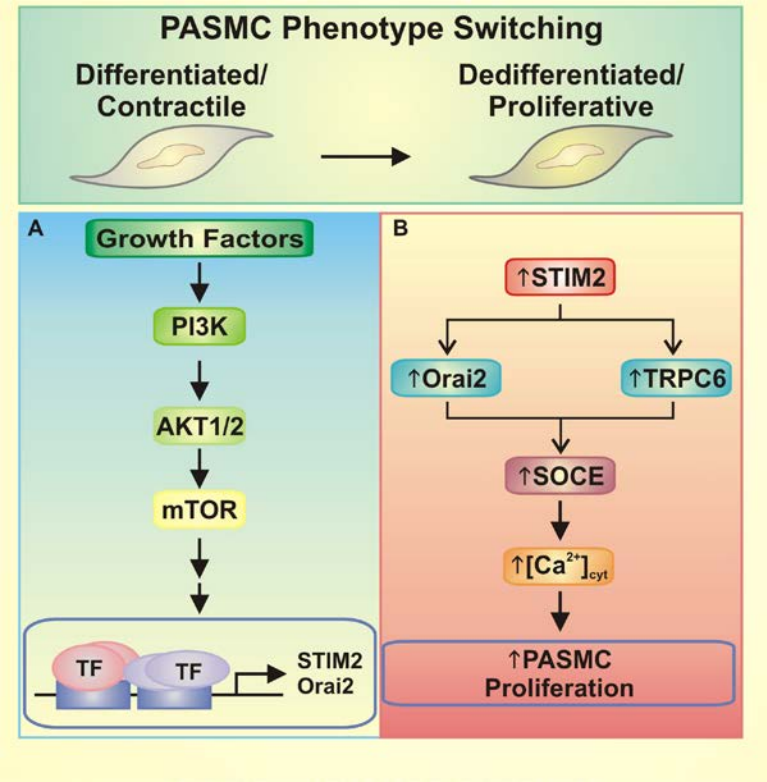

Figure 38: Schematic Diagram of Pathogenic role of STIM2, Orai2 and TRPC6 on PASMC proliferation. Pathogenic role of STIM2 in the development of pulmonary arterial hypertension (PAH). B. Proposed cellular mechanisms involved in excessive increase in $[Ca^{2+}]_{cyt}$ in pulmonary artery smooth muscle cells PASMC. Upregulated STIM2, Orai2, due to genetic mutations and/or environmental stimulation, lead to the increased number of store-operated (SOC) Ca^{2+} channels in the plasma membrane, which enhances store-operated (SOCE) Ca^{2+} entry and increases $[Ca^{2+}]_{cyt}$ in PASMC resulting in increased PASMC proliferation and remodeling. A. Proposed cellular mechanism involved in phenotypic switching of PASMC by activation of the Akt/mTOR pathway.

E. **Future Perspectives**

In the future, we would like to determine whether Orai2 and TRPC6 channels are upregulated in PASMC from HPH rats and MCT-rats and whether they are involved in the enhanced PASMC proliferation in PH. SOCE and ROCE are both of functional importance in the regulation of $[Ca^{2+}]_{cyt}$. It is known that increased $[Ca^{2+}]_{cyt}$ contributes to enhanced proliferation in PASMC. Previous studies have shown that the resting $[Ca^{2+}]_{cyt}$ is increased, while ROCE and SOCE were enhanced, in PASMC from IPAH patients compared to PASMC from normal subjects (Yu et al., 2009; Zhang et al., 2007). Additionally, inhibition of endogenous TRPC6 mRNA and protein expression in rat PASMC using antisense oligonucleotides has been shown to attenuate SOCE and decrease PDGF-induced proliferation (Yu et al., 2003a). Our preliminary data suggests an increase in the expression of Orai2 and TRPC6 channels plays an important role in PASMC proliferation. These observations direct us to hypothesize that upregulation of Orai2 and/or TRPC6, by contributing to the increase in SOCE and ROCE, is an important mechanism that promotes PASMC proliferation in rats with experimental pulmonary hypertension, such as rats with hypoxia-induced pulmonary hypertension (HPH) and monocrotaline (MCT)-induced pulmonary hypertension. To test this hypothesis we will use both HPH rats and MCT-rats as two experimental PH animal models. Hypoxia induces pulmonary vasoconstriction in humans and animals, hypoxic pulmonary vasoconstriction is an important mechanism that maintains an optimal ventilation-perfusion ratio by diverting blood from poorly ventilated areas of the lung to regions with better ventilation for efficient gas exchange (Ward and McMurtry, 2009; Weissmann et al., 2006). MCT is a drug reported to cause inflammation and damage

the endothelium of the pulmonary artery and induce gradual pulmonary hypertension (Rey et al., 2009). In these models we will isolate rat PASMC and measure the gradual increase of protein expression of Orai2 and TRPC6. This will confirm which channels are responsible for enhanced SOCE and ROCE. Additionally, we will determine whether downregulation of Orai2 and TRPC6 inhibits SOCE/ROCE in PASMC from PH induced animal models and whether they attenuate PASMC proliferation. We will also determine whether overexpression of Orai2 and TRPC6 in normal PASMC augments SOCE and enhances PASMC proliferation. Based on our preliminary data showing that STIM2, Orai2, and TRPC6 are upregulated in proliferating PASMC, we hypothesize that these proteins mediate the phenotypic transition seen in PH. We hypothesize that Orai2 and TRPC6 lead to enhance SOCE in PH animal models. Additionally, we believe that these channels play an important role in PASMC proliferation. We will determine whether knockdown Orai2 and TRPC6, by siRNA, will decrease the proliferation in PASMC of HPH-rats and MCT-rats.

We would also like to determine whether pharmacological blockade of SOC prevents the development of PH and reverse the established PH. As previously discussed, we propose that HPH-rats and MCT-rats will have increased expression levels of SOC that impact Ca^{2+} homeostasis. Additionally, previous studies have shown minimal effect of VDCC inhibitors on PAH patients, thus, our aim is to focus on administering pharmacological agents that would inhibit SOCE. We will administer a pharmacological antagonist for Orai channels and determine whether it will exert therapeutic effects on experimental PH animal models by inhibiting the increased $[Ca^{2+}]_{cyt}$. Currently, there are several drugs that could be used to block SOCE current.

The latest and most relevant of these would be 3-fluoropyridine-4-carboxylic acid (2′,5′-dimethoxybiphenyl-4-yl)amide (Synta 66). Synta 66 has been shown to be an Orai1 inhibitor, without affecting TPRC6 (Li et al., 2011; Zheng et al., 2011). Additionally, Synta 66 is also an inhibitor of SOCE in proliferating VSMC and endothelial cells (Li et al., 2011; Zheng et al., 2011). Characterization of the effects of Synta 66 on SOCE *in vitro*, using experiments outlined in Aim 2, will elicit possible mechanisms and targets of Synta 66. This drug may only be obtained as a gift from a pharmaceutical company or synthesized by a medicinal chemist (Beech, 2012). Although the use of SOCE channel blockers are of considerable clinical interest, currently the selective inhibitors are not prescribed to patients, because they are not a selective antagonist and their mechanisms of action are still under investigation. We hope the above approaches will provide new therapeutic applications and lead to prospective drug targets that may alleviate the development of PH. It is possible that Synta 66 only inhibits Orai1 channels, and not Orai2 or Orai3 channels. Currently, the exact mechanism of action and Orai channel specificity of Synta 66 is unknown. Synta 66 has been shown to inhibit SOCE, and therefore it will be used to determine if SOCE is important for the development of experimental PH.

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