SRC KINASE ACTIVATION IN PULMONARY ARTERIAL HYPERTENSION

By

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To my parents, Dennis and Robin, my ever-growing family, and dear friends. I could not

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LIST OF ABBREVIATIONS

BMP	Bone Morphogenetic Protein
BMPR2	Bone Morphogenetic Protein Receptor Type 2
Cav-1	Caveolin-1
CSD	C-term caveolar scaffolding domain
EC	Endothelial cell
eNOS	Endothelial Nitric Oxide Synthase
EPC	Endothelial progenitor cell
HPAH	Heritable Pulmonary Arterial Hypertension
IPAH	Idiopathic Pulmonary Arterial Hypertension
NO	Nitric oxide
PAH	Pulmonary Arterial Hypertension
PH	Pulmonary Hypertension
PVR	Pulmonary vascular resistance
RHC	Right heart catheterization
RV	Right ventricle
SFK	Src family kinase
SMC	Smooth muscle cell
VEGF	Vascular Endothelial Growth Factor
VEGFR2	Vascular Endothelial Growth Factor Receptor Type 2

CHAPTER I

INTRODUCTION

Part I: Pulmonary Hypertension

Definition of Pulmonary Hypertension

Pulmonary hypertension (PH) is a progressive, fatal disease of the lung vasculature. In its simplest definition, PH is described as abnormal and sustained elevation of pulmonary arterial pressures during resting conditions. Traditionally, PH is define as mean pulmonary arterial pressures (mPAP) greater than 25mmHg at rest, as measured by right heart catheterization. However, recent advanced measures of pulmonary capillary wedge pressure (Ppcw), pulmonary vascular resistance (PVR), and cardiac output (CO) enable clinicians to effectively categorize PH heamodyanimic subtype as either pre-capillary (Groups 1, 3, 4, 5) and post-capillary (Group 2- PH due to left heart disease)(Galie, Hoeper, et al., 2009a).

Classification of Pulmonary Hypertension

First discovered by Dresdale in the 1950's (Dresdale et al., 1954), PH has gone through a series of classification systems. Most recently in 2008, the World Health Organization (WHO), at the Fourth World Symposium of Pulmonary Hypertension in Dana Point, California, categorized PH into five major groups listed in Table 1 (Galie, Hoeper, et al., 2009a; PAH-info.com, 2015; Simonneau et al., 2009). We will discuss these groups below.

Table 1: WHO Current Clinical Classification of Pulmonary Hypertension

Group 1		**Pulmonary Arterial Hypertension**
	1.1	Idiopathic pulmonary hypertension
	1.2	Heritable pulmonary arterial hypertension (*BMPR2, ALK1, ENDOGLIN, unknown*)
	1.3	Drug and toxin-induced pulmonary arterial hypertension
	1.4	Associated with: connective tissue diseases, HIV infection, portal hypertension, congenital heart disease, schistosomiasis, chronic hemolytic anemia
	1.5	Persistent pulmonary hypertension of the newborn
	1'	Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis
Group 2		**Pulmonary Hypertension as a result of left heart diseases**
	2.1	Systolic dysfunction
	2.2	Diastolic dysfunction
	2.3	Valvular disease
Group 3		**Pulmonary Hypertension associated with lung disease 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
	3.4	Alveolar hypoventilation disorders
	3.6	Chronic exposure to high altitude
	3.7	Developmental abnormalities
Group 4		**Chronic thromboembolic pulmonary hypertension**
Group 5		**Pulmonary Hypertension with unclear multifactorial mechanisms**
	5.1	Hematologic disorders
	5.2	Systemic disorders
	5.3	Metabolic disorders
	5.4	Others: tumor obstruction, chronic renal failure

Adapted from (PAH-info.com, 2015)

Group 1, which the bulk of this work will discuss, is composed of patients with pulmonary arterial hypertension (PAH). The term PAH differs from PH in that PAH is defined as the presence of pre-capillary PH that is not due to an accompanying lung disease; however, this manuscript will use both terms interchangeably. This group includes the following: 1) heritable forms of the disease associated with germline mutations, predominantly in the *Bone Morphogenetic Protein Receptor Type 2* (*BMPR2*) gene, *Activin-like Kinase Type 1 (ALK1)* gene, and *Endoglin* and familial cases with no known germline mutations; idiopathic, or sporadic, PAH (IPAH); 2) drug and toxin-induced PAH, notably the Fen-Phen epidemic of the late 20th century (Brenot et al., 1993); 3) PAH associated with disorders/diseases including but not limited to connective tissue diseases(Hachulla et al., 2005), HIV (Degano et al., 2010), and portal hypertension(Rodriguez-Roisin et al., 2004); 4) persistent pulmonary hypertension of the newborn (Teixeira-Mendonca et al., 2013); 5) and pulmonary veno-occlusive disease (Montani et al., 2008).

Group 2 makes up the largest population of PH patients and has a high rate of morbidity and mortality. It is the only post-capillary PH group and is composed of patients with PH as a result of left heart disease. Group 2 PH is suspected to occur in accordance with left heart disease due to "passive" pressures emanating from the left heart, though many suspect it is a combination of left heart pressures and preexisting pulmonary pathologies (Guazzi et al., 2012).

Group 3 PH patients develop PH in association with other lung diseases and/or hypoxia. Development of this classification most often arises from alveolar hypoxia, a potent stimulant of vasoconstriction. Perhaps the most well known co-morbidity in this group is chronic obstructive pulmonary disease (COPD); however this group includes a

variety of factors including interstitial lung disease, alveolar hypoventilation disorders, chronic exposure to high altitude (hypoxia), and developmental abnormalities (Sajkov et al., 2013).

Making up Group 4 PH are patients with chronic thromboembolic pulmonary hypertension (CTEPH). Development of CTEPH is the result of increased artery pressures due to obstruction of the pulmonary arteries with thromboembolic materials. While these patients exhibit high morbidity and mortality rates, CTEPH is the only severe PH in which medical intervention other than lung transplant may cure patients (McNeil et al., 2007).

Finally, Group 5 PH is composed of patients with unclear multifactorial mechanisms, including hematologic disorders, systemic disorders, metabolic disorders, tumor obstruction, and chronic renal failure. This group is predominantly a catchall for PH that does not fall into other categories. Given the heterogeneity of this group, Group 5 is the least characterized and studied (Galie, Hoeper, et al., 2009a).

Pathobiology of Pulmonary Hypertension

Several structural and functional abnormalities contribute to the development and progression of PH. Chief among these are structural changes in the pulmonary arteries [loss of the distal pulmonary vasculature (vascular pruning), development of neointima and plexiform lesions, and remodeling of the distal pulmonary arteries (Fig 1)] abnormal vascular reactivity, and right ventricle (RV) hypertrophy due to chronic RV pressure overload (Fig 2). It is this chronic RV overload that eventually results in patient death due to right heart failure (cor pulmonale) (Voelkel et al., 2012).
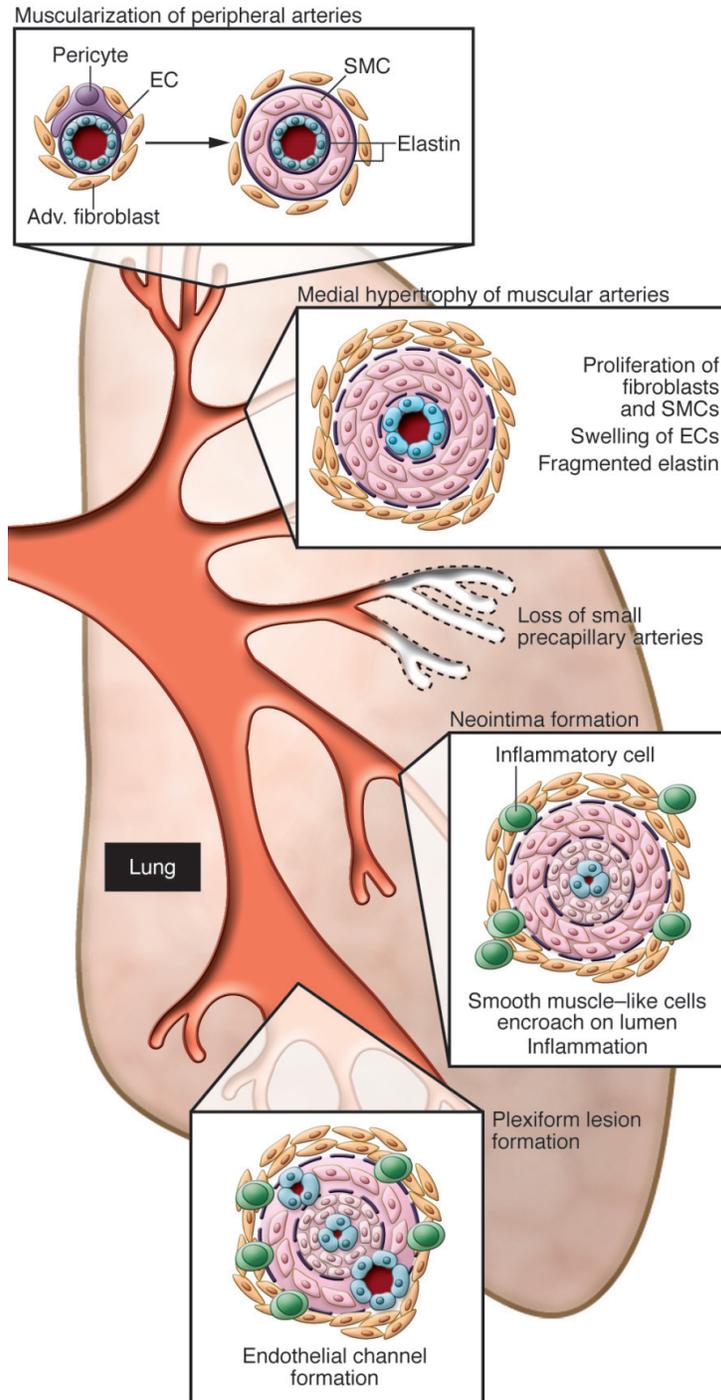
Figure 1: Structural changes associated with pulmonary arterial hypertension. Abnormalities throughout the pulmonary circulation: abnormal muscularization of distal and medial arteries, loss of distal arteries, thickening of large arteries, occlusive neointimal formation and plexiform lesions. From (Rabinovitch, 2012), Copyright Permission: License #3656590755289
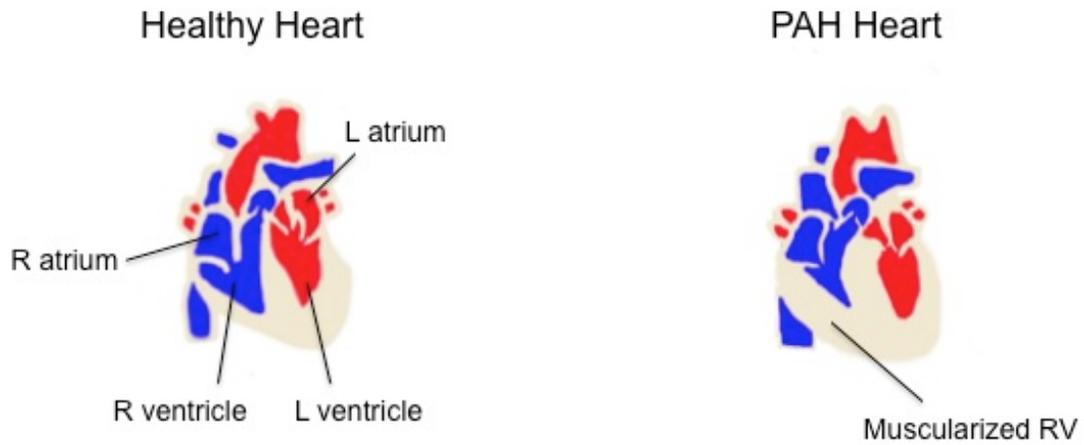
Figure 2: Right heart hypertrophy in pulmonary arterial hypertension. Adaptive remodeling of the PH heart includes thickening right ventricle and reduction of the left ventricle.

Current thinking is that endothelial insult and/or molecular changes within endothelial cells (ECs) initiates a cascade of cellular and environmental changes within the pulmonary structure(Rabinovitch, 2012). The same processes that contribute to vascular lesion formation play a role in the generalized remodeling phenotype and vascular pruning phenomenon. This includes excessive proliferation of apoptotic-resistant ECs (thought to be of monoclonal origins), recruitment of endothelial progenitor cells (EPCs) and inflammatory cells, and the differentiation and proliferation of smooth muscle cells (SMCs)(Cool et al., 1999; S.-D. Lee et al., 1998; Masri et al., 2007; Montani et al., 2011; Perros et al., 2012; Perros et al., 2007; Rabinovitch, 2012; Taraseviciene-Stewart et al., 2002). These events result in remodeling of all three arterial layers (intima, media, and adventitia) and in most cases are not reversible (Sakao et al., 2010).

Exactly how these changes occur within the vascular bed is unclear; however, recent evaluation has prompted comparison to cancer-like mechanisms(Guignabert et al., 2013). Indeed hallmarks of cancer such as increased proliferation and decreased apoptosis are clearly evident in the pulmonary vasculature of PH patients and can be attributed to the increased production and activity of pro-proliferative factors, such as FGF-2 and p130-CAS, and anti-apoptosis factors including Bcl-xl, Bcl-2, and Survivin (McMurtry et al., 2005; Tu et al., 2012; Tu et al., 2011). The result of this excessive proliferation and reduced apoptosis is the development of obstructive and obliterative lesions that prevent free blood flow and contribute to increased pulmonary pressures.

In addition to expansion of existing cell populations within the arterial wall, muscularization of normally non-muscularized distal arteries occurs. Muscularization refers to the addition of SMCs to the normally bare EC layer. Extensively studied, this

process involves the migration of SMCs to the distal arteries, differentiation of precursor cells, and possibly endothelial-to-mesenchymal transition (EMT), all of which results in the thickening of the arterial wall and contributes to abnormal vasoreactivity (Frid et al., 2006; Jones et al., 1998; Meyrick et al., 1980; Morrell et al., 2009; Sakao et al., 2009, 2010; Sheikh et al., 2014).

Further contributing to increased pulmonary artery pressures is paradoxical pulmonary artery pruning. The reduced surface area forces the steady blood volume to pass through fewer blood vessels thereby increasing pressure. It is unclear why the lungs of PH patients undergo this process as there are an abundance of pro-angiogenic factors circulating in the blood of these patients; however, it seems that PH ECs no longer respond to angiogenic factors as normal ECs do (Guignabert et al., 2013; Hernndez et al., 2013).

In addition to the expansive vascular remodeling that occurs in PH patients, the pulmonary arteries also exhibit abnormal vascular reactivity. These abnormalities include altered production of key vascular tone modulators including but not limited to, prostacyclin, endothelin-1, nitric oxide, and serotonin (Budhiraja et al., 2004; Farber et al., 2004), as well as altered responses to vasoactive stimuli (Frank et al., 2008), Together these defects lead to increased vasoconstriction and reduced vasodilation.

Finally, RV hypertrophy and subsequent right heart failure is the main cause of death in PH patients. Despite this, little is known about the mechanisms that drive RV hypertrophy and failure; however, it is appreciated that increased afterload may be the predominant factor driving the progression to right heart failure. Collectively, the above remodeling events and aberrant vasoreactivity cause excessively high pulmonary pressures, forcing the heart to work harder (increase afterload) to push the

same blood volume through reduced vascular space. Due to its inherent compliance, the RV is able to adapt to these changes in volume by increasing contractility. Initially, the RV adapts to increased contractility, undergoing concentric remodeling by increasing muscle mass (hypertrophy). Once the RV has reached its limit of adaptive hypertrophy there is a switch to dilation, which increases wall tension, and thereby oxygen demands. Ultimately, the heart is unable to maintain normal function in the midst of remodeling, and suffering from low oxygen, eventually succumbs to ischemia (Voelkel et al., 2012; Vonk Noordegraaf et al., 2011).

Incidence, Diagnosis, and Treatment of Pulmonary Hypertension

There are an estimated 2.4 to 7.6 new cases of Group 1 PH (PAH) per million people annually, with a markedly higher incidence in high-risk populations (Archer, Weir, et al., 2010; Humbert et al., 2006; Peacock et al., 2007). This large range reflects the difficulty of diagnosing patients, as well as missing data. The difficulty diagnosing PAH stems in part from the nondescript symptoms which patients present, including dyspnea (difficultly breathing), fatigue, syncope (fainting), and chest pain. Given the risks associated with invasive right heart catheterization (RHC) that is required for accurate diagnosis, physicians must play close attention to previous medical conditions, drug use, and family history when pursuing PAH as a diagnosis (Nauser et al., 2001). A series of tests are performed to rule out PH Groups 2-5 and can include any and all of the following: electrocardiograph (ECG), Doppler electrocardiography, chest radiography, pulmonary function tests, high resolution computed tomography (HRCT), perfusion lung scan, CT pulmonary angiography, associated-disease tests (HIV, Sclerosis), and finally, the gold standard, RHC (Fig 3) (PAH-info.com, 2013).

Right heart catheterization

Right heart catheterization is required to confirm the diagnosis of PAH.

PAH is defined by

- mPAP ≥25 mmHg at rest
- PCWP ≤15mmHg

Galié et al. *Eur Heart J* 2009.

Internal jugular vein
Pulmonary artery
Left lung
Right ventricle
Brachial vein
Femoral vein
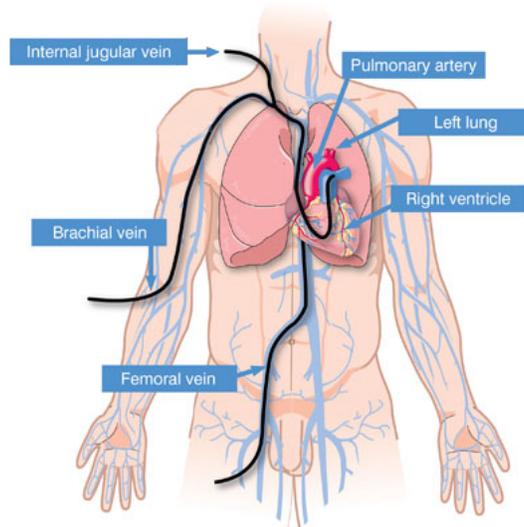
Standard approaches for catheter access

Fig 3: Diagram of right heart catheterization. Right hearth catheterization can be achieved through multiple access points. Here shows entry through the internal jugular vein, brachial vein, and femoral vein.
From: (Galie, Hoeper, Humbert, Torbicki, Vachiery, Barbera, Beghetti, Corris, Gaine, Gibbs, Gomez-Sanchez, Jondeau, Klepetko, Opitz, Peacock, Rubin, Zellweger, Simonneau, et al., 2009) Copyright Permission: License #3662810290910

RHC is required for accurate diagnosis of PAH and can be used to test vasoreactivity and determine if patients will respond to calcium channel blockers (Galie, Hoeper, et al., 2009b; Habib et al., 2010; McLaughlin et al., 2009).

Importantly, non-invasive imaging techniques such as ECG and cardiac magnetic resonance imaging (CMRI) on the heart can yield valuable information on survival and therapy response. For instance, clinicians traditionally measured changes in longitudinal shortening; however, recent indications suggest RV transverse shortening, large RV volume and impaired left ventricular filling, as well as RV ejection fraction are the strongest predictors of survival (Forfia et al., 2006; Mauritz et al., 2012; van de Veerdonk et al., 2011; van Wolferen et al., 2006; Voelkel et al., 2012).

There are various treatments available to PH patients, though none of these treatments are disease modifying. Some of the most basic therapies include oxygen delivery and diuretics and are used to ease symptoms of the disease without affecting patient prognosis. Other therapies that may prolong survival involve the use of vasoactive substances. The most common of these is prostacyclin, calcium channel blockers, Endothelin receptor antagonists, nitric oxide delivery, phosphodiesterase-5 inhibitors, and vasoactive intestinal peptide (Humbert et al., 2004).

Despite decades of research and the availability of various treatment options, survival rates for PAH patients are alarmingly low. Though, Thenappan and associates have determined that these rates are higher than the NIH proposed survival rates that were calculated before the widespread use of therapeutic interventions. Thenappan *et al* used Kaplan-Meier survival method to determine long-term survival outcomes for PH patients and concluded that 5-year survival rates for the entire PH cohort rested at 61% while those with PAH landed at 66% (Thenappan et al., 2010). While this new rate is

double that of pre-1980 NIH estimates, there is vast room for improvement and development of disease modifying PH treatments.

Part II: *BMPR2* Mutations in Heritable Pulmonary Arterial Hypertension

The focus of this dissertation will be on the heritable form of pulmonary arterial hypertension (HPAH), a subsection of Group 1 PH. Originally termed familial PAH due to its tendency to affect entire families despite its rather rare occurrence in the human population, this term adapted to heritable PAH once it was discovered that a large proportion of these families harbored germ line mutations in the Bone Morphogenetic Protein Receptor Type 2 Gene, *BMPR2*(Deng et al., 2000; Lane et al., 2000). Since then, HPAH has come to include idiopathic cases of PAH that do not have *BMPR2* mutations but are hereditary.

Following the discovery of *BMPR2* mutations in HPAH in 2000, *BMPR2* mutations have been identified in 75% of PH patients with a family history of the disease, and nearly 25% of patients with so-called sporadic or idiopathic PH, with over 200 unique mutations identified (Fig 4) (E. D. Austin et al., 2014; J. D. Cogan et al., 2006; Joy D. Cogan et al., 2005; Loyd JE, 2012; Machado et al., 2006). Interestingly, evidence of reduced *BMPR2* expression has been identified in patients without *BMPR2* mutations, further demonstrating the importance of BMPR2 in the development of PH (Atkinson et al., 2002; Morrell et al., 2001; Richter et al., 2003).

In addition to the commonality of *BMPR2* mutations in HPAH, the significance of *BMPR2* mutations in disease progression is apparent as *BMPR2*-mutation positive PH patients present roughly 10 years earlier, have more severe disease, are less likely to respond to vasodilator therapies, and die sooner than non-*BMPR2* PH counterparts

(Elliott et al., 2006; Rosenzweig et al., 2008; Sztrymf et al., 2008). Despite comprehensive research over the past 15 years, how *BMPR2* mutations drive the development of PH remains unclear. One thought is that defects in BMP signaling (the predominant ligand for BMPR2), may be the driving factor. This hypothesis derives in part due to the myriad of other BMP signaling pathways that are disrupted in HPAH including Endoglin, ALK1, Smad4 and Smad8 (Lowery et al., 2010; Nasim et al., 2011; Shintani et al., 2009). Studies using *Bmpr2* mutant mice show that heterozygosity for *Bmpr2* promotes the development of PH in response to hypoxia and is associated with reduced eNOS expression and activity (Hideyuki Beppu et al., 2004; Frank et al., 2008; Gangopahyay et al., 2011). These mice display impaired EC dependent vasodilation and enhanced vasoconstriction in isolated intrapulmonary artery (IPA) preparations (Frank et al., 2008), demonstrating loss of *Bmpr2* expression impairs vascular reactivity within the pulmonary tree. Stimulation of *Bmpr2* mutant IPAs or ECs with BMP2 ligand rescues eNOS expression and activity (Anderson et al., 2010; Gangopahyay et al., 2011), suggesting reduced BMP signaling in these mice deregulates eNOS and promotes endothelial dysfunction and abnormal vascular reactivity. Together these data demonstrate that *Bmpr2* expression is critical for pulmonary vascular function and that mutations that reduce BMPR2 expression or disrupt BMP signaling promote the development of pulmonary hypertension.
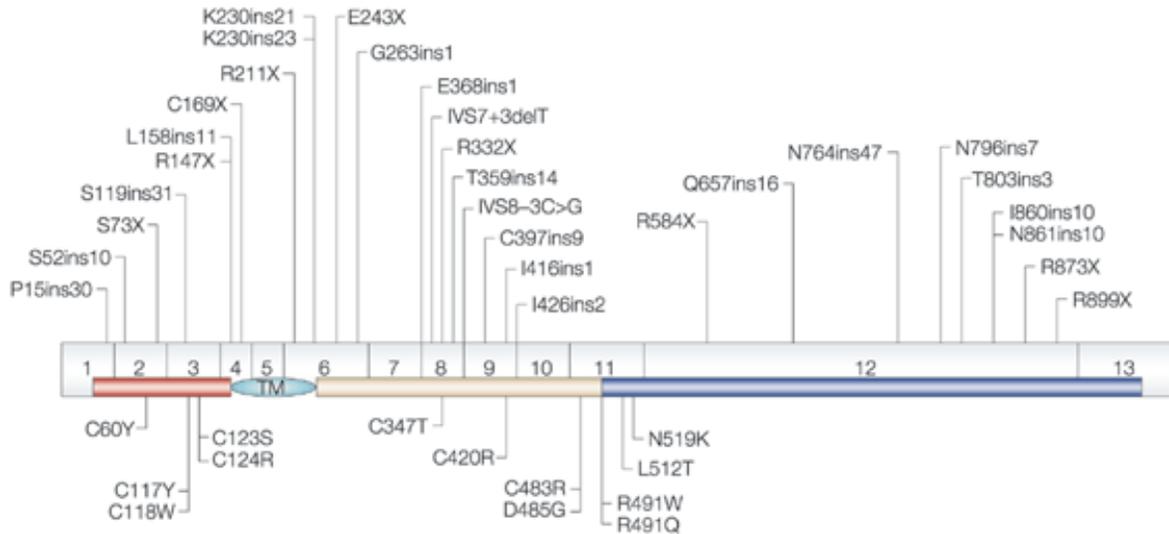
Figure 4: Germline *BMPR2* mutational spectrum in primary pulmonary hypertension. Bone morphogenetic protein receptor type 2 (*BMPR2*) is a 13-exon gene that encodes a ligand-binding domain (red), transmembrane domain (TM), kinase domain (yellow) and cytoplasmic tail (dark blue). Truncating mutations are indicated above and missense mutations below. These are common mutations of the more than 200 known mutations.

Adapted from: (Waite et al., 2003) Copyright Permission: License #3654430510977

Part III: Caveolae and Pulmonary Hypertension

Caveolae are small (50-100nm) invaginations of the plasma membrane. Found abundantly in ECs, adipocytes, and smooth muscle cells, these structures are enriched in sphingolipids and cholesterols and obtain their structure from two core caveolar proteins, Caveolin-1 (Cav-1) and Cavin-1 (Fig 5) (M.-O. Parat, 2009). Both Cav-1 and Cavin-1 are required for caveolae formation, and overexpression of Cav-1 is sufficient to drive formation of caveolae in cells normally devoid of caveolae (Drab et al., 2001; Fra et al., 1995; Hill et al., 2008). Interestingly, overexpression of Cav-1 does not further increase caveolar numbers (Bauer et al., 2005).

Caveolae have a variety of functions within the endothelium. The most obvious function, and the one we will discuss extensively in Chapter II, is endocytosis. Caveolar endocytosis is initiated by Src-mediated phosphorylation of both Cav-1 and Dynamin and is a GTP-dependent process (Fig 6) (Minshall et al., 2002; Shajahan, Timblin, et al., 2004; Shajahan, Tiruppathi, et al., 2004). Endocytosis regulates endothelial barrier permeability, transport of materials into and through the cell (including cholesterol transport and regulation), and signaling transduction and regulation, which we will discuss below in more depth (M.-O. Parat, 2009).

Signal transduction and regulation via caveolae is a double-sided regulation in that both the caveolar body itself sequesters, transports, and recycles signaling receptors, and Cav-1 monomers bind to and often, but not always, repress protein function (Couet et al., 1997; Le Roy et al., 2005; Liu et al., 2002; White et al., 2005). The former is accomplished through the endocytosis of materials, and the latter is through protein-protein interactions involving Cav-1's c-terminal scaffolding domain (CSD). This

domain, composed of 20 amino acids (81-101), is responsible for nearly all of Cav-1's inhibitory protein interactions(S. Li et al., 1996a).

Perhaps most extensively studied is Cav-1's inhibition of Endothelial Nitric Oxide Synthase (eNOS). As it's name implies, eNOS is an enzyme that produces nitric oxide (NO). NO is a potent vasodilator and permeability agent and deregulation of eNOS and NO production has been implicated in PH (Giaid et al., 1995). Caveolae regulate eNOS by controlling its localization in caveolar vesicles and by inhibiting its activity through Cav-1 binding (Sbaa et al., 2005). Indeed in Cav-1 null mice, eNOS is constitutively active and NO production is unregulated; however, despite the abundance of NO, these mice develop PH (Maniatis et al., 2008; Y.-Y. Zhao et al., 2009). This likely stems from aberrant localization of eNOS as the loss of Cav-1 results also in the complete loss of caveolar structures(Sbaa et al., 2005). Recent work using a Cav-1 CSD mutant that is unable to bind eNOS but does not disrupt caveolae results in similar increased NO production; however, its effects on PH is unknown (Bernatchez et al., 2011). Further work using this CSD mutant will prove pivotal for our understanding of the dual caveolar regulation of eNOS.
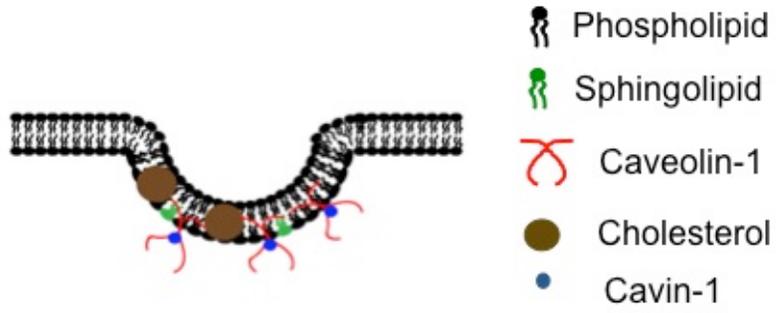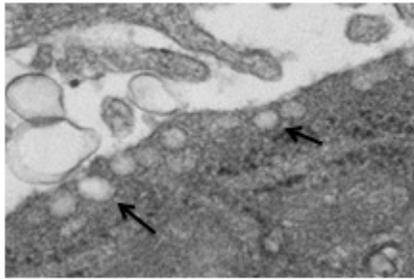
Figure 5: Caveolar structure. Left: EM of caveolar structures at the cell surface. Right: Schematic of caveolar structures.
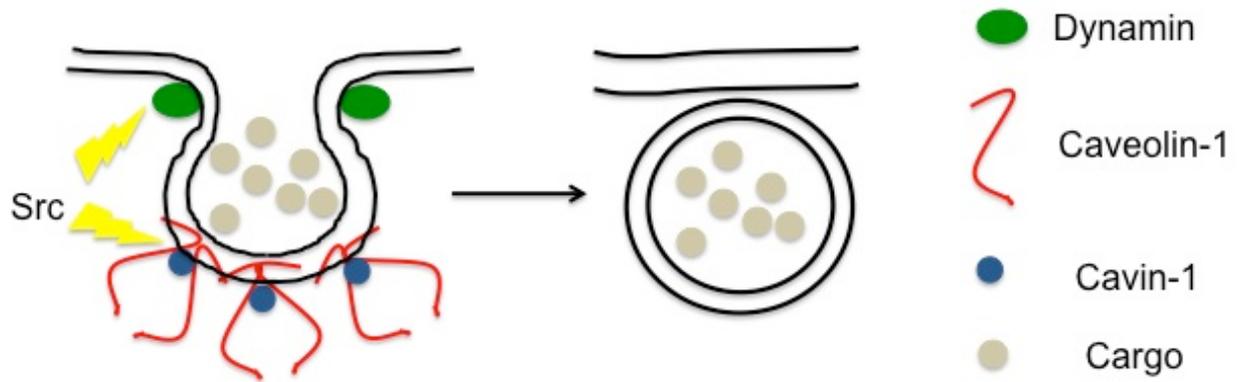
Figure 6: Src-mediated caveolar endocytosis. Src-mediated phosphorylation of Cav-1 and Dynamin, followed by Dynamin-mediated, GTP-dependent caveolar bud scission and endocytosis.

Another noteworthy interaction is Cav-1 and Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2)(Labrecque et al., 2003). VEGFR-2 is an endothelial specific mitogen that regulates angiogenesis and vascular permeability and has been implicated in the development of plexiform lesions in PH patients (Tuder et al., 2001). Interestingly, inhibition of VEGFR-2 through VEGFR-2 receptor inhibitors, most frequently, SU5416, has been used as an experimental PH model, suggesting tight regulation of VEGFR-2 signaling pathways is crucial in the context of PH (Stenmark et al., 2009).

Much work has been done in regards to caveolae and PH. Patel *et al* has shown increased caveolae numbers in IPAH patients lungs, while other groups have shown Cav1 overexpression in mice impairs NO production and endothelial permeability (Bauer et al., 2005; Patel et al., 2007). Interestingly, in the monocrotaline-induced PH model, researchers saw reduced Cav-1 levels in the pulmonary endothelium (Mathew et al., 2004). Other groups note that loss of Cav-1 expression results in increased EC permeability, increased PVR, and induces the development of PH (Lin et al., 2007; Maniatis et al., 2008; Y.-Y. Zhao et al., 2009). And most recently, Austin *et al* discovered two novel frame-shift mutations in Cav-1 in IPAH patients without *BMPR2* mutations (E. Austin et al., 2012). It is also worth noting that there is evidence of Cav1/caveolar-BMPR2 regulatory interactions (Nohe et al., 2005; Ramos et al., 2006; Wertz et al., 2008). BMPR2 localizes to lipid rich caveolar rafts where caveolae regulate BMP signaling. Thus evidence of Cav-1 and caveolar defects in IPAH patients may be related to BMPR2 function. Together these data highlight the importance of caveolae and Cav-1 within the endothelium and suggest that deregulation may drive the development of PH.

Part IV: Src Kinase and Pulmonary Hypertension

Src kinase came to light in 1976, long after the discovery of the transforming proto-oncogene v-Src in 1910 (Rous, 1910; Stehelin et al., 1976). Since then eight additional Src family member kinases (SFKs) have been discovered- Lyk, Frk, Yes, Fyn, Fgr, Hck, Lyn, Blk (Dymecki et al., 1990; J. Lee et al., 1994; Quintrell et al., 1987; Semba et al., 1986; Semba et al., 1985; Thuveson et al., 1995; Tronick et al., 1985; Yamanashi et al., 1987). Their expression patterns can be divided into two classes: widely expressed (Src, Fyn, Yes) and restricted expression (Lck, Hck, Fgr, Blk, Lyn). While the remainder of this work will focus on solely on Src kinase many aspects remain applicable to other SFKs.

Src family kinases have the same basic domain structure (Fig 7a). These domains help define where the protein is localized, proteins with which it may interact, and how it is regulated. At the N-terminus, a 14-carbon myristoylation modification in the Src-homology (SH) 4 domain helps target Src to the plasma membrane (Resh, 1994). Following the SH4 domain, there is a "unique" domain, so-called as this domain differs among the different SFK members. While this domain is dispensable for the regulation of Src (Engen et al., 2008), it may help define which proteins interact with individual SFKs. Extensively involved in the regulation of Src are the SH3 and SH2 domains. These domains, found in a wide array of proteins, act as protein binding domains and function in signal transduction (Pawson et al., 1992). The SH3 domain, composed roughly of 60 amino acids, is a proline rich, left-handed helix predominantly involved in protein interactions (Cicchetti et al., 1992; Ren et al., 1993).
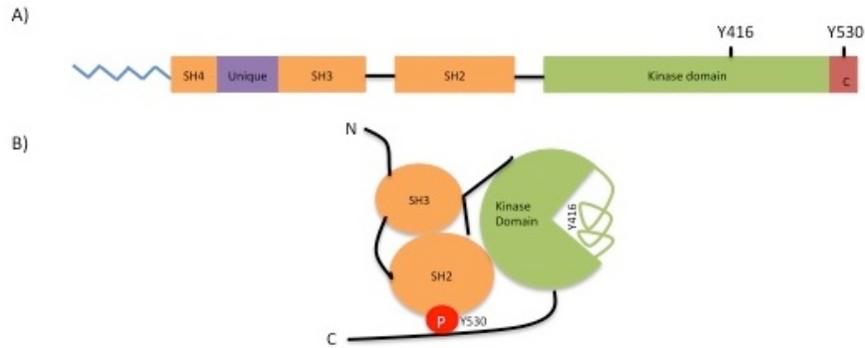
Figure 7. Src kinase structure. A) Open, active Src kinase conformation. This conformation shows the exposed kinase domain. In this conformation Y416 would be phosphorylated while Y530 would remain unphosphorylated. B) Closed, inactive Src kinase conformation. This conformation hides the ATP binding pocket of the kinase domain and blocks Y416 phosphorylation. Notice that Y530 is phosphorylated and bound to the SH2 domain.

The SH2 domain, also involved in protein interactions, recognizes and binds phospho-tyrosine residues, most frequently pYEEI sequences, though other sequences have been identified (Mori et al., 1993; Pawson et al., 1992; Songyang et al., 1994). The SH1, or kinase domain, contains strictly conserved residues that are required for ATP binding and phosphorylation events (Hunter et al., 1985). This domain contains the crucial Tyr 416 residue whose phosphorylation is responsible for Src kinase activation. Finally, there is a C-terminal regulatory domain that is responsible for the majority of Src's negative regulation. This domain contains a few regulatory amino acid residues; however, the most characterized is the negative phosphorylation of Y530.

Src kinase activity is regulated by a combination of catalytic (phosphorylation) and non-catalytic (protein interactions) mechanisms. Though a variety of regulatory phosphorylation sites exist (Table 2) Y416 and Y530 are by far the most extensively characterized and seemingly most crucial. Phosphorylation of the C-term Y530 site results in a conformational change that sequesters the kinase activation loop by folding the protein in on itself (Fig 7b)(Cooper et al., 1986; Kmiecik et al., 1987; Piwnica-Worms et al., 1987). This phosphorylation is mediated by the ubiquitously expressed CSK (c-terminal Src Kinase)(Nada et al., 1991; Okada et al., 1991; Okada et al., 1989) while removal, or dephosphorylation, is mediated by several Protein Tyrosine Phosphatases (PTPs)(Bjorge et al., 2000). Phosphorylation of Y416 is achieved through auto-phosphorylation and is dephosphorylated by PTPs (Bjorge et al., 2000; Smart et al., 1981). Phosphorylation of this residue requires an open conformation to allow access to the tyrosine residue (Fig 7a). Importantly, phosphorylation of Y416 is required for Src activation, so while many non-catalytic mechanisms exist to negatively regulate Src, non-catalytic activation also requires catalytic Src auto-phosphorylation.

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Table 2: Phosphoregulation of Src Kinase

Amino Acid Residue	Protein Kinase	Regulatory Effect
Ser12	PKC	No direct effect
Ser17	PKA	Unclear
Thr34	CDK/cdc12	Activation
Thr46	CDK/cdc12	Activation
Ser72	CDK/cdc12	Activation
Tyr138	PDGFR	No direct effect
Tyr213	PDGFR, ErbB2/HER2	Activation
Tyr416	Src	Activation
Tyr530	CSK	Inhibition

Adapted from: (Roskoski, 2005)

Non-catalytic Src inhibitors exert their repression by inducing conformational changes that block access to the kinase domain. But in addition to physical disruption, non-catalytic inhibitors often recruit catalytic inhibitors in order to achieve long-term repression (Chong et al., 2005). One non-catalytic inhibitor is unique in that is also functions catalytically. CHK, a CSK homologue, physically binds to Src and represses its activity while actively phosphorylating the c-terminal tail. This repression does not require the dephosphorylation of Y416 and simply overrides the activating phosphorylation. It is considered a non-catalytic inhibitor as it is able to repress Src activity even when Y530 is mutated to phenylalanine and therefore can no longer be phosphorylated (Chong et al., 2004). Another non-catalytic inhibitor is RACK1 (Fig 8a). RACK1, an intracellular receptor for Protein Kinase C (PKC), binds to and inhibits Src. Interestingly, as RACK1 binds to both Src and PKC, one hypothesis is that RACK1 may also function as a dual inhibitor by facilitating the phosphorylation of Src by PKC (Chong et al., 2005; Mochly-Rosen et al., 1995; Yaciuk et al., 1989). However, the role of PKC-mediated Ser12 phosphorylation on Src activity is unclear (Roskoski, 2005). A true, single mechanism, non-catalytic Src inhibitor is WASP (Fig 8c). WASP inhibits Src activity by binding to both the SH3 and kinase domains thereby inducing conformational change and blocking the kinase domain(Schulte et al., 2003). And finally, and of special note, Cav-1 functions as a dual non-catalytic Src inhibitor (Fig 8b). Cav-1 binds Src kinase via Cav-1's CSD while actively recruiting CSK to the c-terminus of Src (S. Li et al., 1996a). Together, Cav-1 is able to exert immediate and long-term inhibition of Src kinase.
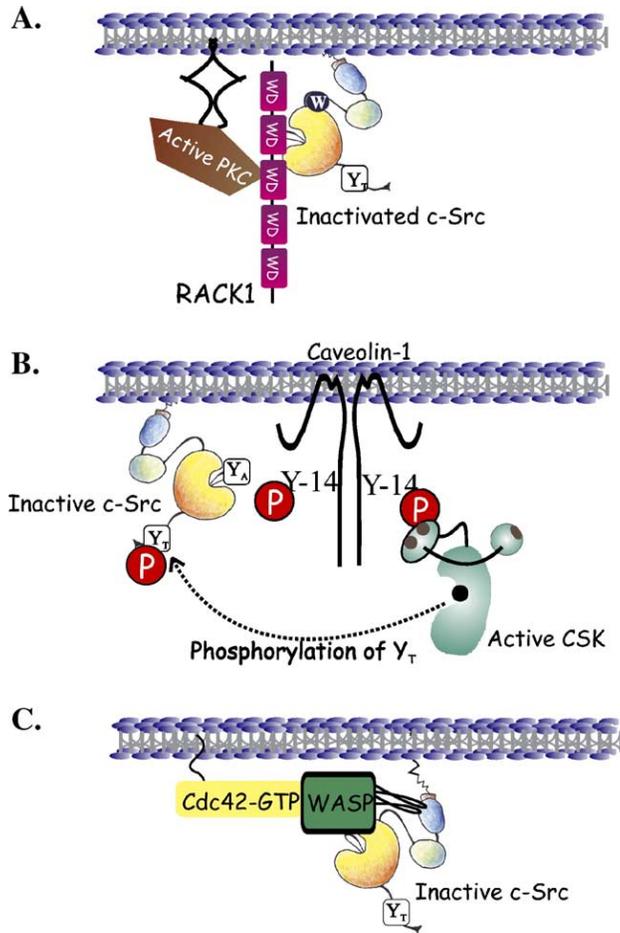
Figure 8: Non-catalytic Src kinase inhibitors. These non-catalytic inhibitors bind to and inhibit Src kinase activity. (A) RACK1 binds to both Src and PKC. (B) Cav-1 binds to Src and recruits CSK to the c-terminus of Src. (C) WASP binds to and inhibits the SH3 and kinase domains of Src.
Adapted from: (Chong et al., 2005) Copyright Permission: License #3654460133472

These catalytic and non-catalytic measures work in concert to keep Src activity under proper control; however, aberrant regulation of Src kinase in PH is apparent and has become an active field of investigation. This may not be of surprise as Src activation has been implicated in many forms of cancer and may be exerting similar pro-proliferative, anti-apoptotic functions in the pulmonary endothelium in PH (Kim et al., 2009). Indeed, groups have identified Src activation in the pulmonary vasculature of PH patients (Paulin et al., 2014; Paulin et al., 2011). Using animal models of PH, other groups have shown that Src is activated in both monocrotaline and hypoxia induced PH and that inhibition of Src improved several parameters of PH including reduced RV pressures, RV hypertrophy, muscularization, and proliferation and increased apoptosis (Paulin et al., 2014; Pullamsetti et al., 2012). Insights as to how these effects may be mediated come from earlier studies that showed that increased Src activation in PH patients was associated with a downregulation of miR-204 via STAT3 (Brock et al., 2009; Courboulin et al., 2011). They show miR-204 downregulation correlates with PH severity and that delivery of miR-204 to animal lungs reduced PH severity in these animals (Courboulin et al., 2011). Interestingly, activation of Src-STAT3-miR-204 pathway led to a reduction of BMPR2 expression, providing a potential mechanism for reduced BMPR2 expression in idiopathic PAH (Brock et al., 2009). All together these data provide evidence of the importance of Src kinase regulation and suggest Src kinase activation may be a critical step in the development of PH.

Summary

In summation, pulmonary hypertension is a lethal disease with no known cure. There is evidence of *BMPR2* mutations in a large subset of HPAH patients and reduced *BMPR2* expression in IPAH patients, but how these changes drive the development of the disease is unclear. This work aims to elucidate how *BMPR2* mutations contribute to the pathogenesis of PH and show how BMPR2 intersects with other known mediators of PH, specifically Src Kinase and caveolae.

CHAPTER II

BMPR2$^{+/-}$ MUTATIONS PROMOTE SRC KINASE DEPENDENT CAVEOLAR TRAFFICKING DEFECTS AND ENDOTHELIAL DYSFUNCTION

Introduction

Despite intensive research, there is no clear consensus on the role of *BMPR2* in development of PH. However, given *BMPR2's* high expression level in the pulmonary endothelium, it is reasonable to suspect the endothelium may be the primary target of vascular injury in HPAH patients (Atkinson et al., 2002; Frank et al., 2008; Ramos et al., 2006; Takahashi et al., 2006). This hypothesis is supported by animal studies that show mice with conditional deletion of *Bmpr2* in the endothelium develop spontaneous PH and have endothelial barrier dysfunction associated with increased pulmonary vascular leak and peri-vascular inflammation, a common occurrence in patients with PH (Burton, Ciuclan, et al., 2011; Burton, Holmes, et al., 2011; Hong et al., 2008) (Dorfmuller et al., 2003; Savai et al., 2012; Stacher et al., 2012). Additional studies show heterozygous *Bmpr2* mutant mice have pulmonary endothelial cell (PEC) dysfunction with decreased endothelial-dependent relaxation in isolated intrapulmonary pulmonary artery preparations(Frank et al., 2008). These data suggest that endothelial dysfunction associated with abnormal endothelium-dependent vasodilatation and with decreased endothelial barrier function contribute to pulmonary vascular pathophysiology in patients with HPAH.

In the pulmonary vasculature, loss of Caveolin-1 (Cav-1) expression promotes PH in mice(Maniatis et al., 2008), and is associated with chronic activation of endothelial

NOS and enhanced pulmonary vasoconstriction(Y. Y. Zhao et al., 2009). Additionally, caveolar numbers are deregulated in the pulmonary vasculature of patients with idiopathic PAH(Patel et al., 2007), and recent studies have identified *BMPR2* negative HPAH patients with frame shift mutations in the *CAV-1* gene(Asosingh et al., 2012; E. Austin et al., 2012). These data suggest that caveolar defects can promote pulmonary vascular disease, but the relationship between caveolae and the pathogenesis of *BMPR2* mutation-associated HPAH has not been established.

In these studies we have used PECs derived from heterozygous null *Bmpr2*$^{+/-}$ mutant mice to establish that loss of a single *Bmpr2* allele gives rise to enhanced, Src kinase-dependent caveolar trafficking. Late Outgrowth Endothelial Progenitor Cells (LO-EPCs) isolated from the peripheral blood of an HPAH patient have a similar defect in Src activation. We also show that *Bmpr2*$^{+/-}$ mutant PECs have decreased barrier function, and that treatment with a Src kinase inhibitor reverses the caveolar trafficking defect and reduces permeability in *Bmpr2*$^{+/-}$ PECs. These data establish for the first time a relationship between *BMPR2* mutations and caveolar trafficking defects that may promote pulmonary vascular disease in HPAH, and suggest that Src kinase inhibitors may be used therapeutically to ameliorate these effects.

Materials and Methods

Chemicals and Reagents

Recombinant human BMP2 (R&D Systems); 3-(4-chlorophenyl)-1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine [PP2] (Cayman Chemical); Dynasore hydrate, 70kD dextran-Rhodamine and FITC-conjugated albumin, SKI606 [Bosuntinib] (Sigma-Aldrich); AlexaFluor-555 Albumin, AlexaFluor-488 ConA (Molecular Probes); interferon

gamma (IFNγ) (Peprotech); Dio-Ac-LDL (Biomedical Technologies Inc); PP3 (Millipore). Monoclonal antibodies include pY14-Cav1 [clone 56], Cav1 [clone 2234], Bmpr2 [clone 18] and β-catenin [clone 14] (BD antibodies), and β-actin [clone AC-74] (Sigma). Polyclonal antibodies include Cav1, pY416-Src, and Src (Cell Signaling) and Cavin-1 (Bethyl Laboratories). Antibodies for FACs analysis include CD31 [clone WM59], CD146 [clone P1H12], CD14 [M5E2] and CD45 [clone HI30] (BD Biosciences).

Mouse pulmonary endothelial cell (PEC) isolation, characterization and culture

Six independent lines of PECs were generated from 3 wild type (W1, W2, W3) and 3 $Bmpr2^{\Delta ex4-5/+}$ ($Bmpr2^{+/-}$) (N1, N3, N6) mice (H. Beppu et al., 2005) as described above. For this, $Bmpr2^{+/-}$ mice were maintained on a C57Bl/6 background (>10 generations backcrossed) and crossed with C57Bl/6 H-2Kb-tsA58 SV40 large T Ag transgenic mice (Charles Rivers "immortomice") to generate wild type and $Bmpr2^{+/-}$ immortomice. Genotype was confirmed by PCR using primers and conditions outlined in previous studies(Anderson et al., 2010; H. Beppu et al., 2000). To isolate PECs, mice were anesthetized with isoflurane prior to sacrifice by cervical dislocation. Lungs were perfused with a mixture of PBS/2mM EDTA followed by 0.25% Trypsin/2mM EDTA via right ventricle. Heart and lungs were removed en bloc and incubated at 37°C for 20 minutes. Finally, lungs were perfused again in complete endothelial microvascular media EGM-2MV (Lonza) and the perfusate recovered for isolated cells. Cells were grown under permissive conditions in EGM-2MV + 10units/mL INFγ at 33°C before being transferred to 37° C without INFγ for 3-5 days to inhibit SV40 large-T antigen activity for phenotyping and before conducting experiments. Endothelial cell phenotype was confirmed for all isolates with >90% VCAM and EPCR positive expression by FACS using mouse anti-

VCAM-AlexaFluor 647 [clone 429] and EPCR-APC [clone eBio1560] (eBioscience), and by the ability to form tubes in 3D culture in Collagen 1, as described(Anderson et al., 2010; Frank et al., 2005). For PP2 treatment, experiments were performed in complete media. Dynasore treatment was performed in serum-free basal EBM2 media. For BMP2 treatment, cells were first serum starved in basal EBM2 media with 0.1% Bovine Serum Albumin (Sigma) for 18 hours.

Isolation and characterization of late outgrowth endothelial progenitor cells (LO-EPCs)

Late outgrowth endothelial progenitor cells (LO-EPCs) were isolated from peripheral blood samples as previously described(Mead et al., 2008). Roughly 60mL of blood was collected from each patient and aliquoted into 50mL falcon tubes containing 3mLs 3.8% sodium citrate. Samples were collected from normal volunteers and PAH patients attending the Vanderbilt Pulmonary Hypertension Clinic after obtaining informed consent under a Vanderbilt University Institutional Review Board approved protocol (IRB #9401 "Genetic and Environmental Pathogenesis of PPH"). The blood was then diluted 1:1 with phosphate buffered saline (PBS) and slowly layered atop 10mL of Ficoll (GE healthcare) in a separate tube. Samples were then spun at 400g for 35 minutes at room temperature with brake and accelerator turned off. The mononuclear cell layer was then collected from the Ficoll density gradient and diluted 1:1 in PBS followed by centrifugation for 20 minutes at 300g at room temperature. The supernatant was discarded and the cell pellet was re-suspended in EGM-2MV + 20% ES grade fetal bovine serum (FBS, Hyclone). The cell suspension was then plated into T-75 flasks coated with 5µg/cm^2 collagen I (BD Biosciences). Media was changed every two days, and LO-EPC colonies pooled 2-3 weeks after plating, as described (Mead et al.,

2008). Endothelial cell phenotype was confirmed by Dio-Ac-LDL uptake and by flow cytometry for EC markers CD31 and CD146, and negative for leukocyte and macrophage markers CD45 and CD14, respectively. Briefly, cells were plated on gelatin-coated coverslips before being incubated in 10ug/ml Dio-Ac-LDL for 4 hours at 37°C. Cells were then rinsed in PBS and fixed in 4% PFA for imaging. For fluorescence-activated cell sorting (FACS) cells were trypsinized, centrifuged and cell pellets re-suspended in 100uL of EGM-2 basal serum with desired antibody. After incubation on ice for one hour, samples were centrifuged, re-suspended in basal EGM-2 media and evaluated by FACS using BD FACSCantoIIs.

Characterization of caveolae numbers

For transmission electron microscopy (TEM) PECs were fixed in 2.5% glutaraldehyde and 0.1M sodium cacodylate prior to ethanol dehydration. Cells were subsequently pelleted by gravity in propylene oxide and embedded in resin for imaging on Philips FEI T-12 transmission electron microscope. For tissue fixation, we performed tracheal perfusion with the same fixative. Total PEC caveolae were counted in 3 randomly selected images per mouse lung. Caveolae numbers were quantified by a blinded observer counting caveolar-like structures of 50-100nm size per micron of endothelial plasma membrane.

Cav-1 and Cavin-1 localization in isolated PECs

PECs were grown to confluence in non-permissive conditions on gelatin-coated coverslips. Cells were then fixed in 4% paraformaldehyde (PFA) (Electron Microscopy Sciences) and permeabilized in 0.2% Saponin (Sigma) in PBS at room temperature.

Coverslips were incubated in primary antibody in 3% donkey serum in PBS overnight at 4°C, followed by secondary antibody for 1 hour at room temperature. Nuclei were stained with Topro3 (Molecular Probes). Digital images were obtained by confocal microscopy using a Zeiss LSM510-Meta microscope. To quantify subcellular localization of Cav-1 and Cavin-1, x-axis digital slices were taken across the middle of each cell, and immunofluorescence localization of Cav-1 and Cavin-1 quantified by generating intensity plots using ImageJ software "plot profile" function. To correct for differing cell size during fluorescent quantification, the distance across the cell was normalized to 100 arbitrary units. Intensity of Cav-1 and Cavin-1 fluorescence at the plasma membrane was quantified by averaging the fluorescent intensity of the first and last 20 distance units and dividing by the average intensity of the middle 21-79 distance units. We evaluated between 10 and 30 cells for each cell line under each condition, pooling results for each genotype or treatment condition, as indicated in the figure legends. Serial z-stack sections were also gathered at the time of analysis and reconstructed using LSM510 software to evaluate the intracellular distribution of Cav-1.

Western blots

Cells were lysed in 1x lysis buffer containing 150mM NaCl, 25mM HEPEs, 5mM EDTA, 1% TritonX-100, 10% glycerol and phosphatase and protease inhibitors (1:100 Sigma) on ice for 15 minutes before centrifugation at 5000xg for 30 minutes. Western blots were blocked in 5% milk before incubation in primary antibody in either 5% milk or bovine serum albumin (BSA) for phosphorylation site-specific antibodies overnight at 4°C. Primary antibodies were detected using species-specific HRP-conjugated secondary antibodies and Perkin-Elmer Western Lightning Plus ECL reagent. Films were exposed

over a range of times, digitally scanned and densitometry performed using ImageJ on films exposed for intermediate time periods in order to avoid non-linear ECL signals associated with very short or long exposure times.

Albumin endocytosis in isolated PECs

Cells were grown to confluence in non-permissive conditions on gelatin-coated coverslips in complete EGM-2MV media before being switched to basal EGM-2 media immediately prior to start of experiment. Cells were incubated with or without Dynasore for 30 minutes at 37°C prior to the addition of 1mg/ml AlexaFluor555-Albumin in chilled basal EGM-2. Cells were then incubated at 4°C for 30 minutes to inhibit endocytosis(Brickman et al., 1995; Rode et al., 1997; Silverstein et al., 1977) while allowing fluorophore attachment before being transferred to 37°C for 5 minutes to initiate endocytosis. Cells were then place on ice and stripped of all membrane bound flourophore by three rounds of acid stripping in 100mM glycine pH 2.0 followed by Hanks Balanced Salt Solutions (HBSS) pH 7.4 each for 5 minutes on ice. Cells were then transferred to 37°C for 10 seconds in HBSS pH 7.4 to remove any remaining fluorophore. Immediately following, cells were washed two times in PBS and fixed in 4% PFA. Following fixation cells were processed for confocal imaging.

Albumin exocytosis in isolated PECs

Cells were grown to confluence in non-permissive conditions on gelatin-coated coverslips in complete EGM-2MV media. Cells were incubated in chilled media containing 1mg/ml AlexaFluor555-Albumin and subsequently placed at 4°C for 30 minutes to allow fluorophore binding. Cells were then transferred to 37°C for 5 minutes

to initiate endocytosis. Following endocytosis cells were immediately incubated on ice and received one round of acid stripping (100mM glycine, pH2.0, HBSS pH 7.4 for five minutes each) to remove fluorophore bound to the cell surface. Cells were rinsed twice in PBS before addition of complete media. Time zero was collected at this point and placed on ice while the remaining dishes were returned to 37°C to allow exocytosis of the remaining internalized fluorophore. Cells were placed on ice at the indicated time points before receiving two final rounds of acid stripping and subsequent fixation in 4% PFA.

Endothelial Permeability

Transwell filters, 12-mm in size with 0.4mm pore size, were coated in 0.1% gelatin in PBS and further prepared according to manufacturer recommendations. PECs were plated and grown to confluence in EGM-2MV on these filters 3-4 days at 37°C prior to treatment. Transendothelial electrical resistance (TEER) was tracked throughout the experiment using EVOM2 voltometer and STX2 electrode (WPI). Permeability assessments began once TEER measurements plateaued. FITC-Albumin (10mg/ml) and 70kd Rhodamine-Dextran (1mg/ml) were added to the upper chamber and complete media without additional fluorophores added to the lower chamber. For parallel 4°C/37°C studies, cells were grown to confluence in EGM-2MV media and transferred to 4°C 30 minutes prior to fluorophore addition, to block endocytosis(Brickman et al., 1995; Rode et al., 1997; Silverstein et al., 1977). Samples were taken from the lower chamber at specified time points and FITC-Albumin and Rhodamine-Dextran measured on the Molecular Probes SpectraMax at Em488/Ex530 and Em570/Ex590. Studies were

performed using 1-3 wild type and *Bmpr2$^{+/-}$* PEC lines, as indicated in the figure legends, and repeated in triplicate for each line.

Statistical Analyses

Statistical analyses were performed by Student's T-Test for paired group comparisons, or One-Way or two-way ANOVA for multiple between group comparisons using Dunnett's or Sidek's correction for multiple post-hoc between group comparisons, as indicated. The minimal level of significance was set at $p<0.05$. Statistical analyses were performed using Graph Pad Prism 5 software.

Results

Altered Cav-1 and Cavin-1 localization in Bmpr2$^{+/-}$ PECs

In order to evaluate the effects of heterozygous *Bmpr2* mutations on caveolar localization and function in PECs, we first isolated and characterized 6 separate lines of conditionally immortalized PECs from 3 wild type mice and 3 mice carrying heterozygous null *Bmpr2* mutations (*Bmpr2$^{+/-}$* mice). When cultured under non-permissive temperatures, all 6 of these cell lines have typical endothelial cobblestone morphology, rapidly form tube-like structures in 3D cultures, and >90% express endothelial-specific markers VCAM and EPCR by FACS. These cells are advantageous over primary cultured endothelial cells since they can be maintained and expanded over numerous passages at permissive temperatures, but re-differentiate and express endothelial specific markers at non-permissive temperatures(Anderson et al., 2010; Frank et al., 2005; Frank et al., 2008).

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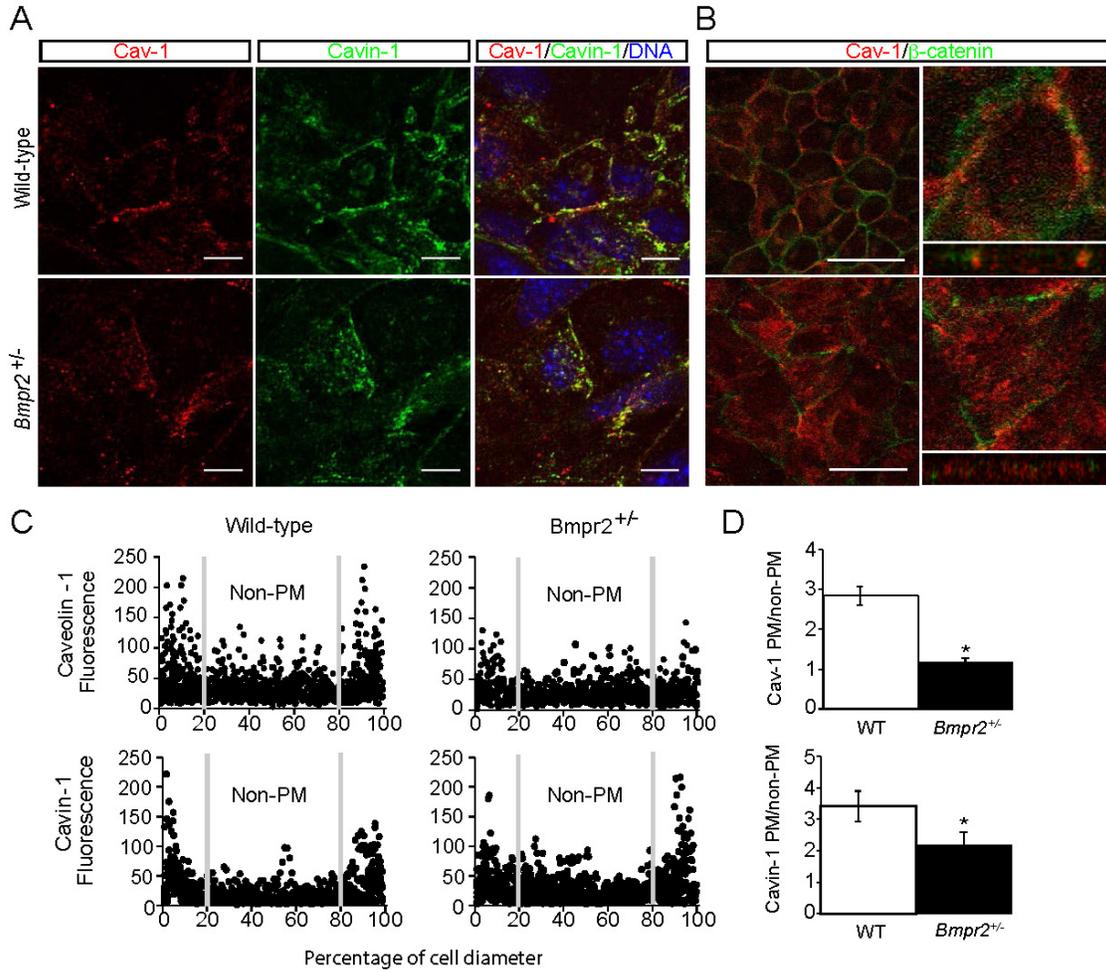
Figure 9: Mis-localization of caveolar structural proteins in *Bmpr2*+/- PECs. (A) Representative immunofluorescence images of Caveolin-1 (Cav-1/red) and Cavin-1 (green) in wild type and *Bmpr2*+/- PECs. Scale bars: 10μm. (B) Left panels: Single x/y plane from z-stack images. Caveolin-1 (red) and beta-catenin (green). Scale bar: 50μm. Right panels: Orthogonal views reconstructed from z-stack images depicting caveolin-1 localization in x/z and y/z planes of a single cell magnified from left panels. (C) Fluorescent intensity plots depicting Cav-1 and Cavin-1 distribution across the cells. Grey lines delineate plasma membrane (PM) from non-PM. Plots generated from multiple cells from 3 wild type (n=12) and 3 *Bmpr2*+/- (n=14) cell lines. Fluorescent in arbitrary units and distance in percentage of cell diameter (D) Quantitative analysis of Cav-1 and Cavin-1 localization. Values are expressed as the mean +/- SEM ratio of PM to non-PM fluorescence intensities. T-test *p<0.05 vs. wild type controls.

Using these cells we show that there is altered localization of Cav-1 and Cavin-1 in *Bmpr2*$^{+/-}$ PECs from a predominantly plasma membrane localization in wild type PECs to an intracellular localization in *Bmpr2*$^{+/-}$ PECs (Fig. 9). This is apparent both from representative immunofluorescence images (Fig. 9A) and from fluorescence intensity plots obtained across multiple PECs lines isolated from wild type and *Bmpr2*$^{+/-}$ mice (Fig. 9C). Quantitative analysis of these fluorescence intensity plots confirms that there is a significant reduction in Cav-1 and Cavin-1 localization at the plasma membrane in *Bmpr2*$^{+/-}$ PECs (Fig. 9D). This effect is not associated with a change in cell shape or size as shown in orthogonal slices constructed from serial z-stacks of the entire cell (Fig 9B) and is not associated with a generalized defect in plasma membrane markers since the adherens junction marker β-catenin was similarly membrane localized in both wild-type and *Bmpr2*$^{+/-}$ PECs (Fig. 9B). Taken together, these data indicate that there is an increase in intracellular localization of Cav-1 and Cavin-1 in *Bmpr2*$^{+/-}$ PECs.

Increased number of caveolae in Bmpr2$^{+/-}$ *mouse lungs and PECs*

We used transmission electron microscopy (TEM) to determine whether changes in Cav-1 and Cavin-1 localization are associated with alterations in caveolar structures in *Bmpr2*$^{+/-}$ mouse PECs. There are increased numbers of intracellular caveolar-like structures in PECs from *Bmpr2*$^{+/-}$ mice (Fig. 10A). Quantitative analysis of caveolar numbers in lung sections indicates that there are also increased numbers of caveolar-like structures in the intact pulmonary endothelium of *Bmpr2*$^{+/-}$ mice (Fig. 10B/C). These data indicate that changes in intracellular localization of Cav-1 and Cavin-1 are associated with increased numbers and intracellular localization of caveolae in *Bmpr2*$^{+/-}$ PECs.
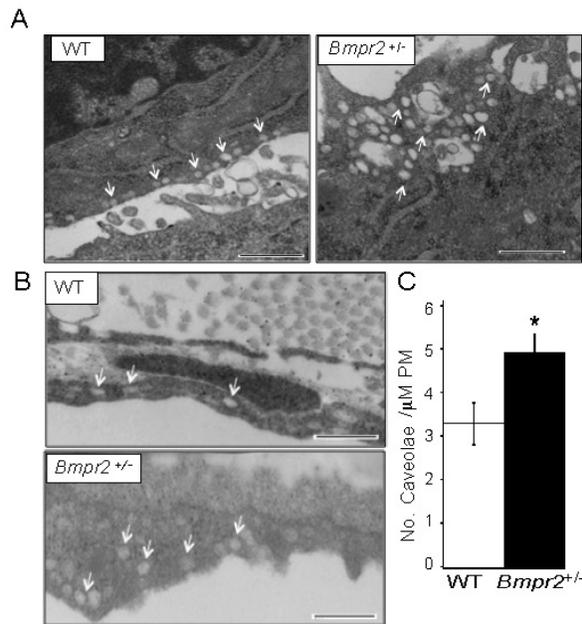
Figure 10. Increased caveolar structures in *Bmpr2*^{+/-} PECs and lungs. (A) Transmission electron microscopy (TEM) of cultured PECs from wild type and *Bmpr2*^{+/-} mice. Arrows indicate caveolar structures. (B) TEM of lung sections from wild type and *Bmpr2*^{+/-} mice. Arrows indicate caveolar structures in the endothelium. Scale bars: 500nm. (C) Quantitative analysis of caveolar structures in the lung endothelium from wild type and *Bmpr2*^{+/-} mice (5 mice per group/3 images per mouse). Values are expressed as the number of caveolae per μm of endothelium. Mean +/- SEM. T-test *p<0.01 vs. wild type controls.

Dynamin-2 inhibition restores Cav-1 localization to the plasma membrane

Increased numbers of intracellular caveolae may result from enhanced Dynamin-2-mediated caveolar scission and endocytosis(Henley et al., 1998). We therefore evaluated the effect of Dynasore, a selective cell permeable Dynamin-2 inhibitor(Kirchhausen et al., 2008; Macia et al., 2006), on Cav-1 localization in $Bmpr2^{+/-}$ PECs. Dynasore restores Cav-1 localization to the plasma membrane in $Bmpr2^{+/-}$ PECs (Fig. 11A/B). These data suggest that increased intracellular localization of Cav-1 in $Bmpr2^{+/-}$ PECs results from increased caveolar endocytosis.

Increased caveolar endocytosis in Bmpr2$^{+/-}$ PECs

To determine if $Bmpr2^{+/-}$ PECs have increased endocytosis we evaluated the uptake of fluorescently label albumin in wild-type and $Bmpr2^{+/-}$ PECs. Using the assay outlined in Fig12A, we show $Bmpr2^{+/-}$ PECs have increased uptake of Albumin-AlexaFluor-555 and that this is blocked in cells pre-treated with Dynasore (Fig. 12B/C), suggesting $Bmpr2^{+/-}$ PECs have increased caveolar endocytosis. However, this assay does not rule out potential defects in exocytosis. To exclude this possibility, we evaluated exocytosis in wild-type and $Bmpr2^{+/-}$ PECs as outlined in Fig12D. We demonstrate $Bmpr2^{+/-}$ PECs have no defect in exocytosis as intracellular albumin levels return to levels similar to wild-type as early as 15 minutes following endocytosis of albumin (Fig. 12E/F). These data demonstrate $Bmpr2^{+/-}$ PECs have increased endocytosis contributing to mislocalization of Cav1 and accumulation of intracellular albumin while exhibiting no defects in exocytosis.
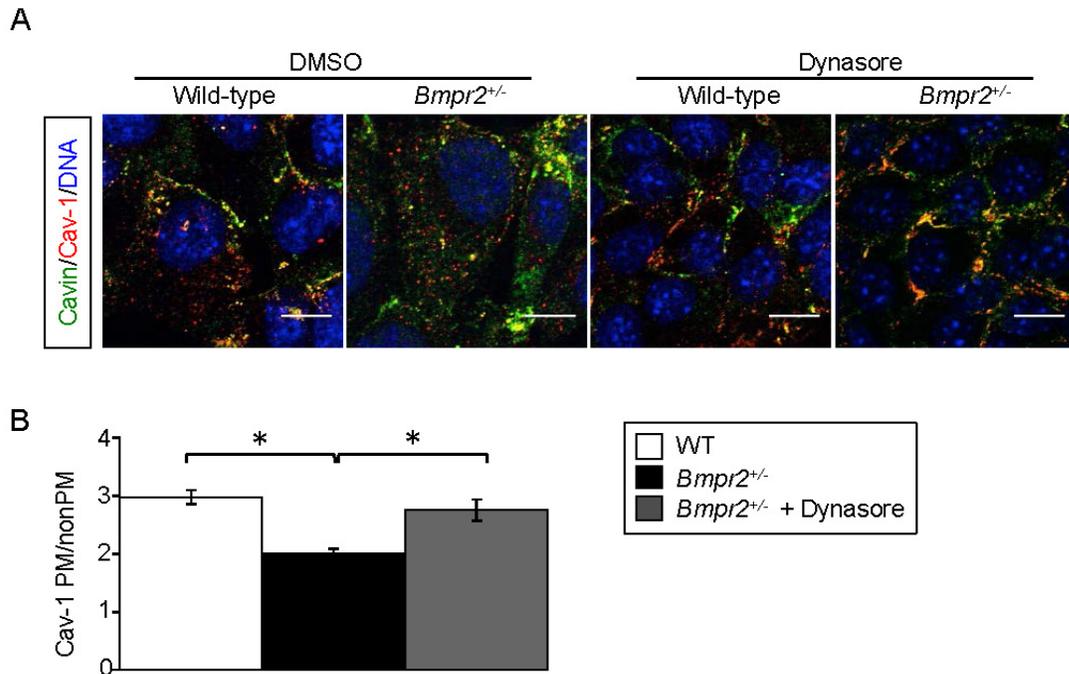
Figure 11: Dynamin-2 inhibition restores Cav-1 localization to the plasma membrane in *Bmpr2+/-* PECs. (A) Cav-1 and Cavin-1 localization after Dynasore treatment. Representative immunofluorescence images demonstrating Cav-1 and Cavin-1 localization following vehicle or 80μM Dynasore treatment for 30 minutes. Scale Bar: 10μM (B) Quantitative analysis of Cav-1 localization after Dynasore treatment. Values expressed as the Mean +/- SEM ratios of PM to non-PM fluorescence intensities in multiple cells from 3 wild-type lines (n=100), 3 untreated *Bmpr2+/-* lines (n=100) and 2 *Bmpr2+/-* lines after Dynasore treatment (n=60). One-way ANOVA with Dunnett's correction for repeated comparisons with untreated *Bmpr2+/-* PECs, *p<0.001.

A

Dynasore Treatment @37°C	Albumin-AF555 @4 °C	Endocytosis 37°C	Acid wash	Fix
30 minutes	30 minutes	5 minutes	3x	

B

WT   Bmpr2+/-

Albumin-AlexaFluor-555

DMSO   DMSO   Dynasore

C

WT
Bmpr2+/-
Bmpr2+/- + Dynasore

D

Albumin-AF555 @4°C	Endocytosis 37°C	Acid wash	Exocytosis @37°C	Acid wash	Fix
30 minutes	5 minutes	1x	0, 5, 15, 30 minutes	2x	

E

0 minutes   5 minutes   15 minutes   30 minutes

Wild-type

Albumin-AlexaFluor-555

Bmpr2+/-

F

wt
null

Figure 12: *Bmpr2*+/- PECs have increased caveolar endocytosis but exhibit normal exocytosis. (A) Schematic illustrating experimental setup in (B). (B) Increased Albumin uptake in *Bmpr2*+/- PECs is ablated in *Bmpr2*+/- PECs pre-treated with Dynasore. Representative immunofluorescence images demonstrating albumin localization following vehicle or 80μM Dynasore treatment for 30 minutes. Cells were incubated in 0.1mg/ml Albumin-AlexaFluor-555 for 5 minutes at 37°C in DMSO or Dynasore as indicated followed by acid stripping to removed membrane bound albumin as outlined in (A). Scale bars: 50μm. (C) Quantitative analysis of Albumin uptake in (B). Values are expressed as the mean fluorescent intensity divided by total cell number +/- SEM. One-way ANOVA with Dunnett's correction for multiple comparisons with untreated *Bmpr2*+/- PECs, *p<0.05. (D) Schematic illustrating experimental setup in (E). (E) Representative immunofluorescence images demonstrating albumin exocytosis following 5 minutes endocytosis. Cells were incubated in 0.1 mg/ml Albumin-AlexaFluor-555 for 5 minutes at 37°C, followed by one round of acid stripping. Cells were then returned to 37°C for the indicated times and subsequently fixed. Scale bars: 50μm. (F) Quantitative analysis of Albumin exocytosis in (E). Values are expressed as the mean fluorescent intensity divided by total cell number +/- SEM. Two-way RM ANOVA with Sidak's correction for multiple comparisons with *Bmpr2*+/- PECs, **p<0.0001, *p<0.01, ns= not significant.

Increased Src kinase activity in Bmpr2$^{+/-}$ PECs

Dynamin-dependent caveolar endocytosis is initiated by the Src-mediated phosphorylation of Cav-1 on Tyr14(Hu et al., 2009; M.-O. Parat, 2009; Shajahan, Timblin, et al., 2004). Western blot analysis indicates that there is increased phosphorylated (pY14) Cav-1, as well as increased expression of activated phosphorylated (pY416) Src kinase in *Bmpr2$^{+/-}$* PECs (Fig. 13A/B).

Previous studies have shown that BMPR2 interacts with Src, and that BMPs reduce basal pY416-Src expression in pulmonary artery smooth muscle cells(Wong et al., 2005). We were unable to detect interaction between Src and Bmpr2 in wild type PECs after immunoprecipitation with Src or Bmpr2 antibodies (S.G, data not shown). Moreover, BMP2 had no effect on basal pY14-Cav-1 or pY416-Src expression in wild type PECs (Fig. 14A/B). However, treatment with BMP2 reduces pY14-Cav1 and pY416-Src expression in *Bmpr2$^{+/-}$* PECs, indicating that BMP2 reverses aberrant Src-dependent Cav-1 phosphorylation in *Bmpr2$^{+/-}$* PECs. Since treatment with BMP ligands activates basal BMPR2 signaling, these data suggest that defective BMPR2 signaling promotes constitutive Src activation in *Bmpr2$^{+/-}$* PECs.

Increased Src activation in HPAH patient late outgrowth endothelial progenitor cells (LO-EPCs)

To determine whether Src activation also occurs in endothelial cells from HPAH patients carrying germ-line heterozygous null mutations at the *BMPR2* locus, we isolated late outgrowth endothelial progenitor cells (LO-EPCs) from an HPAH patient with a known *BMPR2* mutation [Family 164, *BMPR2 V299 FsX1* (*BMPR2 893 ins GG*)] and an IPAH patient without a known *BMPR2* mutation. As previously described(Reinisch et al., 2009),
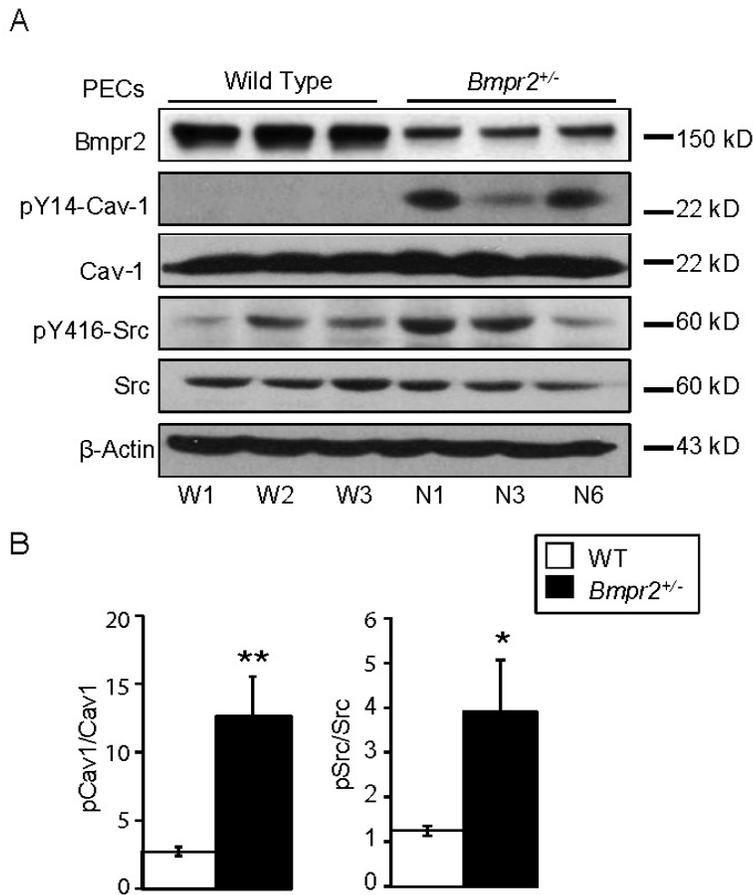
Figure 13: Increased Src and Cav-1 phosphorylation in *Bmpr2*⁺/⁻ PECs. (A) Western blots demonstrating basal expression of phosphorylated Y14-Cav-1 and Y416-Src, and total Bmpr2, Cav-1 and Src kinase from 3 wild-type (W1, W2, W3) and 3 *Bmpr2*⁺/⁻ (N1, N3, N6) PEC lines. β-actin as a loading control. (B) Quantitative analysis of Western blots band densitometry values from 4 independent experimental replicates for a total of twelve samples per genotype. Values expressed as the Mean +/-SEM ratios of band densities, as indicated. T-test, *p <0.0001 and **p<0.001 vs. wild type controls. Gels were run and probed as follows: Gel 1-Bmpr2; Gel 2- pY14-Cav1, pY416-Src, β-actin; Gel 3- Cav1, Src.
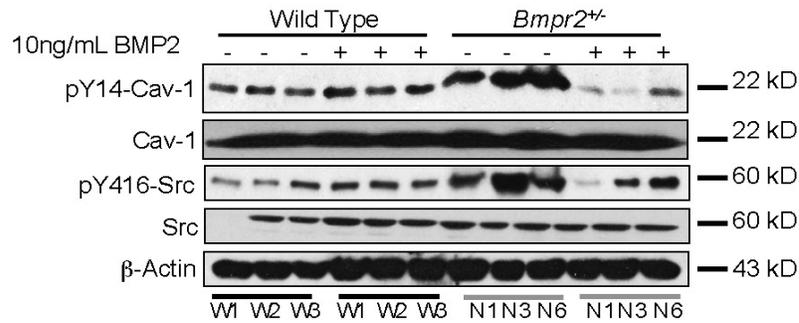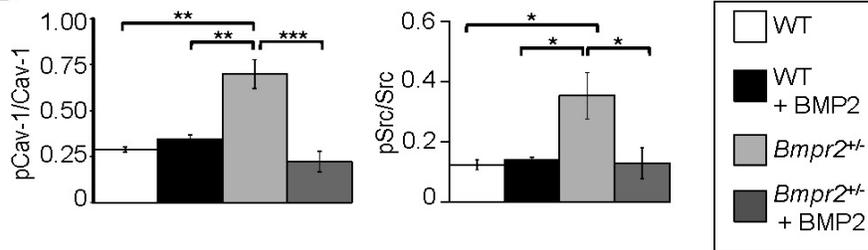
Figure 14. BMP2 stimulation reduces Src activity in *Bmpr2*+/- PECs. (A) Western blot analysis of pY14 and total Cav-1, and pY416 and total Src following vehicle or 10ng/mL BMP2 ligand treatment for 1 hour in 3 wild-type (W1, W2, W3) and 3 *Bmpr2*+/- (N1, N3, N6) PEC lines. (B) Quantification of band densitometry in (A). Values expressed as the mean +/-SEM ratios of band densities, as indicated. One-way ANOVA with Dunnett's correction for comparisons with untreated *Bmpr2*+/- PECs, $*p<0.05$, $**p<0.01$, $***p<0.001$. Gels were run and probed as follows: Gel 1- pY14-Cav1, pY416-Src, β-actin; Gel 2- Cav1, Src.

LO-EPCs are rapidly proliferating cells with endothelial morphology that express endothelial cell markers CD31 and CD146 and take up Dio-Ac-LDL but unlike early outgrowth EPCs, do not express markers of the macrophage lineage (Fig 15A/B). They therefore provide a readily accessible, renewable source of endothelial cells from patients with this rare genetic disorder. Using these cells we show there is an increase in pY416-Src and pY14-Cav1 expression in the HPAH patient-derived LO-EPCs compared with two normal controls and the IPAH patient-derived cells (Fig 15C), suggesting defects in Src kinase and Cav-1 may be applicable to human disease.

Src inhibition restores Cav-1 localization to the plasma membrane in Bmpr2$^{+/-}$ PECs

To determine whether increased intracellular localization of Cav-1 results from constitutive activation of Src in *Bmpr2$^{+/-}$* PECs, we first determined whether inhibition of Src kinase activity reduces basal Y14-Cav-1 phosphorylation in *Bmpr2$^{+/-}$* PECs. Treatment with the selective Src family kinase inhibitor, PP2 (Hanke et al., 1996), decreases Cav-1 Y14 phosphorylation in *Bmpr2$^{+/-}$* PECs (Fig. 16A/B). These findings indicate that increased Src kinase activity causes the increase in basal Cav1 phosphorylation in *Bmpr2$^{+/-}$* PECs. Since Src-dependent phosphorylation of Y14-Cav-1 promotes caveolar endocytosis(Hu et al., 2009; M.-O. Parat, 2009; Shajahan, Timblin, et al., 2004), we sought to determine whether constitutive activation of Src kinase also increases intracellular localization Cav-1 in *Bmpr2$^{+/-}$* PECs. Treatment with PP2 restores plasma membrane localization of Cav-1 in *Bmpr2$^{+/-}$* PECs (Fig. 16C/D), suggesting that caveolar defects result from increased Src-dependent caveolar endocytosis in *Bmpr2$^{+/-}$* PECs.
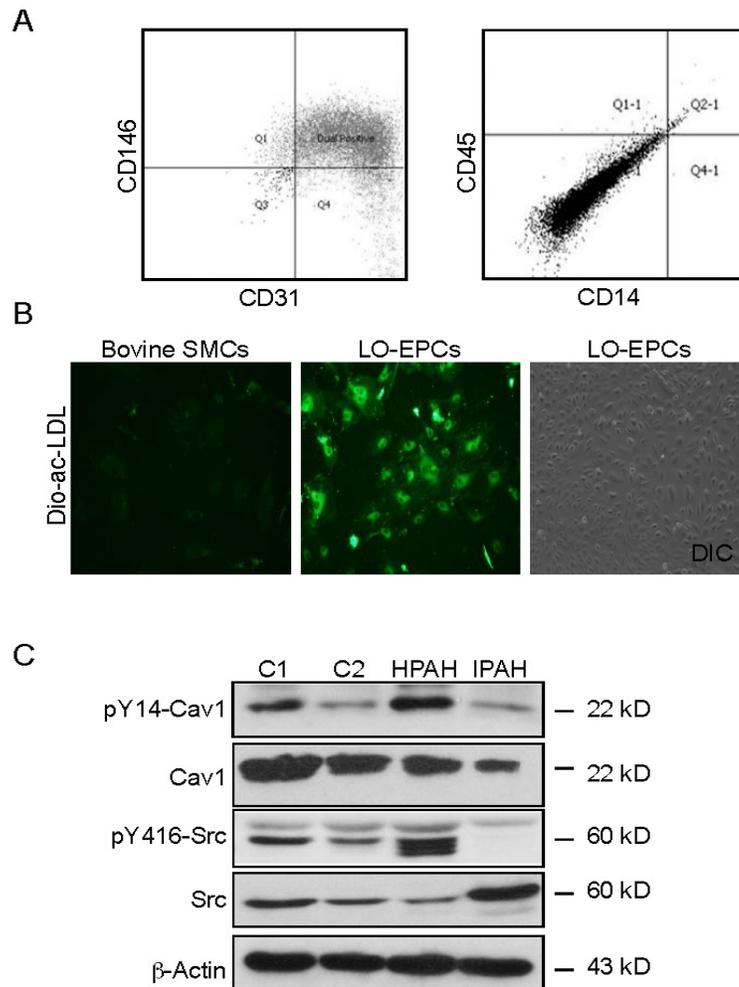
Figure 15. Increased Src activity in HPAH patient-derived late-outgrowth endothelial progenitor cells (LO-EPCs). (A) LO-EPCs express endothelial cell markers. Fluorescence-assisted cell sorting (FACS) of patient-derived LO-EPCs are positive for two endothelial cell markers CD146 and CD31 and negative for leukocyte and macrophage markers CD45 and CD14. (B) Dio-Ac-LDL uptake. LO-EPCs take up endothelial specific Dio-Ac-LDL and exhibit cobblestone-like morphology. Bovine aortic smooth muscle cells (SMCs) serve as negative controls. (C) Increased Src activity in HPAH patient-derived LO-PECs. Western blot depicting basal protein expression of pY14 and total Cav1 and pY416 and total Src in LO-PECs from two disease-free controls (C1, C2), one HPAH patient (*BMPR2 V299, FsX1*), and one IPAH patient negative for *BMPR2* mutations. β-actin as a loading control. Gels were run and probed as follows: Gel 1- pY14-Cav1, pY416-Src, β-actin; Gel 2- Cav1, Src.

To confirm these effects were due to inhibition of Src kinase and not off-target effects, we used an additional Src kinase inhibitor, SKI606 (Vultur et al., 2008), and a PP2 analogue with no activity, PP3. We show SKI606 reduces Src phosphorylation at Y416 in *Bmpr2*^{+/-} PECs, while PP3 has no effect (Fig. 16E). Furthermore, we show that SKI606 is able to rescue Cav1 localization to the plasma membrane in *Bmpr2*^{+/-} PECs, similar to PP2, while PP3 had no effects on Cav1 localization (Fig. 16C/D), demonstrating restoration of Cav1 to the plasma membrane is due to inhibition of Src kinase activity.

Impaired endothelial barrier function in Bmpr2^{+/-} *PECs*

Since enhanced caveolar endocytosis promotes increased endothelial cell permeability(Minshall et al., 2003; Y. Sun et al., 2011) we determined whether *Bmpr2*^{+/-} PEC monolayers have decreased barrier function. There is a significant reduction in trans-endothelial electrical resistance (TEER) across confluent monolayers of *Bmpr2*^{+/-} PECs compared with wild type controls (Fig. 17A), indicating endothelial barrier dysfunction. To determine whether impaired endothelial barrier function in *Bmpr2*^{+/-} PECs is associated with enhanced transcellular permeability, we used Transwell assays to assess permeability to two high molecular weight solutes that are transported through the endothelium by caveolar endocytosis, Albumin and 70kD Dextran(Minshall et al., 2002). *Bmpr2*^{+/-} PECs have increased permeability to both Albumin and 70kD Dextran compared to wild type PECs (Fig. 17B/C). Taken together, these data suggest that there is a defect in paracellular and transcellular barrier function in *Bmpr2*^{+/-} PECs. To determine whether increased endothelial permeability to high molecular weight solutes result from differences in active endocytic trafficking rather than a paracellular defect in permeability, we repeated this assay in cells cultured at 4°C, since

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endocytosis is a temperature-sensitive process that is completely blocked at 4°C(Brickman et al., 1995; Rode et al., 1997; Silverstein et al., 1977). As anticipated, when cultured at 4°C wild type and *Bmpr2*$^{+/-}$ PECs exhibit reduced permeability to 70kD Dextran, but there is no discernable difference between genotypes (Fig. 17D). Similar effects were seen with FITC-Albumin (data not shown A.P.). These data indicate that enhanced permeability to high molecular weight solutes results from enhanced endocytic transcellular and not paracellular transport in *Bmpr2*$^{+/-}$ PECs.

Src inhibition partially restores endothelial barrier function in Bmpr2$^{+/-}$ *PECs*

To determine whether defective endothelial barrier function is due to increased Src activity, we assessed whether Src inhibition would ameliorate endothelial barrier dysfunction in *Bmpr2*$^{+/-}$ PECs. TEER and permeability to 70kD dextran in *Bmpr2*$^{+/-}$ PECs is restored to wild type levels following Src inhibition with PP2 (Fig. 18A/B). There is also a reduction in permeability Albumin after treatment with PP2, but these levels do not return to those of wild-type PECs (Fig. 18C). These data indicate *Bmpr2*$^{+/-}$ PECs have impaired barrier function due in part to constitutive activation of Src kinase. Since Src inhibition restores the caveolar trafficking in *Bmpr2*$^{+/-}$ PECs, our findings also suggest that abnormal Src-mediated caveolar trafficking plays a role in promoting decreased barrier function in *Bmpr2*$^{+/-}$ PECs.

A

Wild Type   *Bmpr2*⁺ᐟ⁻

30μM PP2   -   -   -   +   +   +   -   -   -   +   +   +

pY14-Cav-1   ▬ 22 kD

Cav-1   ▬ 22 kD

pY416-Src   ▬ 60 kD

Src   ▬ 60 kD

β-Actin   ▬ 43 kD

B

☐ WT
■ WT + PP2
▨ *Bmpr2*⁺ᐟ⁻
▨ *Bmpr2*⁺ᐟ⁻ + PP2

P-cav1/cav1

C

Wild-type   *Bmpr2*⁺ᐟ⁻

DMSO

PP2

PP3

SKI606

Cavin/Cav-1/DNA

D

☐ WT
■ *Bmpr2*⁺ᐟ⁻
▨ *Bmpr2*⁺ᐟ⁻ + PP2

Cav-1 PM/non-PM

☐ WT
■ *Bmpr2*⁺ᐟ⁻
▨ *Bmpr2*⁺ᐟ⁻ + PP3

Cav-1 PM/non-PM

☐ WT
■ *Bmpr2*⁺ᐟ⁻
▨ *Bmpr2*⁺ᐟ⁻ + SKI606

Cav-1 PM/non-PM

E

DMSO   SKI606   PP2   PP3   DMSO   SKI606   PP2   PP3

pY416-Src   ▬ 60 kD

Src   ▬ 60 kD

β-Actin   ▬ 43 kD

Wild-type   *Bmpr2*⁺ᐟ⁻

51
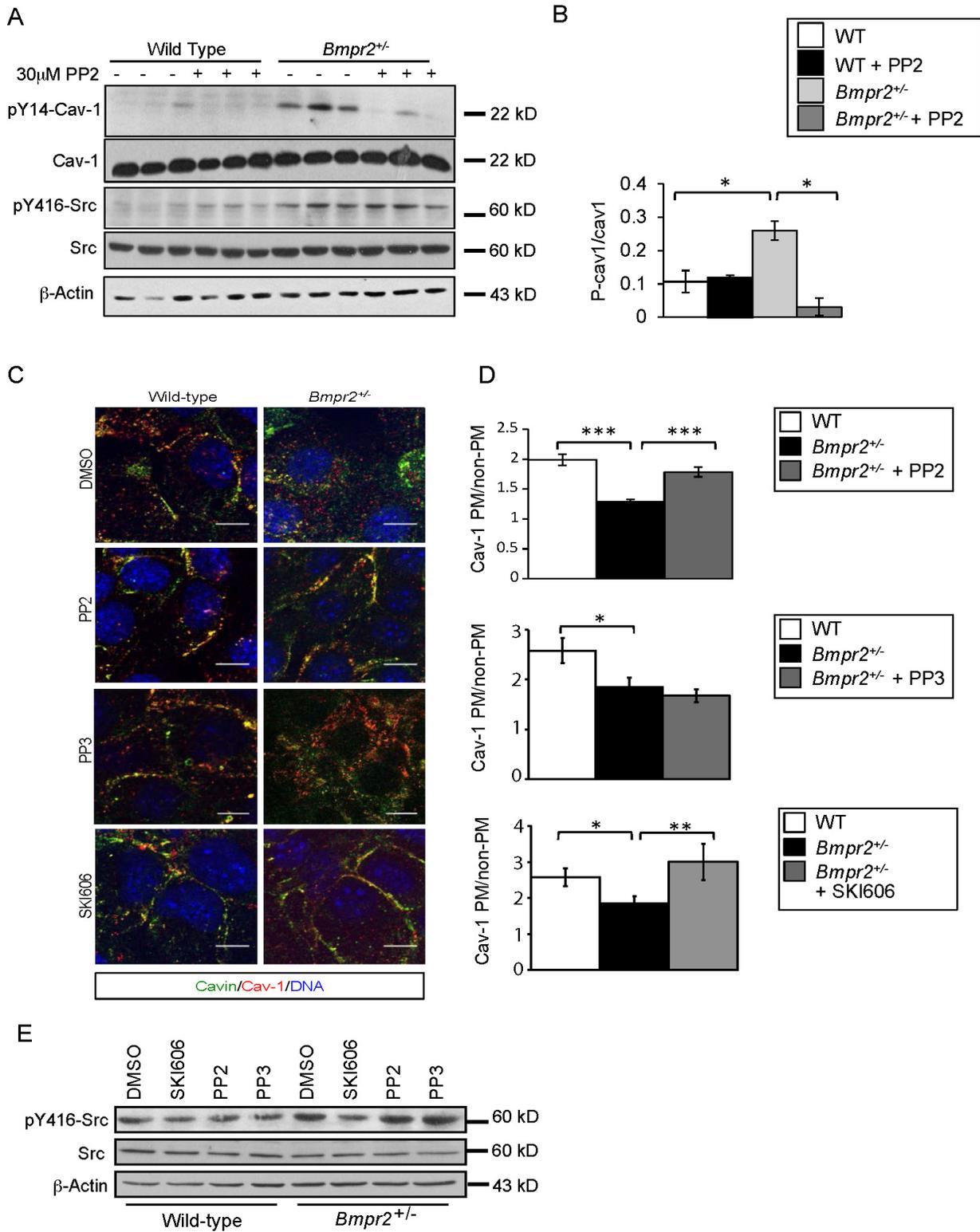
Figure 16: Src inhibition with PP2 reduces pY14-Cav-1 and restores Cav-1 localization to the plasma membrane in *Bmpr2*^{+/-} PECs. (A) PP2 inhibits Tyr[14]-CAV-1 phosphorylation. Cells treated with 30μM PP2 for 30 minutes prior to cell lysis. Performed in triplicate in one wild-type line (W1) and one *Bmpr2*^{+/-} line (N1). SRC inhibition demonstrated by reduced phosphorylation of SRC target Tyr(P)[14]-CAV-1. (B) Quantification of band densitometry in (A). Values expressed as the Mean ratios of band densities, as indicated. Error bars represent S.E. T-test, *p <0.01 vs. wild type controls. (C) CAV-1 and Cavin-1 localization after PP2, PP3, or SKI606 treatment. Representative immunofluorescence images showing CAV-1 and Cavin-1 localization after treatment. Scale bars: 10μm. (D) Quantitative analysis of CAV-1 localization after treatment. Values are expressed as the ratio of PM to non-PM fluorescence intensities in multiple cells from one wild type (W1, n=45) and one *Bmpr2*^{+/-} line (N1, n=75) before and after PP2 treatment, and (W1, n=33; N1, n=35; N1 PP3, n=10; N1 SKI, n=14) mean. Error bars represent S.E. One-way ANOVA with Dunnett's and Holm-Sidak correction for comparisons with untreated *Bmpr2*^{+/-} PECs, respectively, *p<0.05, **p<0.01, ***p<0.0001. (E) SKI606 inhibits SRC phospohorylation at Tyr[416]. Cells treated with 30μM PP2, 30μM PP3, or 1μM SKI606 for 30 minutes prior to cell lysis. Gels were run and probed as follows: (A) Gel 1- Tyr(P)[14]-CAV-1, Tyr(P)[416]-SRC, β-actin; Gel 2- CAV-1, SRC. (E) Gel 1- Tyr(P)[416]-SRC; Gel 2- SRC, β-actin.
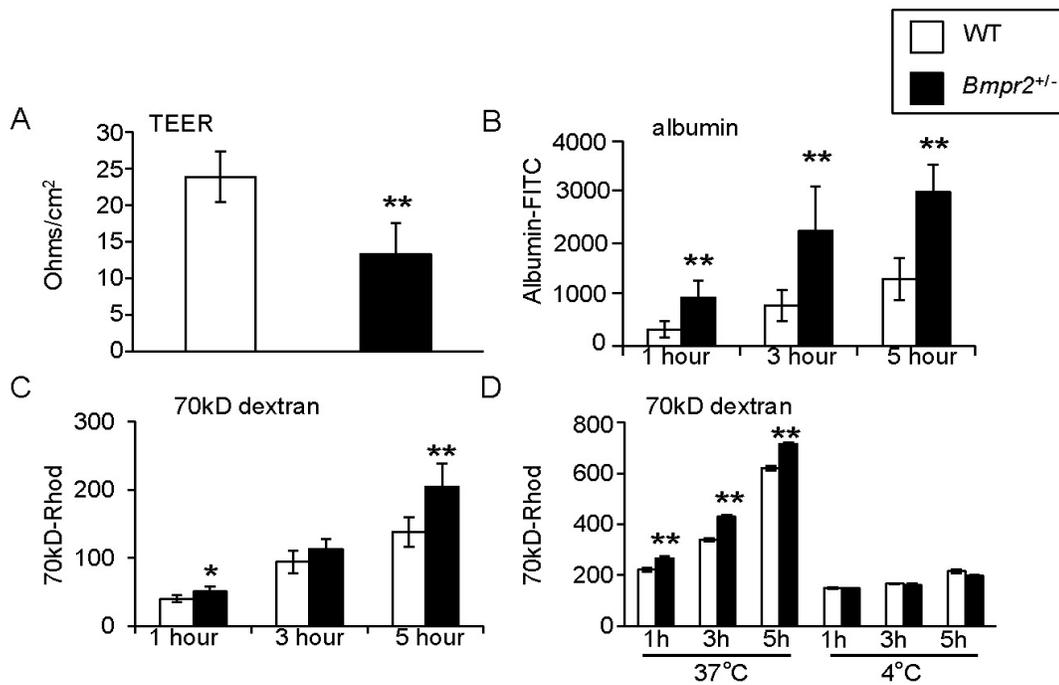
Figure 17: Impaired endothelial barrier function in *Bmrp2*⁺/⁻ PECs. (A) Trans-endothelial electrical resistance (TEER) in 3 wild type and 3 *Bmpr2*⁺/⁻ PEC lines. (B/C) Transwell permeability assays for (B) FITC-albumin, and (C) Rhodamine 70kDa dextran. Studies performed in triplicate in 3 wild-type lines and 3 *Bmpr2*⁺/⁻ lines. (D) Comparison of permeability to Rhodamine 70kDa dextran at 37°C and 4°C. Transwell permeability assays in wild type (W1) and *Bmpr2*⁺/⁻ (N6) PECs, 6 replicates/genotype/condition. All results expressed as mean+/-SEM. T-test, *p<0.01, **p<0.001 vs. wild type controls.
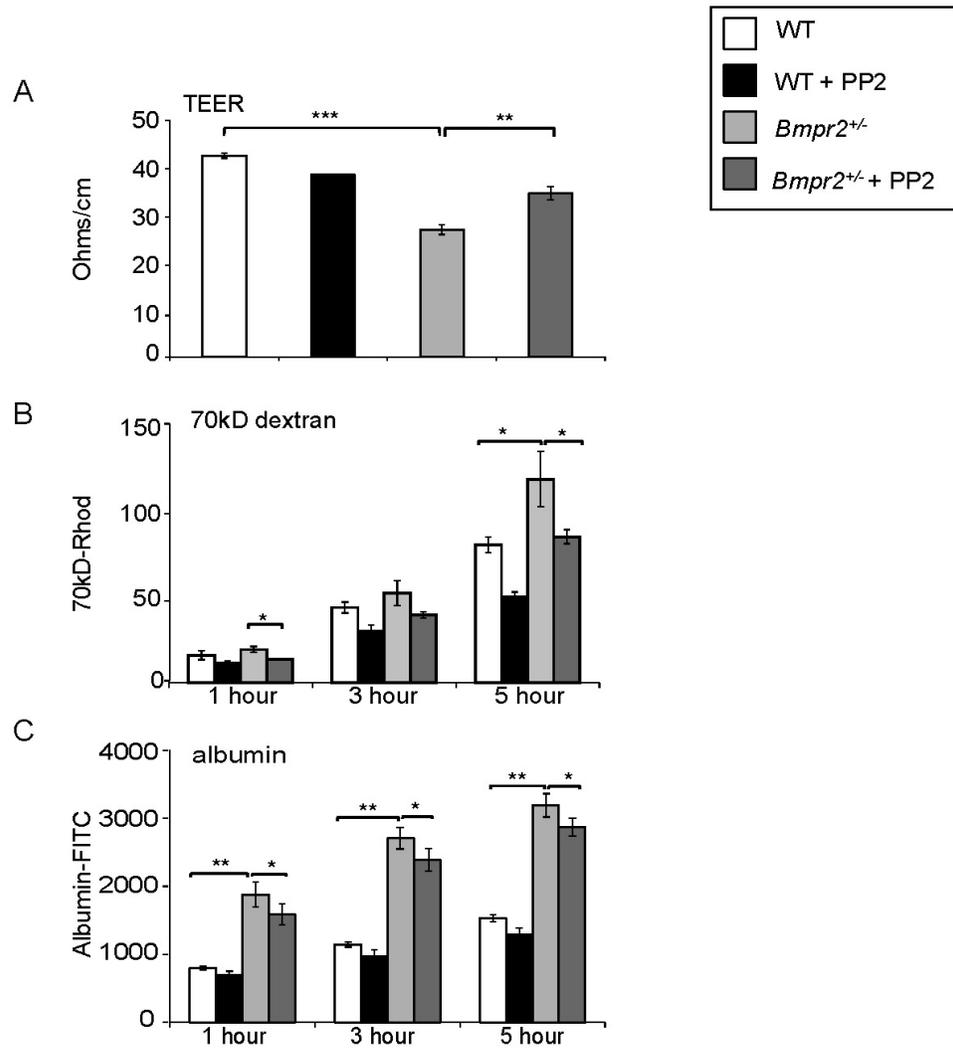
Figure 18: Src inhibition with PP2 improves endothelial barrier function in *Bmrp2*+/- PECs.
(A) TEER. TEER was evaluated 30 minutes after treatment with 30μM PP2. (B/C) Transwell permeability for (B) Rhodamine 70kD-dextran, and (C) FITC-Albumin. Cells treated with 30μM PP2 for 30 minutes before adding fluorophore-conjugated solutes. Studies performed in wild type (W1) and *Bmpr2*+/- PECs (N6) in triplicate. Results expressed as mean+/-SEM. T-test, *p<0.05, **p<0.01 vs. untreated *Bmpr2*+/- PECs.

Discussion

Several studies implicate altered caveolae and Cav-1 in endothelial dysfunction and in the pathogenesis of PH(Asosingh et al., 2012; E. Austin et al., 2012; Maniatis et al., 2008; Mathew et al., 2004; Patel et al., 2007), but to date the link between caveolar dysfunction and *BMPR2* mutations in HPAH has not been established. In these studies we have used PECs from *Bmpr2*^{+/-} mice to show that a heterozygous null *Bmpr2* mutation gives rise to increased numbers of internalized caveolae and core caveolar structural proteins, Cav-1 and Cavin-1 in the pulmonary endothelium. We have also shown that aberrant intracellular localization of Cav-1 in *Bmpr2*^{+/-} PECs is restored to the plasma membrane after treatment with either a Dynamin-2 or Src kinase inhibitor. This suggests that increased numbers caveolae in *Bmpr2*^{+/-} PECs results from increased dynamin-dependent caveolar endocytosis, and that this defect is the result of constitutive activation of Src kinase that we have also observed in *Bmpr2*^{+/-} PECs. We also show increased Src activity in late-outgrowth endothelial progenitor cells (LO-EPCs) isolated from HPAH patients. These findings are consistent with previous studies showing increased numbers of caveolae and increased Src activity in the lungs of patients with idiopathic PAH(Courboulin et al., 2011; Paulin et al., 2011), and suggest a mechanism by which *BMPR2* mutations give rise to endothelial dysfunction in HPAH.

There is evidence that endothelial barrier dysfunction and perivascular inflammation contribute to pathogenesis of PAH(Dorfmuller et al., 2003; Savai et al., 2012; Stacher et al., 2012). Our findings that PECs from *Bmpr2*^{+/-} mice have decreased barrier function are also consistent with data showing that mice with conditional *Bmpr2* deletion in the endothelium develop spontaneous pulmonary hypertension and have increased vascular leak and peri-vascular inflammation(Burton, Ciuclan, et al., 2011; Burton,

Holmes, et al., 2011; Hong et al., 2008). However the mechanism by which *Bmpr2* deficiency decreases endothelial barrier function was unknown. Caveolae regulate endothelial cell permeability by promoting endocytic transcellular transport of macromolecules(Minshall et al., 2003; Sowa, 2012; Y. Sun et al., 2011), and to a lesser extent by recycling components of the endothelial tight junctions to increase paracellular transport(Shen et al., 2005; Stamatovic et al., 2009). Our data show that both paracellular and transcellular barrier function is impaired in *Bmpr2*$^{+/-}$ PECs. While there is cross talk between transcellular and paracellular permeability pathways in endothelial cells(Driessche et al., 2007), we also show that increased permeability to the high molecular weight solutes is diminished by culturing *Bmpr2*$^{+/-}$ PECs at 4°C, indicating that *Bmpr2*$^{+/-}$ PECs have a defect in endocytic transcellular permeability. However, Src kinase also increases paracellular permeability in endothelial cells by promoting cytoskeletal contraction and by inducing dissociation of junctional complexes(Kim et al., 2009), so it is possible that some of the effects of the Src family kinase inhibitor, PP2, on paracellular permeability in *Bmpr2*$^{+/-}$ PECs are mediated through non-caveolar mechanisms.

We also show that there is increased intracellular accumulation of another core caveolar structural protein, Cavin-1, and that there are increased numbers of intracellular caveolar structures in the pulmonary endothelium of *Bmpr2*$^{+/-}$ mice. These findings suggest that mis-localization of Cav-1 results from abnormalities in caveolae rather than mis-trafficking of individual protein monomers in *Bmpr2*$^{+/-}$ PECs. In addition, we show that blocking phosphorylation of Y14-Cav-1 with PP2 restores Cav-1 localization to the plasma membrane in *Bmpr2*$^{+/-}$ PECs, suggesting that intracellular accumulation of endogenously expressed Cav-1 in *Bmpr2*$^{+/-}$ PEC is dependent on Src-

mediated pY14-Cav-1 phosphorylation. This effect is distinct from the intracellular accumulation of Cav-1 that occurs when Cav-1 levels are increased, as this has been shown to occur without Y14-Cav-1 phosphorylation(Hanson et al., 2013).

The mechanism by which heterozygous loss of Bmpr2 expression in $Bmpr2^{+/-}$ PECs promotes constitutive activation of Src kinase remains to be established. However, there is evidence that Src kinase is activated in the lungs of patients with idiopathic PAH(Courboulin et al., 2011; Paulin et al., 2011). Furthermore, published data suggests the C-terminus of BMPR2 binds to and inhibits Src kinase activity, and that BMP stimulation reduces Src activity in pulmonary artery smooth muscle cells(Wong et al., 2005). We were unable to reproduce these findings in wild type PECs. This discrepancy is likely to reflect transient and weak interactions between Src and Bmpr2, and/or differences in molecular behavior of these proteins in two distinct cell types. However we did observe a marked reduction of pY416 Src and pY14 Cav-1 in $Bmpr2^{+/-}$ PECs after treatment with BMP2, suggesting that restoration of BMP signaling in these cells is sufficient to reverse these effects. These findings have therapeutic implications since they suggest that activation of the BMP signaling pathway with BMP agonists, either secreted ligands or small molecule activators such as THR-123 or FK506 (Spiekerkoetter et al., 2013; Sugimoto et al., 2012), might be used to reverse Src-dependent endothelial dysfunction in HPAH patients with *BMPR2* mutations.

Therapeutic strategies targeted towards restoring endothelial barrier integrity may be beneficial for the treatment of patients with PAH. Here we have shown Src kinase inhibition with the pharmacological inhibitor PP2 improves endothelial barrier function in $Bmpr2^{+/-}$ PECs, suggesting that the constitutive activation of Src kinase in pulmonary endothelium of HPAH patients carrying heterozygous *BMPR2* mutations may provide an

attractive therapeutic target for this disease. Src activation has also been identified in patients with idiopathic PAH(Courboulin et al., 2011; Paulin et al., 2011) suggesting that therapeutic inhibition of Src activity may be applicable to a wider range of patients with this disease. Furthermore, since Src-dependent caveolar dysfunction may also promote endothelial dysfunction through de-regulation of eNOS activity(M. O. Parat, 2009; Sbaa et al., 2005), this strategy may have more extensive beneficial effects on pulmonary vascular disease pathophysiology in patients with HPAH.

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CHAPTER III

BMPR2 REGULATION OF SRC KINASE ACTIVATION

Introduction

Several studies have implicated Src kinase activation in PH; however until recently, the relationship between BMPR2 and Src kinase in PH had not yet been established(Brock et al., 2009; Courboulin et al., 2011; Paulin et al., 2014; Paulin et al., 2011; Pullamsetti et al., 2012). Studies from our lab now show $Bmpr2^{+/-}$ mutant mice have increased Src kinase activity; however, the mechanism for this increased activity remains unknown (Prewitt et al., 2015). Published data suggests the C-terminus of BMPR2 binds to and inhibits Src kinase activity, and that BMP stimulation reduces Src activity in pulmonary artery smooth muscle cells(Wong et al., 2005). While we were unable to reproduce these findings in wild type pulmonary endothelial cells (PECs) (data not shown), we did observe a marked reduction of pY416 Src and pY14 Cav-1 in $Bmpr2^{+/-}$ PECs after treatment with BMP2, suggesting that restoration of BMP signaling in these cells is sufficient to reverse these effects(Prewitt et al., 2015). Interestingly, mice overexpressing a BMPR2 C-term tail domain mutation develop PH, while PH patients express a higher ratio of Exon 12 BMPR2 splice variants which lack the tail domain(J. Cogan et al., 2012; Johnson et al., 2012; West et al., 2008), suggesting the BMPR2 tail domain may play a critical role in preventing PH. Here, we have set out to determine how BMPR2 regulates Src kinase activity. Using BMPR2 truncation mutation constructs and $Bmpr2^{f/+}$ PECs, we will assess BMPR2 dose responses and define the domains responsible for Src regulation.

Materials and Methods

Chemicals and Reagents

Interferon gamma (INFγ) (Peprotech). Monoclonal antibodies include Bmpr2 [clone 18] (BD antibodies) and β–actin [clone AC-74] (Sigma). Polyclonal antibodies include pY416-Src, pY530-Src and Src (Cell Signaling). FACS antibodies include anti-VCAM-AlexFluor 647 [clone 429] and EPCR-APC [clone eBio1560] (eBioscience).

PHBI patient endothelial cells

Distal, resistance levels PECs (EC3s) from control and IPAH patients were obtained from and characterized by the PHBI Cell Core. Cells were grown in complete EGM-2MV media (Lonza) and culture at 37°C. Src kinase activation was assessed between passages 3-5 as detailed below.

Mouse pulmonary endothelial cell (PEC) isolation, characterization and culture

PECs from $Bmpr2^{\Delta ex2/+}$ mice had been previously generated and characterized in the lab(Frank et al., 2008). Four independent lines of PECs were generated from 2 wild type (++2, ++3) and 2 $Bmpr2^{fl/+}$ ($Bmpr2^-$flox) (F/+ 10, F/+ 12) mice (H. Beppu et al., 2005). For this, $Bmpr2$-flox mice were crossed with C57Bl/6 H-2Kb-tsA58 SV40 large T Ag transgenic mice (Charles Rivers "immortomice") to generate wild type and $Bmpr2$-flox immortomice. Genotype was confirmed by PCR using primers and conditions outlined in previous studies(H. Beppu et al., 2005). To isolate PECs, mice were anesthetized with isoflurane prior to sacrifice by cervical dislocation. Lungs were perfused with a mixture of PBS/2mM EDTA followed by 0.25% Trypsin/2mM EDTA via right ventricle. Heart and

lungs were removed en bloc and incubated at 37°C for 20 minutes. Finally, lungs were perfused again in complete endothelial microvascular media EGM-2MV (Lonza) and the perfusate recovered for isolated cells. Cells were grown under permissive conditions in EGM-2MV + 10units/mL INFγ at 33°C before being transferred to 37° C without INFγ for 3-5 days to inhibit SV40 large-T antigen activity for phenotyping and before conducting experiments. Endothelial cell phenotype was confirmed for all isolates with VCAM and EPCR positive expression by FACS using mouse anti-VCAM-AlexaFluor 647 [clone 429] and EPCR-APC [clone eBio1560] (eBioscience). *Bmpr2* was excised from *Bmpr2-flox* PECs using adenoviral cre transfection. Briefly, for each 50% confluent 100mm dish, 2ml Basal EBM-2 (Lonza) + 20uL Ad-Cre were prepared. Media was removed from each dish and cells were washed in HBSS. The virus/media mixture was added to each dish and incubated over night 16-18 hours at 33°C. Following incubation, media was removed and complete EGM-2MV was added.

Western blots

Cells were lysed in 1x lysis buffer containing 150mM NaCl, 25mM HEPEs, 5mM EDTA, 1% TritonX-100, 10% glycerol and phosphatase and protease inhibitors (1:100 Sigma) on ice for 15 minutes before centrifugation at 5000xg for 30 minutes. Western blots were blocked in 5% milk before incubation in primary antibody in either 5% milk or bovine serum albumin (BSA) for phosphorylation site-specific antibodies overnight at 4°C. Primary antibodies were detected using species-specific HRP-conjugated secondary antibodies and Perkin-Elmer Western Lightning Plus ECL reagent. Films were exposed over a range of times, digitally scanned and densitometry performed using ImageJ on

films exposed for intermediate time periods in order to avoid non-linear ECL signals associated with very short or long exposure times.

Results

Previous studies in our lab have shown increased Src kinase activation in $Bmpr2^{+/-}$ mouse PECs and late-outgrowth endothelial progenitor cells (LO-EPCs) from IPAH patients (Prewitt et al., 2015). We now show that distal, small resistance pulmonary endothelial cells (EC3s) isolated from multiple IPAH patients also have increased Src activation (Fig 19), suggesting deregulated Src kinase may be a common disease mechanism in PAH.

The mechanism of increased Src activation in $Bmpr2^{+/-}$ PECs is unclear; however, we see no changes in Src activation in an additional $Bmpr2$ mutant, $Bmpr2^{\Delta ex2/+}$ (Fig 20). This mutant lacks 56 amino acids in the extracellular domain but expresses similar levels of the cytoplasmic domain (kinase and tail domain) as wild-type BMPR2(Frump et al., 2013), suggesting it is dosing of these domains that regulate Src kinase activity.

As discussed in the main introduction, Src kinase is regulated by catalytic and non-catalytic means. The major inhibitory catalytic regulation is through phosphorylation of Y530 which induces conformational change and blocks the activating Y416 site. Given the dramatic increase in Y416 phosphorylation in $Bmpr2^{+/-}$ PECs, we assessed pY530-Src levels in control and $Bmpr2^{+/-}$ PECs. We show no difference in Y530-Src phosphorylation in $Bmpr2^{+/-}$ PECs (Fig 21), suggesting BMPR2 is not regulating Src activity by acting upon Src's C-terminal Y530 regulatory domain. Given that BMPR2 does not appear to affect Y530-Src phosphorylation status and $Bmpr2^{\Delta ex2/+}$ mutants, which have intact kinase and tail domains, do not have increased Src kinase

activation, we reasoned that the levels of these cytoplasmic domains may regulate Src kinase. To test this we have developed a new *Bmpr2* mutant cell line in which BMPR2 has been deleted to yield *Bmpr2$^{f/+}$* cells (*Bmpr2-flox)*. These cells express endothelial cell markers and exhibit cobblestone-like morphology (Fig 22 and data not shown). Following adenoviral cre transfection, we show loss of BMPR2 expression and increased expression of phosphorylated Y416-Src in *Bmpr2-flox* PECS, similar to that of *Bmpr2$^{+/-}$* PECs (Fig 23). These data suggest that BMPR2 dosing is critical for Src kinase regulation. This is an ongoing project for which we will discuss future directions and implications below.
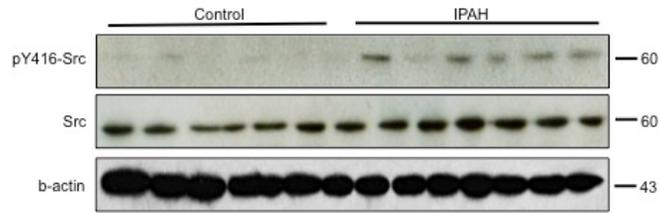
Figure 19: Src kinase activation in distal, resistance endothelial cells from IPAH patients.
Western blots demonstrating basal expression of phosphorylated Y416-Src and total Src kinase from control patients and IPAH patients. β-actin as a loading control.
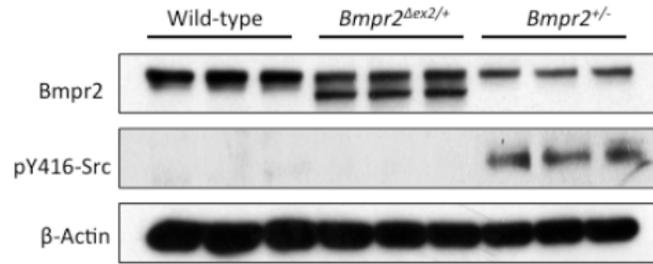
Figure 20: Src kinase is activated in *Bmpr2$^{+/-}$* PECs but not in *Bmpr2$^{\Delta ex2/+}$* PECs. Western blots demonstrating basal expression of phosphorylated Y416-Src from control, *Bmpr2$^{+/-}$*, and *Bmpr2$^{\Delta ex2/+}$* PECs. β-actin as a loading control.
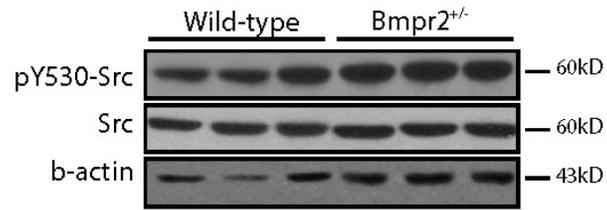
Figure 21: *Bmpr2*$^{+/-}$ **PECs exhibit no change in pY530-Src expression.** Western blots demonstrating basal expression of phosphorylated Y530-Src and total Src kinase from control and *Bmpr2*$^{+/-}$ PECs. β-actin as a loading control.
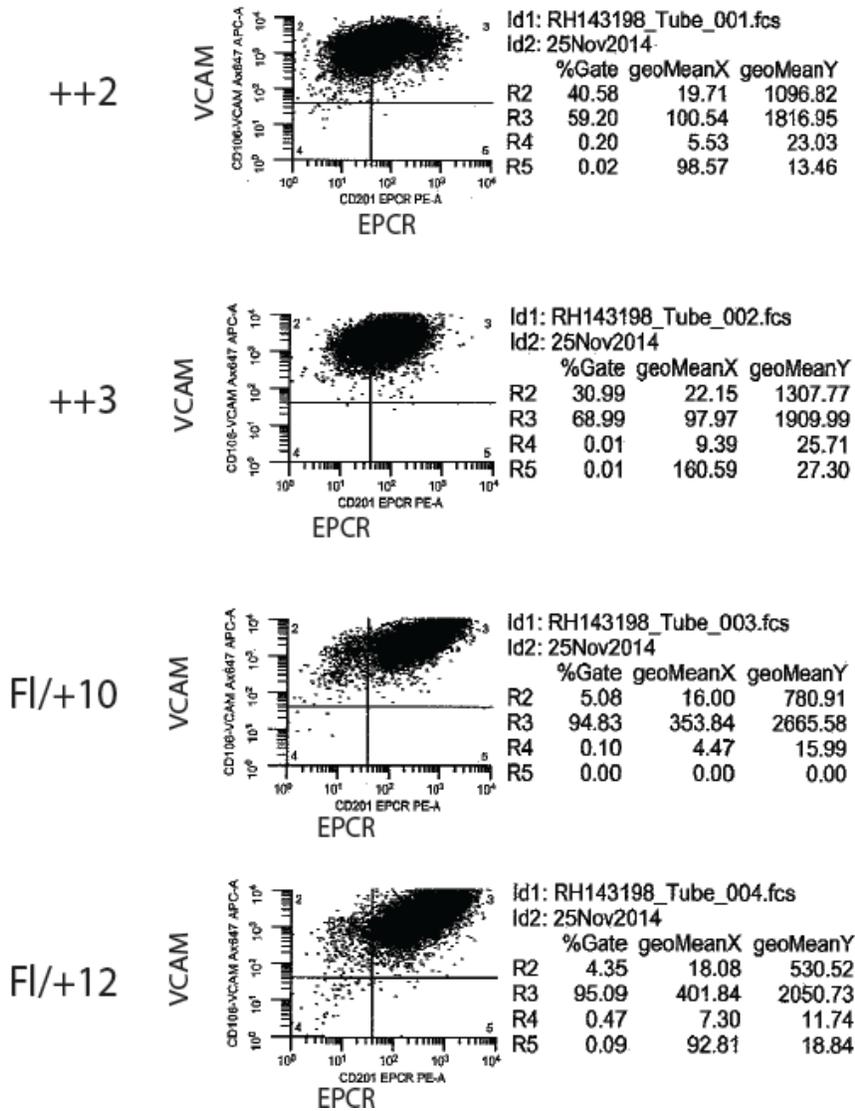
Figure 22: *Bmpr2*-flox cells express endothelial specific markers. FACS analysis of *Bmpr2* wild-type and flox cells expressing VCAM and EPCR. Two wild-type lines (++2 and ++3) and two *Bmpr2 flox* lines (F/+ 10 and F/+ 12).
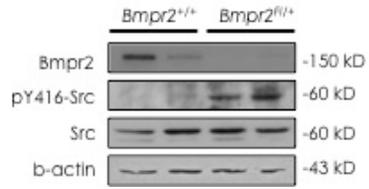
Figure 23: Increased Src activation in *Bmpr2-flox* PECs. Western blots demonstrating basal expression of phosphorylated Y416-Src and total Src kinase from control, $Bmpr2^{+/+}$, and $Bmpr2^{Fl/+}$ PECs. β-actin as a loading control.

Discussion

BMPR2 mutations and Src kinase activation in PH have previously been identified, but the relationship between BMPR2 and Src kinase was only recently established(Prewitt et al., 2015). In this current work, we aim to establish how *BMPR2* mutations regulate Src kinase activity. We have developed a new system using *Bmpr2-floxed* PECs and BMPR2-PiggyBac expression constructs to help us define the domains necessary for Src kinase regulation and determine the dosing of these domains required to inhibit Src kinase activity. Initial data from these studies show *Bmpr2-flox* PECs have increased Src kinase activation (pY416) that is not due to reduced Y530 inhibitory phosphorylation, suggesting that BMPR2 may be regulating Src through non-catalytic mechanisms (ie by inducing conformational change through direct binding). Given that *Bmpr2$^{\Delta ex2/+}$* PECs, which have intact kinase and tail domains, do not exhibit increased Src activation and previous studies that suggest BMPR2 tail domain interacts with Src(Wong et al., 2005), we hypothesize that BMPR2 tail domain physically interacts with Src kinase and that decreased expression of BMPR2 tail domain results in increased Src kinase activation. Insights from these studies are significant as unintended toxicities from global Src kinase inhibition may preclude their use in clinical settings. For example, Dasatinib, a broad Src and Abl kinase inhibitor that reverses PH in experimental models, also induces sever pre-capillary PAH in a subset of patients treated with this for CML (Montani et al., 2013; Pullamsetti et al., 2012). Therefore there is a need to understand the mechanisms regulating Src kinase to develop more selective therapies that only target the pathological Src activation in the pulmonary vasculature of PH patients.

To determine the role of BMPR2 in Src regulation, we have developed four BMPR2 expression constructs including full-length and three mutation constructs that

represent three common BMPR2 mutations found in PH patients (Fig 24). These constructs are based on a novel PiggyBac transposon based system in which Myc-tagged BMPR2 constructs have been inserted into a cumate controlled transposon flanked plasmid (Fig 25). Expression remains 'off' until cumate is added to the system and can be tightly titrated (Fig 26) (Mullick et al., 2006). Using the Virofect system, we will transfect the BMPR2-PiggyBac constructs along with the PiggyBac transposase vector to integrate the Myc-Tagged BMPR2 constructs into the genomic DNA (Fig 27). We will then select for puromycin resistant clones and flow sort for the extracellular Myc tag in cells in which BMPR2 has been properly trafficked to the cell surface.

The tight control of the PiggyBac system means we will be able to accurately assess BMPR2 dosing effects on Src Kinase activation. Our initial results suggest that the level of BMPR2 expression may be critical for Src kinase regulation as *Bmpr2*^{+/-} PECs have increased Src activation while *Bmpr2*[Δ/ex2] PECs, which have similar BMPR2 expression levels as wild-type PECs, do not show this increase. Using BMPR2 rescue constructs we will definitely test whether the level of BMPR2 expression effects Src kinase activation in *Bmpr2-flox* PECs. Our initial studies will utilize full-length BMPR2 to establish amount of BMPR2 expression required to reduce Src Y416 phosphorylation. Following these studies, we will test our additional BMPR2 constructs to determine which domain is required for this inhibition. Our constructs include a BMPR2 truncation mutation just downstream of the Myc-tag that eliminates the transmembrane domain, kinase domain, and tail domain (R147X); a kinase domain point mutation that renders the kinase domain inactive (K230R); and a tail domain deletion (R899X). These mutations will allow us to effectively define which domain is required for Src kinase inhibition.

Once we have defined BMPR2 dosing requirements and defined the domains

necessary, we will elucidate the mechanism by which BMPR2 exerts these effects. Previous studies suggest BMPR2 and Src physically interact and that this interaction inhibits Src kinase. We were unable to detect interactions between BMPR2 and Src in our cells and suspect this may be due to the transient nature of the interaction. To get a better understanding of potential interactions between BMPR2 and Src we will utilize complementary approaches to evaluate the interactions between full-length BMPR2 and any constructs that reduced Src activation and determine if these interactions are disrupted in BMPR2 constructs that did not reduce Src kinase activation. These approaches include co-immunoprecipitation using low stringency lysis buffers and reversible crosslinkers, immunolocalization, and cell fractionation studies. The co-immunoprecipitation studies will determine if BMPR2 is exerting control over Src kinase activity through direct inhibitory mechanisms, while subsequent studies will determine whether BMPR2 may be exerting indirect inhibitory control. For instance, BMPR2 is often localized to caveolae and therefore may be helping sequester Src into Src-Cav-1 inhibitory complexes within caveolae via its relationship with Cav-1 (S. Li et al., 1996a; Nohe et al., 2005; Ramos et al., 2006; Wertz et al., 2008). We have evidence that BMP2 stimulation reduces Src activation in $Bmpr2^{+/-}$ PECS, suggesting that BMP signaling may strengthen these interactions. Therefore we will perform the above experiments both with and without BMP2 stimulation.

In summary, we have shown $Bmpr2^{+/-}$ PECs have increased Src kinase activation while $Bmpr2^{\Delta ex/2}$ PECs do not exhibit this increase. We go on to show that increased Src activation in $Bmpr2^{+/-}$ PECs is not due to reduced phosphorylation of the Y530 regulatory domain, suggesting that BMPR2 is regulating Src activation through non-catalytic means and maybe inhibiting Src through direct binding or through sequestration into

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inactivating complexes (ie Src-Cav-1 inhibitory complexes). We previously showed that BMP2 stimulation reduces Src activation in *Bmpr2*$^{+/-}$ PECs, suggesting a role for BMP signaling in BMPR2-Src interactions. Interestingly, recent work has shown stimulation with BMP9 in *Bmpr2*$^{+/R899X}$ mutant mice, which also show increased Src activation, reverses established PH in these mice (Johnson et al., 2012; Long et al., 2015). These data suggest that BMP agonists may be an attractive therapeutic option for reducing Src-mediated development of PH. Our current studies will help to define the relationship between BMPR2 and Src kinase and may provide clues for more direct interventions that precisely target the BMPR2-Src axis in PH.
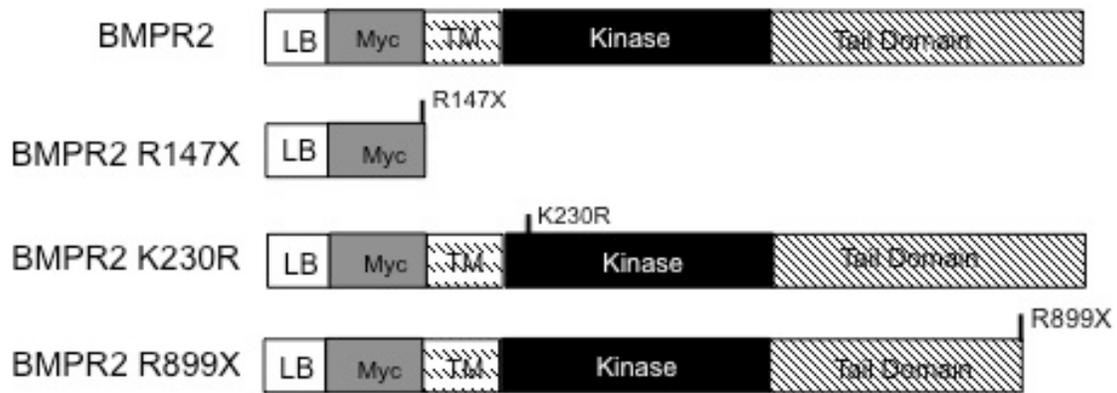
Figure 24. BMPR2 PiggyBac expression constructs. Schematic of BMPR2 expression constructs inserted into the PiggyBac transposon vector. Constructs include full-length BMPR2, transmembrane, kinase, and tail domain truncation (BMPR2 R147X), kinase domain point mutation (BMPR2 K230R), and tail domain truncation (BMPR2 R899X). LB= ligand binding domain, Myc= Myc tag, TM= transmembrane domain.
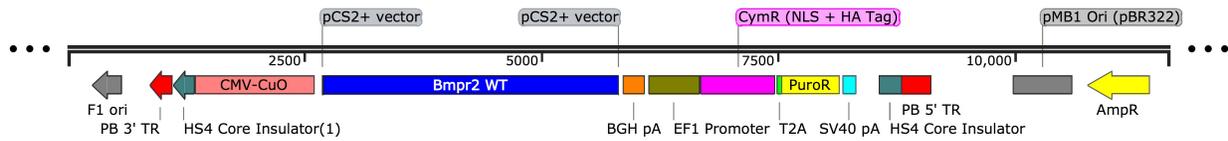
Figure 25: Linearized BMPR2-Piggybac constructs. *BMPR2* constructs were excised from PCS2+ vectors (Zefrang) using EcoRI and BamHI restriction enzymes (New England Biolabs). BMPR2 inserts were PCR amplified using primers with roughly 15 bp extensions homologous to the PiggyBac vector (Table 3). The PiggyBac vector was linearized with NotI (New England Biolabs). BMPR2 inserts were subsequently ligated into the linearized PiggyBac vector using the In-Fusion cloning kit (Clonetech Laboratories). The newly formed BMPR2-PiggyBac constructs were then transformed into competent *E. coli* and screened for ampicillin resistance. Plasmid constructs were purified using plasmid purification kits (Qiagen). The transposon-based PiggyBac system (PB 3' TR and PB 5' TR) contains a puromycin resistance cassette (PuroR) and CymR repressor. Wild-type BMPR2 is shown here for simplicity. Image generated using SnapGene.

BMPR2 construct	Primer
BMPR2	Forward: agcacagtggcggccgatccGCCACCatggctt Reverse: tagactcgagcggccctcacagacagttcattcctatatctttagacac
BMPR2 R147X	Forward: agcacagtggcggccgatccGCCACCatggctt Reverse: tagactcgagcggccctcacagacagttcattcctatatctttagacac
BMRP2 K230R	Forward: agcacagtggcggccgatccGCCACCatggctt Reverse: tagactcgagcggccctcacagacagttcattcctatatctttagacac
BMPR2 R899X	Forward: agcacagtggcggccgatccGCCACCatgGctt Reverse: tagactcgagcggccctcacagacagttcattcctatatctttagacac

Table 3: BMPR2 PCR amplification primers. Bmpr2 amplification primers were designed with ~15bp PiggyBac homologous overhangs.
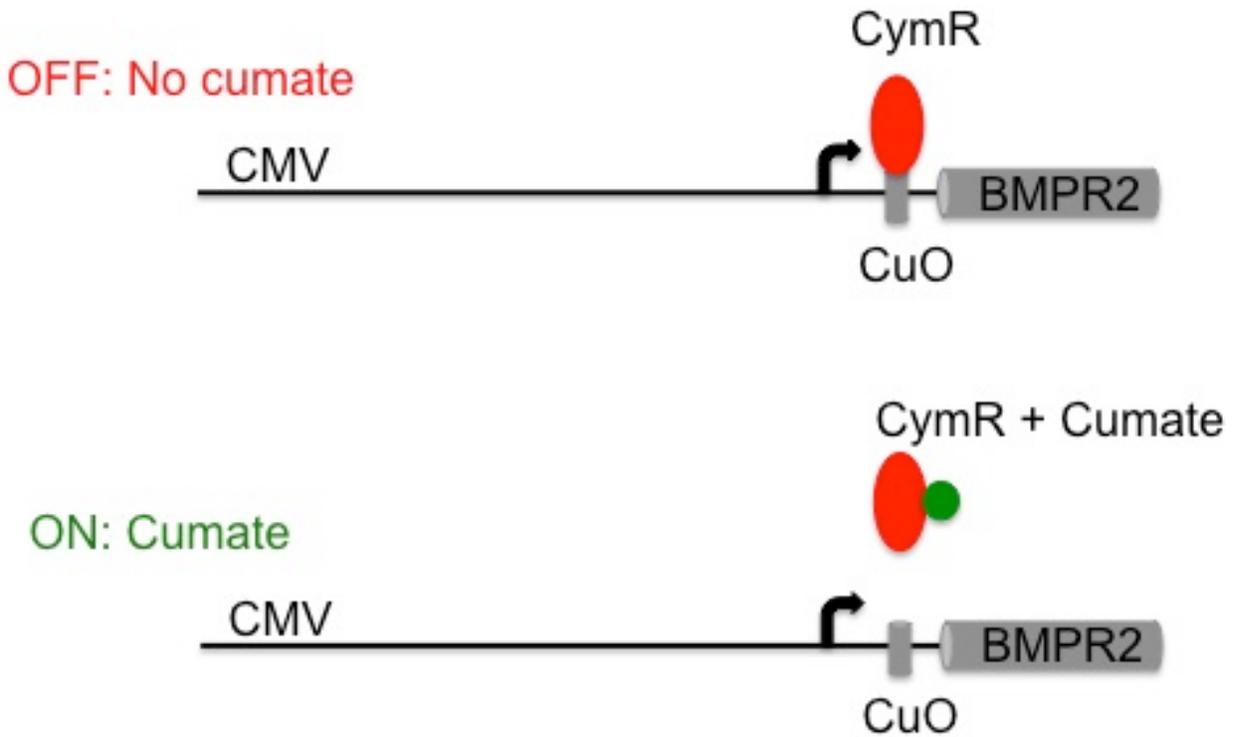
Figure 26: Schematic of CymRepressor configuration. The bacterial repressor, CymR, can bind to the operator sequence (CuO) placed downstream of CMV5, a strong viral promoter that is active in mammalian cells. Once bound, CymR blocks transcription from the CMV5 promoter. CymR bound to cumate is unable to bind to CuO. Transcription from CMV5 can proceed unhindered.
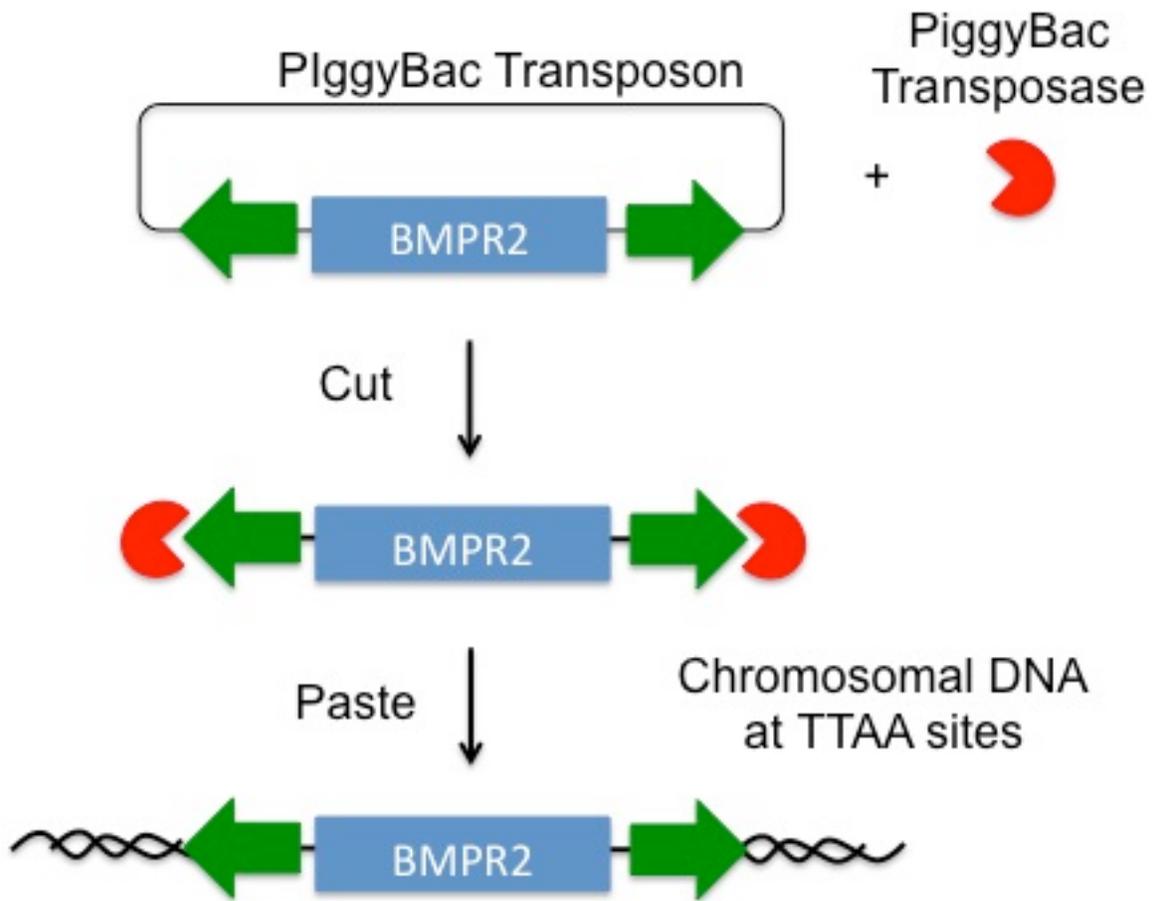
Figure 27: BMPR2-PiggyBac schematic. BMPR2-PiggBac transposon constructs are co-transfected with the PiggyBac Transposase to insert BMPR2 constructs into the genome.

CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

Src Kinase Activation and Endothelial Dysfunction

The studies outlined in this dissertation were designed to identify a mechanism by which *BMPR2* mutations may give rise to the development of Pulmonary Arterial Hypertension (PAH). Our studies show for the first time a link between *BMPR2* mutations and Src-mediated endothelial permeability. We show that increased Src activation is a common feature in both Heritable and Idiopathic PAH, suggesting that aberrant Src regulation may be an underlying cause for multiple forms of PH.

Endothelial permeability defects have been identified in mouse models of PH in which BMPR2 has been deleted in the endothelium of mice(Burton, Ciuclan, et al., 2011; Hong et al., 2008; Soon et al., 2015), while PH patients show impaired endothelial barrier function giving rise to increased inflammatory infiltration (Burton, Holmes, et al., 2011; Hong et al., 2008; Stacher et al., 2012) (Dorfmuller et al., 2003; Savai et al., 2012). It is increasingly appreciated that inflammation may be a driving factor for PH. Indeed, exposure of underlying the pulmonary tissues to various growth factors, blood proteins, and inflammatory cytokines may dictate the proproliferative and antiapoptotic phenotype seen in endothelial cells and smooth muscle cells (Guignabert et al., 2013). While these studies demonstrate endothelial permeability defects in BMPR2 mutants, the mechanism for increased permeability was not fully understood. Here we have shown that endothelial barrier dysfunction in *Bmpr2*$^{+/-}$ PECs is due to enhanced Src-

mediated caveolar endocytosis and that these defects can be reversed using Src kinase inhibitors(Prewitt et al., 2015).

Src has been shown to mediate permeability in a variety of situations in addition to its role in initiating caveolar endocytosis. For instance, Src has been implicated in neutrophil extravasion in endothelial cells via disruption of endothelial junctions, predominantly β-catenin, leading to a state of hyperpermeability (Tinsley et al., 2002). Additionally, Src mediates oxidant induced endothelial hyperpermeability via both caveolar endocytosis and endothelial junction disruption (Hu et al., 2008). Oxidant exposure is a common theme in EC dysfunction and may be linked to defects in mitochondrial function, which we will discuss in more detail later on. Src is also required VEGF induced permeability and participates in a positive feedback loop in which VEGF induces activation of Src kinase and Src kinase promotes VEGF production via HIF1α (Abu-Ghazaleh et al., 2001; Cheranov et al., 2008; Chou et al., 2002; Z. Sun et al., 2012; Weis et al., 2004). Interestingly we have noted increased VEGF receptor activation in $Bmpr2^{+/-}$ PECs, suggesting Src may be promoting endothelial permeability through this pathway as well (data not shown). All together, Src activation in the pulmonary endothelium gives rise to endothelial barrier dysfunction via a variety of mechanisms. Our data suggest Src activation in $Bmpr2^{+/-}$ PECs promotes endothelial permeability through increased caveolar endocytosis, and possibly also through paracellular pathways and VEGFR2 activation.

Though we have mentioned the role of caveolae in promoting endothelial permeability through the direct uptake of materials in caveolar bodies, less has been discussed on caveolae's role in promoting paracellular permeability through endocytosis. The scission and internalization of caveolae results in dramatic remodeling

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of the actin cytoskeleton which in turn results in tension on endothelial junctions and often results in gaps between endothelial cells(Shen et al., 2005). Thus, in addition to the direct modification of endothelial junctions by Src, Src-mediated caveolar endocytosis also promotes permeability through paracellular pathways.

In addition to regulating endothelial permeability through endocytosis, caveolae regulate the critical enzyme, eNOS, a potent vasodilator and permeability factor. Several studies have detailed the relationship of caveolae and eNOS, demonstrating that caveolae negatively regulate eNOS activity and therefore NO production (Sbaa et al., 2005). This is a two-part regulation in that caveolae sequester eNOS into inactive domains while Cav-1 physically binds to and inhibits eNOS activity (Y. Xu et al., 2008). Importantly, inhibition of NO production impairs endothelial barrier function and results in increased leukocyte infiltration, both hallmarks of PH (Burton, Ciuclan, et al., 2011; Giaid et al., 1995; Kubes et al., 1992; Stacher et al., 2012). Therefore, increased caveolae numbers and caveolar endocytosis in $Bmpr2^{+/-}$ mice may be further contributing to the endothelial permeability phenotype by actively inhibiting eNOS activity.

The significance of eNOS inhibition extends beyond the permeability phenotype since in addition to regulating endothelial permeability eNOS also regulates vascular tone by promoting smooth muscle cell relaxation (vasodilation)(Frank et al., 2008). Deregulation of eNOS has been implicated in various vascular diseases not limited to hypertension (H. Li et al., 2000; Shesely et al., 1996). NO protects against platelet aggregation and leukocyte adhesion, preventing exposure of underlying smooth muscle cells to cytokines that promote proliferation (H. Li et al., 2000). Thus in addition to promoting vasodilation, NO production protects against vascular remodeling in the

pulmonary bed, another common PH phenotype that contributes to increased pulmonary pressures.

Together these data demonstrate that increased Src kinase activation in *Bmpr2$^{+/-}$* PECs may play a significant role in the development of PH. Here we have shown that increased Src activation leads to endothelial barrier dysfunction and present evidence that increased Src activation may promote endothelial dysfunction in a variety of pathways. Information from these studies provides a potential link between *BMPR2* mutations and endothelial dysfunction in PAH and thereby lays the groundwork for future therapeutic intervention in the clinical setting.

Future studies for this project should include further characterization of the paracellular permeability defect. Our studies indicate that *Bmpr2$^{+/-}$* PECs have endothelial barrier dysfunction at the site of endothelial junctions and that inhibition of Src kinase with PP2 rescues this defect. These data suggest increased paracelluar permeability in *Bmpr2* mutants is Src mediated; however, the mechanism has not yet been elucidated. One promising avenue is through the direct phosphorylation of junctional proteins by Src kinase. This includes β-catenin and VE-Cadherin in adherens junctions and Occludin and ZO1 in tight junctions(Dorfel et al., 2012; Hu et al., 2008). However, as caveolar endocytosis remodels the actin cytoskeleton and thus creates tension and breaks of endothelial junctions, it is prudent to investigate actin remodeling and cytoskeletal defects in these mutants as well.

Additional studies could include investigating the consequences and mechanism of VEGFR2 activation in *Bmpr2$^{+/-}$* PECs. VEGF-the predominant ligand for VEGFR2- is critical for angiogenesis and permeability of the vasculature(Hoeben et al., 2004). Increased VEGFR2 activation is implicated in the development of plexiform lesions in PH

patients(Tuder et al., 2001), while VEGFR2 receptor blockade is an emerging animal model for PH(Stenmark et al., 2009). These data suggest tight regulation of VEGFR2 signaling is crucial for vascular homeostasis and prevention of PH related phenotypes. Preliminary data in our lab show VEGFR2 is activated in $Bmpr2^{+/-}$ PECs suggesting VEGF signaling is deregulated in $Bmpr2$ mutants. Additional studies could include investigating how activated VEGF signaling affects vascular remodeling and endothelial permeability in $Bmpr2^{+/-}$ mice. Additionally, as VEGF and Src are known to participate in a positive feedback look with each other, further work elucidating how Src activation may contribute to VEGFR2 activation and vice versa is worth exploring.

And finally, studies elucidating the effects of enhanced Src-mediated caveolar endocytosis on eNOS regulation are key studies for the field. Unfortunately, a consequence of conditionally immortalized endothelial cells is that they quickly lose their expression of eNOs after a few passages. As such, studies involving eNOS were not immediately feasible. Future experiments using either isolated primary cells or low passage conditionally immortalized cells will overcome this barrier and allow accurate assessment of eNOS localization and activity- aspects regulated by caveolae/Cav-1.

Src-Mediated Mitochondrial Dysfunction in PAH

There is increasing evidence of metabolic reprogramming in PAH(Archer, Marsboom, et al., 2010; Bonnet et al., 2006; Fessel et al., 2012; Fijalkowska et al., 2010; Marsboom et al., 2012; Tuder et al., 2012). Under normoxic conditions, cells typically favor oxidative phosphorylation over aerobic glycolysis, as this pathway yields up to 38 ATP per glucose molecule versus 2 under glycolysis(Tuder et al., 2012). However, several studies using PH mouse models as well as PAH patient samples indicate that pulmonary

vascular cells undergo a shift in metabolic pathways from oxidative phosphorylation to aerobic glycolysis, even with sufficient oxygen(Archer, Marsboom, et al., 2010; Fessel et al., 2012; Fijalkowska et al., 2010; Marsboom et al., 2012; W. Xu et al., 2007). This phenomenon is considered a hallmark of cancer progression, and indeed, PAH vascular cells exhibit a malignancy-like state in which proliferation abounds and apoptosis is minimal(Tuder et al., 2012). Recent evidence suggests that this shift in metabolism is due in part to upregulation of Hypoxia Inducible Factor 1 (Hif1α)(Fijalkowska et al., 2010; Tuder et al., 2012). Importantly, Src kinase upregulates Hif1α expression in malignant conditions, and may be playing a similar role in PAH(Jiang et al., 1997; Karni et al., 2002). Additionally, Src activation in cultured cells increases aerobic glycolysis and inhibition of Src by PP2 decreases Hif1α expression and reverses aerobic glycolysis induction in cells(Carroll et al., 1978; Karni et al., 2002). Taken together, these data suggest regulation of Src kinase through Src kinase inhibitors may be an attractive therapeutic option for metabolic reprogramming in PAH.

The role of *BMPR2* mutations in metabolic reprogramming is an emerging area of study. Recent work suggests *BMPR2* mutants exhibit a range of metabolic defects(Fessel et al., 2012). Given the role of Src in promoting metabolic shift, we hypothesize that Src kinase activation in *Bmpr2*$^{+/-}$ mice as well as IPAH patients without *BMPR2* mutations leads to metabolic reprogramming. To test this hypothesis we first would need to establish that metabolic reprogramming occurs in *Bmpr2* mutants. These studies would include assessing cellular bioenergetics in *Bmpr2*$^{+/-}$ PECs and IPAH patient samples using the Seahorse Bioanalyzer in the Vanderbilt High Throughput Sequence Core. This machine analyzes, among other things, oxygen consumption and extracellular acidification rates- both markers of mitochondrial dynamics. Further studies would

elucidate the role of Src kinase activation on metabolic reprogramming, if any, in *Bmpr2* mutants. These studies would be instrumental in advancing our collective understanding of mitochondrial dynamics, PH, and *BMPR2* mutations.

Mechanisms of Src Activation

The mechanism of increased Src kinase activation in *Bmpr2*$^{+/-}$ PECs has not yet been elucidated. Src is regulated in two distinct, yet complementary ways: catalytically and non-catalytically. Catalytic regulation refers to activation or repression by phosphorylation of key residues (see Table 2), while non-catalytic regulation refers to activation or repression by conformational change caused by direct protein-protein binding. Non-catalytic inhibitors often exploit catalytic inhibitors to ensure long-term inhibition of Src kinase activity. This long-term repression is achieved through the phosphorylation of the c-terminal Y530 residue. Under basal conditions, 90-95% of src is phosphorylated at Y530(Doerks et al., 2002), suggesting that phosphorylation of this residue is the predominant mechanism for negative Src regulation. Indeed, loss of Y530 is the defining feature of transforming v-Src (Parsons et al., 1989). This led us to investigate whether Y530 phosphorylation was reduced in *Bmpr2*$^{+/-}$ PECs. Our results demonstrate there is no change in Y530 phosphorylation in *Bmpr2*$^{+/-}$ PECs compared to wild-type, indicating BMPR2 does not promote Src activation through deregulation of Src's c-terminal domain. Therefore, BMPR2 is likely regulating Src through non-catalytic mechanism. There is limited evidence that BMPR2 physically interacts with Src and that this interaction represses Src activity(Wong et al., 2005). Thus, future work will aim to identify and detail these interactions in wild-type PECs and in the context of *Bmpr2* mutations. We have detailed these experiments in Chapter III.

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In our initial studies we were unable to detect interactions between BMPR2 and Src in wild-type PECs (data not shown). Though this may be the result of technical difficulties, we are aware that these results may indicate BMPR2 and Src do not physically interact in our cell type. Therefore, alternative mechanisms for BMPR2 regulation of Src should be explored. One such mechanism is BMPR2 acting as an indirect non-catalytic inhibitor, that is, BMPR2 may promote the interaction and establishment of non-catalytic inhibition between Src and an additional protein. A candidate for this interaction is Cav-1. Cav-1 and BMPR2 have been shown to physically interact in caveolae (Nohe et al., 2005; Ramos et al., 2006; Wertz et al., 2008), and it is well established that Cav-1 inhibits Src through Cav-1's CSD(S. Li et al., 1996b). Thus, BMPR2 may promote Cav-1/Src inhibitory complexes within caveolae and a reduction of BMPR2 expression in *Bmpr2*$^{+/-}$ PECs reduces these interactions. This hypothesis could be readily tested using the experimental setup outlined in Chapter III.

In addition to direct physical interactions and indirect inhibition through a secondary protein, BMPR2 may be inhibiting Src activation through downstream BMP signaling. Our studies show that treatment of *Bmpr2*$^{+/-}$ PECs with BMP2 reduces Src Y416 to levels similar to wild-type, suggesting that BMP signaling regulates Src kinase activity. This could conceivably be accomplished through modulation of PDGFR signaling. PDGFR is a known activator of Src kinase (Roskoski, 2005; Stover et al., 1996), and BMP signaling has been shown to inhibit PDGFR downstream signaling (Ghosh-Choundhury et al., 1999). Therefore, the reduction in BMP signaling in *Bmpr2*$^{+/-}$ PECs may enhance Src kinase activation by reduced inhibition of PDGFR. Future studies could assess Src kinase and PDGFR dynamics in *Bmpr2*$^{+/-}$ mutants.

Alternatively, BMP signaling may work to help strengthen BMPR2-Src interactions or

BMPR2-Cav-1-Src interactions. Wong *et al* demonstrate that BMP stimulation reduced Src kinase activation in pulmonary arterial smooth muscle cells with wild-type BMPR2 but not in cells with BMPR2 tail domain truncations in which BMPR2 and Src no long interact (Wong et al., 2005). These data support our hypothesis that BMPR2 inhibits Src activity by physically interacting with Src and that BMP stimulation strengthens or stabilizes these interactions. Future studies detailed in Chapter III will effectively test this hypothesis.

Summary

The work presented in this dissertation adds to our understanding as to how *BMPR2* mutations may promote the development of pulmonary hypertension. Here we show that *BMPR2* mutations cause increased Src Kinase activation in pulmonary endothelial cells and that this activation, in turn, enhances caveolar endocytosis. We go on to show that *Bmpr2*$^{+/-}$ PECs have endothelial barrier defects, due in part, to enhanced Src-mediated caveolar endocytosis. We show that inhibition of Src kinase with pharmacological inhibitors reverses these barrier defects, suggesting treatment with Src kinase inhibitors may be a viable option for therapeutic intervention in PH. However, recent evidence suggests Src inhibitors in some contexts actually promote the development of PH in some patients (Montani et al., 2012). Therefore, the need for alternative therapeutic strategies is apparent. Our proposed work in Chapter III addresses how we may overcome this complication by defining the mechanism by which *BMPR2* mutations promote Src kinase activation in the pulmonary endothelium. All together these data contribute to our understanding of the development of PH and lay the groundwork for future studies that may provide new and effective therapeutic strategies for PH.

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