The Role of EBP50 in Regulating Endothelial-To-Mesenchymal Transition in Pulmonary Hypertension

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The Role of EBP50 in Regulating Endothelial-To-Mesenchymal Transition in Pulmonary Hypertension

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Pulmonary hypertension (PH) is a cardiopulmonary disease that manifests in a chronic elevation of mean pulmonary arterial pressure, pulmonary vascular remodeling, and right ventricular hypertrophy, and arises from a combination of various factors including endothelial dysfunction. Among the many signs of endothelial dysfunction in PH, increased production of vasoconstrictive signaling molecules and growth factors, proinflammatory shift, and endothelial-to-mesenchymal transition (EndMT) are increasingly recognized as important events potentiating endothelial reprogramming and impaired vascular homeostasis. Despite a critical need to identify molecular players that regulate endothelial reprogramming and the process of EndMT in PH, progress in that direction has been limited.

By utilizing lung samples from PH patients as well as three experimental rodent models of PH, we show that expression of the scaffolding PDZ protein ezrin-radixin-moesin-binding phosphoprotein 50 (EBP50, NHERF1) is downregulated in PH pulmonary vessels and isolated pulmonary vascular endothelial cells. Our in vitro studies indicate that PH-relevant downregulation of endothelial EBP50 expression can be induced by hypoxia and inflammatory cytokine interleukin-1 beta (IL-1β) in a time-dependent manner. Phenocopy of EBP50 reduction using siRNA was found to exert regulatory control over EndMT transcription factors Snail, Zeb1 and Slug, and mesenchymal phenotype transition in pulmonary endothelial cells, and to regulate endothelial cell proliferation and barrier function. Further, the downregulation of EBP50 appeared to potentiate hypoxia-driven EndMT transcription factor Slug upregulation. In vivo studies on

EBP50$^{+/-}$ mice demonstrated that downregulation of EBP50 exacerbates hemodynamic manifestations of chronic hypoxia PH.

In our work, we identify EBP50 as a key regulator of EndMT in PH whose expression is downregulated in PH patient pulmonary endothelium as well as animal models of PH, and whose partial deletion exacerbates PH disease manifestations in rodents. Taken together, our findings support the importance of this protein in PH and highlight the rescue of EBP50 levels in this disease as a promising therapeutic approach.

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Preface

Моим бабушкам, Екатерине Львовне Антышевой и Тамаре Алексеевне Козловой.

To my grandmothers, Yekaterina Lvovna Antysheva and Tamara Aleksyevna Kozlova.

Over the past five and a half years, there were many times when I did not think I will get to sit down and write these words, reflecting on the journey I have made since my arrival to Pittsburgh. These years were immensely challenging, but also incredibly rewarding, both personally and scientifically. When I started my first rotation as a freshman IBGP student, I could not have imagined how far I will come, and how much I will grow as a scientist. For my accomplishments, I have many people to thank.

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As I grew to be more independent and take more ownership in my scientific thinking, I got to work alongside many fantastic undergraduate and graduate students. Seeing them grow scientifically under my guidance was by far the most fulfilling experience of my time in Al Ghouleh lab. Mariah, Anas, Kyle, Carlos – I have learned so much from you, and I hope I have left a good impression in your hearts.

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My family is the reason why I found strength and courage to pursue my career aspirations, even if it meant moving 5000 miles away from home. I could not have come this far without the support of my mom, dad and brother. Having parents who encouraged me to take risks and be bold in my thinking and ambitions is a true gift, and I do not take it for granted. Even if we do not see each other often, I always know that they are standing behind me, ready to hold me up. *Мама, папа, Коля, спасибо вам за всё.*

Finally, I am very fortunate to have navigated my time in graduate school in the company of my boyfriend, Paul. Whenever I get anxious and stressed, your calmness is what gets me through it. And also, silly jokes. Those help too.

Commonly Used Abbreviations

5-HT	**Serotonin**
ALK1, ACVRL1	Activin A Receptor Type I-Like Kinase-1
Ang II	Angiotensin II
α-SMA	Alpha-Smooth Muscle Actin
BMP	Bone Morphogenetic Protein
BMPR2	Bone Morphogenetic Protein Receptor 2
CAV1	Caveolin-1
CDK1 (Cdc2)	Cyclin-Dependent Kinase 1
CTD	Connective Tissue Disease
DPP-4	Dipeptidyl Peptidase-4
EB	C-Terminal ERM-Binding Region
EBP50, NHERF1	Ezrin-Radixin-Moesin-Binding Phosphoprotein 50, Sodium / Hydrogen Exchanger Regulatory Factor 1
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-To-Mesenchymal Transition
EndMT	Endothelial-To-Mesenchymal Transition
ENG	Endoglin
ERM	Ezrin / Radixin / Moesin
ET-1	Endothelin-1
FBN	Fibronectin

FGF	Fibroblast Growth Factor
FGF-2	Fibroblast Growth Factor-2
FGFR	Fibroblast Growth Factor Receptor
FoxM1	Forkhead Box M1
FoxO1	Forkhead Box O1
GLP-1	Glucagon-Like Peptide 1
HAT	Histone Acetylase
HDAC	Histone Deacetylase
HIF-1α and HIF-2α	Hypoxia-Inducible Factors 1 Alpha and 2 Alpha
HMGA1	High Mobility Group AT-Hook 1
HPAECs	Human Pulmonary Artery Endothelial Cells
HRE	Hypoxia Response Element
Hx	Hypoxia
ICAM-1	Intercellular Adhesion Molecule 1
IL-1β	Interleukin-1 Beta
IL-6	Interleukin-6
iPAH	Idiopathic PAH
KCNK3	Potassium Channel Superfamily K Member-3
LPS	Lipopolysaccharide
LV	Left Ventricle
MAPK	Mitogen-Activated Protein Kinase
Max RVP	Maximum Right Ventricular Pressure

MCT	Monocrotaline
MCTP	Monocrotaline Pyrrole
miRNA	MicroRNA
MMP	Matrix Metalloproteinase
mPAP	Mean Pulmonary Arterial Pressure
MSC	Mesenchymal Stem Cells
mTOR	Mammalian Target of Rapamycin Pathway
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
Nox	NADPH Oxidase
PAEC	Pulmonary Arterial Endothelial Cells
PAH	Pulmonary Arterial Hypertension
PAWP	Pulmonary Arterial Wedge Pressure
PECAM-1, CD31,	Platelet Endothelial Cell Adhesion Molecule, Cluster of Differentiation 31
PDGF	Platelet-Derived Growth Factor
PDGFR	Platelet-Derived Growth Factor Receptors
PDZ domain	Postsynaptic Density 95 / Disc Large / Zona Occludens Domain
PGI2	Prostacyclin
PH	Pulmonary Hypertension

PHD	Prolyl Hydroxylase Domain-Containing Protein
PI3K	Phosphatidylinositol 3-Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
PMVEC	Pulmonary Microvascular Endothelial Cells
PPARɣ-1	Peroxisome Proliferator-Activated Receptor Gamma-1
PV loops	Pressure-Volume Loops
PVR	Pulmonary Vascular Resistance
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinases
RV	Right Ventricle
S100A4, FSP1	S100 Calcium Binding Protein A4, Fibroblast-Specific Protein 1
SM22α	Transgelin (TAGLN)- not sure if you need both abbreviations.
Smads	Mothers Against Decapentaplegic Proteins
SMC	Smooth Muscle Cell
SOD	Superoxide Dismutase
SSc	Systemic Sclerosis
Su-Hx	Sugen-Hypoxia

TGF-β	Transforming Growth Factor-Beta
TGFβR	Transforming Growth Factor Beta Receptor
TKI	Tyrosine Kinase Inhibitor
TNFα	Tumor Necrosis Factor Alpha
VCAM-1	Vascular Cell Adhesion Molecule 1
VE-cadherin	Vascular Endothelial Cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptors
vWF	Von Willebrand Factor

1.0 Introduction

1.1 Pulmonary Hypertension

1.1.1 Definition and Characterization of Pulmonary Hypertension (PH)

Since the 1st and 2nd World Symposiums on Pulmonary Hypertension (WSPH) in 1973 and 1998, the term pulmonary hypertension (PH) has been widely used to describe diseases of heart and lung circulation of various etiologies, where elevated mean pulmonary arterial pressure (mPAP) ≥ 25 mmHg measured by right heart catheterization at rest presents as a primary or secondary symptom to other underlying pathological conditions [1,2]. Within the term PH, a current WSPH classification discriminates five disease subgroups, grouped according to shared similarities in pathophysiological and hemodynamic characteristics and clinical disease management [3,4]. Pulmonary arterial hypertension (PAH), classified as Group I PH, is defined as an elevation of pulmonary arterial pressure associated with maladaptive right heart dysfunction and hypertrophy [5]. Whereas PAH is considered a primary disease [5], the remaining four PH subgroups are classified as secondary diseases developing as a result of other cardiovascular complications: PH due to left heart disease (Group II PH), PH due to lung diseases and / or hypoxia (Group III), PH due to pulmonary artery obstructions (Group IV), and PH with unclear and / or multifactorial mechanisms (Group V) [4]. Most recently, a task force on hemodynamic definitions and clinical disease classification at the 6th WSPH proposed to change the cutoff value for the clinical definition of all forms of pre-capillary PH from mPAP ≥ 25mmHg to mPAP > 20 mmHg

and to include pulmonary vascular resistance (PVR) ≥ 3 Wood Units (WU) in the diagnosis of pre-capillary PH [4].

1.1.2 Pathophysiology of Pulmonary Arterial Hypertension (PAH)

PAH is a rare disease that remains deadly despite the steadily increasing number of FDA-approved therapies [6]. For more than a decade, since the 3rd WSPH, PAH has been defined clinically by elevated mPAP ≥ 25 mmHg and elevated pulmonary vascular resistance (PVR) ≥ 3 WU with normal pulmonary arterial wedge pressure (PAWP) ≤ 15 mmHg [7]. However, because of the nonspecific disease symptoms, such as fatigue, dyspnea, or syncope, initial diagnosis of PAH is often delayed by over 2 years [8] and up to a median of 44 months (3 years and 8 months) [9]. With mismanaged medical treatment, or in patients non-responsive to available therapies, chronic elevation of mPAP can result in increased afterload on the right ventricle (RV), leading to hypertrophy and, eventually, right-sided heart failure, a major cause of death in PAH patients [10,11]. Based on a statistical definition of the upper limit of normal mPAP (97.5 percentile, two standard deviations above the normal range of mPAP at rest, 14.0±3.3 mmHg [12]), the new WSPH guidelines, as mentioned earlier, propose to lower the cutoff value for elevated mPAP to > 20mmHg in the new definition of all precapillary forms of PH, PAH included.

In addition to idiopathic PAH (iPAH), heritable PAH (hPAH), drug- and toxin-induced PAH, persistent PH of the newborn (PPHN), and PAH associated with connective tissue disease (CTD), HIV infection, portal hypertension (portopulmonary PH), congenital heart disease or schistosomiasis infection, the clinical classification of PAH subgroups has now been expanded to include PAH in long-term responders to calcium channel blockers and PAH with overt features of venous or capillary involvement, such as pulmonary veno-occlusive disease (PVOD) and

pulmonary capillary hemangiomatosis (PCH) [4]. Of the various forms of PAH, hPAH presents a special interest because of its associations with mutations in the bone morphogenetic protein receptor 2 (BMPR2), an essential member of the transforming growth factor-beta (TGF-β) signaling family. It is now established that 53-86 % of patients with hPAH and 20% of patients with iPAH carry mutations in the BMPR2 [13,14]. Additionally, although more rare, hPAH has also been associated with mutations in other genes of the TGF-β family [i.e., activin A receptor type II-like kinase-1 (ACVRL1, also known as ALK1), endoglin (ENG), and mothers against decapentaplegic proteins (Smads)], as well as mutations in potassium channel superfamily K member-3 (KCNK3), caveolin-1 (CAV1), and eukaryotic translation initiation factor 2 alpha kinase 4 (EIF2AK4) [15–22].

The higher prevalence of PAH in females over males has been widely documented across international PAH registries and ranges between 1.7 : 1 (USA / NIH, 1981-1985 [23]) and 3.9 : 1 (USA / REVEAL, 2006-2007 [24]) in all subgroups with the exception of HIV-associated PAH and portopulmonary PH [25]. It appears that CTD-associated PAH tends to affect women overwhelmingly more often than men, with reported sex differences as high as 9 : 1 (USA / REVEAL [24]). It is notable that many autoimmune diseases associated with PAH development are female-dominant, including scleroderma (women to men ratio, 12 : 1), Hashimoto's thyroiditis (19 : 1), systemic lupus erythematosus (7 : 1), and mixed connective tissue disease (4 : 1), among others [26]. An association between estrogen levels and immune cell activation has been extensively studied over the years and demonstrates that estrogen can promote dendritic cell maturation [27], activate B-cells and increase T-cell response, as well as reduce BMPR2 expression on immune cells [28], all of which might contribute to increased prevalence of PAH in females.

At the tissue level, patients with PAH present with signs of vasoconstriction, extensive pulmonary vascular remodeling, perivascular inflammation, vascular wall thickening, and vessel narrowing, occlusion, rarefaction, and stiffening [29–31]. Pulmonary vascular remodeling in PAH affects the entire vascular wall, spanning endothelial vascular lining (intima), smooth muscle cells (media), as well as adventitial fibroblasts and connective tissue, and impaired intercellular communication between pulmonary vascular cells represents a key feature of disease progression [32,33]. Remodeling of pulmonary vessels is also characterized by distal muscularization of precapillary arterioles and emergence of cells expressing smooth muscle markers within the intimal layer of those vessels, resulting from inward migration of medial smooth muscle cells (SMCs) into the intima and cellular transdifferentiation of endothelial cells (endothelial-to-mesenchymal transition, EndMT) [30]. In severe PAH cases, unrestricted proliferation of endothelial cells results in a formation of obliterative plexiform lesions which consist of endothelial- and smooth muscle-like cells and dramatically restrict normal blood flow [34,35]. However, even in the absence of plexiform lesions, pulmonary arterial and microvascular endothelial cells (PAECs and PMVECs) show discernable signs of activation and dysregulated signaling, including heightened hyperproliferative and anti-apoptotic response, reduced anticoagulant properties, imbalanced production of vasoreactive compounds and reactive oxygen species (ROS), and metabolic shift from oxidative phosphorylation to glycolysis (Warburg effect) [36,37]. An imbalanced production of vasoreactive compounds by endothelial cells contributes to an aberrant local microenvironment within the pulmonary vascular wall and tends to favor production of vasoconstrictive signaling molecules [endothelin-1 (ET1), angiotensin II (Ang II), and serotonin, (5-HT)], growth factors (i.e. fibroblast growth factor-2, FGF-2), cytokines (interleukin-1 and -6, IL-1 and IL-6), chemokines (monocyte chemoattractant protein-1, MCP-1), and adipokines (leptin). At the same time, the

production and secretion of vasodilatory agents, such as nitric oxide (NO) and prostacyclin (PGI2), are decreased [31]. The altered vascular microenvironment, in turn, tends to promote smooth muscle hyperplasia, immune cell infiltration, and sustained inflammation [32,38].

1.1.3 Signaling pathways contributing to PH

Cellular phenotypes associated with PH, including Group I PH (PAH), are driven by complex interactions between multiple cell types and signaling pathways and governed by changes in transcription factor activation, epigenetic dysregulation, mitochondrial dysfunction, as well as dysfunctional receptor- and non-receptor-activated kinase signaling [39,40].

Dozens of transcription factors and transcriptional coregulators controlling the expression of pro- and anti-proliferative and inflammatory genes are implicated in pulmonary vascular dysfunction in PH [41]. Multiple studies associated the downregulation of protective transcription factors with a decrease in anti-proliferative and pro-apoptotic gene expression in total lung tissues of PH patients and rodents, as well as in vascular cells subjected to PH-relevant treatments in vitro. Much of those insights were gathered from the in vivo models of PAH and PH, which will be discussed in more detail in the **Section 1.1.5**. Expression of peroxisome proliferator-activated receptor gamma-1 (PPARγ-1) is decreased in lung tissue and complex vascular lesions of patients with severe PH [42]. Loss of PPARγ potentiates the emergence of hyperproliferative endothelial [42] and smooth muscle cells [43], and treatment with PPARγ agonists can reverse experimental PAH and protect from PAH-associated RV failure [44,45]. Endothelial-specific deletion of myocyte enhancer factor 2 (MEF2) exacerbates experimental PH in mice subjected to chronic hypoxia [46], and its activity, linked to increased endothelial migration and proliferation, is decreased in PAH-patient-derived PAECs [47]. Moreover, in PH, multiple pro-proliferative and pro-inflammatory

signaling axes converge on transcription factor Forkhead box O 1 (FoxO1) [48]. FoxO1 expression is decreased in lungs and pulmonary SMCs of PH animals and human PH patients [48,49], and is linked to increased proliferation of PAH pulmonary artery SMCs (PASMCs) [48] and decreased apoptosis of PAH PASMCs and PH PAECs [48,49]. Furthermore, global genetic deficiency or chronic pharmacological inhibition of the tumor suppressor protein p53 can promote hypoxia-induced PH and vascular remodeling in mice [50] or induce spontaneous PH in rats [51]. Conversely, activation of p53 prevents and reverses chronic hypoxia PH and PAH induced by a combination of vascular endothelial growth factor receptor (VEGFR) antagonist Sugen (SU5416) and hypoxia (Su-Hx) in mice [52]. Finally, expression and activity of the protective vascular endothelial transcription factor Krüppel-like factor 4 (KLF4), is downregulated in the lungs of PAH patients and PH rats [53,54], and mice with endothelial KLF4 knockdown develop exacerbated PH symptoms when subjected to chronic hypoxia [54].

As opposed to a decreased expression of protective transcription factors, expression and activity of other transcription factors and coregulators that induce pro-proliferative, pro-inflammatory, and pro-apoptotic genes is commonly upregulated in PH lung tissues. Whereas chronic hypoxia exposure alone can induce PH in the absence of additional pathological complications [55], a generalized hypoxic environment in the lung often accompanies severe vasoocclusion and pulmonary remodeling in various forms of PH (primarily Group III and Group IV PH) [4], contributing to disease severity. Effects of hypoxia on surrounding tissues are typically mediated through the oxygen-sensitive transcription factors hypoxia-inducible factors 1 alpha and 2 alpha (HIF-1α and HIF-2α), structurally similar homologs with overlapping but non-redundant functions [56]. Under baseline conditions of atmospheric concentration of oxygen (normoxia; 20.9% of sea-level air; O_2 partial pressure = 21.136 kPa), HIF proteins are targeted for proteasomal

degradation by hydroxylation of proline residues by prolyl hydroxylase domain-containing (PHD) proteins [57–59]. In contrast, under hypoxia (~ 10% O_2 concentration), the activity of PHD proteins is suppressed, allowing HIF proteins to escape ubiquitination and undergo nuclear translocation [60], where they bind to hypoxia response elements (HREs) of hypoxia-inducible genes and transactivate their expression [61]. In turn, by transactivating expression of erythropoietin [62], vascular endothelial growth factor (VEGF) [63], vascular endothelial growth factor receptor (VEGFR) [64], inducible nitric oxide synthase (iNOS) [65], and ET-1 [66] among others, HIF proteins mediate an adaptive response to hypoxia at the cellular level. Interestingly, as will be further described in **Section 1.3.3**, many of the proteins upregulated by hypoxia have previously been implicated in PH development. While the global homozygous deletion of HIF-1α or HIF-2α is embryonically lethal, HIF-1α and HIF-2α heterozygous mice have long been known to be protected from hypoxia-induced PH [67,68]. More recent studies demonstrated the crucial role of endothelial HIF-2α in PH development, where endothelial-specific HIF-2α knockout (KO) mice, but not endothelial HIF-1α KOs or smooth muscle HIF-2α KOs, were protected from hypoxia-induced PH [69,70]. Further, therapeutic targeting of HIF-2α using HIF-2α-selective pharmacological inhibitors or targeted antisense oligonucleotides proved to be protective against hypoxia-induced PH in mice and rats and Su-Hx and plant alkaloid monocrotaline (MCT)-induced PAH in rats [70,71].

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is another important transcription factor contributing to PH-associated pathologies. The NF-κB pathway is one of the key signaling pathways initiated during inflammation [72] and in the pulmonary vessels of patients with idiopathic PAH [73]. Insights from animal models of PAH and PH further indicated that pharmacological inhibition of NF-κB improves disease symptoms and decreases their severity [74–76]. Recent studies have implicated FoxO1 homolog, Forkhead box M1 (FoxM1), in promoting

proliferation and apoptosis resistance in PAH PASMCs, mediated by an impaired endothelial – smooth muscle cell cross-talk [77,78]. More specifically, upregulation of FoxM1 in SMCs is induced by multiple endothelial-derived factors upregulated in PH lung tissue, such as platelet-derived growth factor-beta (PDGF-B) and endothelin-1 (ET-1), and, in turn, activates pro-proliferative and anti-apoptotic signaling cascades in SMCs leading to medial vascular hypertrophy [77]. Expression of FoxM1 is upregulated in the lungs of iPAH patients, as well as in chronic hypoxia-, MCT- and Su-Hx-exposed mice, and MCT- and Su-Hx rats [77–79]. Interestingly, expression of smooth muscle FoxM1 can be induced by the endothelium-derived factors, in particular, stromal cell-derived factor 1 (SDF1), also known as C-X-C motif chemokine 12 (CXCL12) [77]. Further, SMC-specific inhibition of FoxM1 prevents hypoxia-induced PH in mice, and inducible SMC-specific FoxM1 knockout reverses pulmonary artery remodeling [79], whereas pharmacological inhibition of FoxM1 improves symptoms of MCT- and Su-Hx-induced PAH in rats [77,78]. Moreover, expression of transcriptional corepressor C-terminal binding protein 1 (CtBP1) is upregulated in the adventitial fibroblasts of patients with iPAH and in PH mice, and linked to metabolic reprogramming, proliferation and increased expression of pro-inflammatory genes [80]. In addition, the expression of pyruvate kinase muscle isoform 2 (PKM2), a regulator of glycolysis and a transcriptional coactivator, is upregulated in pulmonary adventitial fibroblasts of patients with severe PH and controls their glycolytic status and proliferation [81]. Other transcription factors, induction of which has been implicated in PH pathogenesis, include signal transducers and activators of transcription-3 (STAT3) [82], octamer-binding protein 4 (OCT4) [83], cAMP response element-binding protein (CREB) [84,85], serum response factor (SRF) [86], nuclear factor of activated T cells (NFAT) [87], Runt-related transcription factor 2 (RUNX) [88], Twist-related protein 1 (Twist-1) [89,90], and zinc finger protein SNAI2 (Slug) [91,92].

In addition to changes in expression and activity of various transcription factors and transcriptional regulators, a number of reports indicate the involvement of epigenetic alterations in pulmonary vascular cells in PH. Changes in chromatin structure and expression of histone deacetylases (HDACs) and bromodomain-containing proteins, dysregulated microRNA and long non-coding RNA networks present among the changes associated with PH pathogenesis [40,93–96]. DNA methylation is an epigenetic modification mediated by DNA methyltransferases that results in the tight packing of DNA and histones, and represses gene transcription long-term [97]. Genomic sequencing of PASMCs explanted from fawn-hooded rats with PAH indicates the presence of selective hypermethylation in the enhancer region and promoter of gene encoding mitochondrial superoxide dismutase-2 (SOD2). Epigenetic SOD2 deficiency leads to impaired redox signaling and potentiates the development of hyperproliferative and apoptosis-resistant PASMCs in PAH [98]. Patterns of hypermethylation in the BMPR2 promoter, as well as in 1,743 promoter regions of genes controlling cell proliferation, inflammation, and migration, are identified in patients with hPAH [99] and in PASMCs from CTEPH patients (Group IV PH) [100]. Histone acetylation and deacetylatiosn are two of the most common enzymatic histone modifications and are mediated by histone acetylases (HATs) and HDACs, respectively. Histone acetylation promotes binding of bromodomain and extra-terminal (BET) proteins and enhances DNA transcription, whereas histone deacetylation inhibits it [97]. Increased acetylation of histones H3 and H4 at the promoter site of endothelial nitric oxide synthase (eNOS) is associated with increased eNOS expression in persistent pulmonary hypertension of the newborn (PPHN, Group I PH) [101]. Decreased expression of sirtuin 3 (a Class III HDAC) is associated with diminished mitochondrial function and PAH in humans and mice [93], and increased expression of smooth muscle HDAC6 promotes PAH by stimulating proliferation and apoptosis resistance of PASMCs [94]. These and other findings [102]

spurred the development of novel therapeutic strategies implementing broad and selective HDAC modulators for the treatment of PAH [94,102–105]. Likewise, BET mimics that can effectively prevent binding of BETs to acetylated histones are being studied for potential use as a future PAH treatment strategy [106]. Finally, dysregulated microRNA (miRNA) and long non-coding RNA (lncRNA) networks are universally recognized in PAH progression [40,95,96]. Namely, activation of the miRNA clusters miR-143/145 and miR130/301 regulates endothelial and smooth muscle cell cross-talk and extracellular matrix remodeling in PAH and PH [107,108], and miR-27a and miR-126a-5p promote PAH-associated EndMT [109,110].

Dysregulated receptor- and non-receptor kinase signaling, along with impaired Wnt / β-catenin [111] and Notch- [112,113] signaling, are other important contributors to pulmonary vascular remodeling and dysfunction in PH. Many pathways maintaining vascular homeostasis signal through receptor kinases or induce activation of downstream non-receptor kinases, such as mitogen-activated protein kinase pathway (MAPK/ERK), phosphatidylinositol 3-kinase pathway (PI3K/AKT), and mammalian target of rapamycin pathway (mTOR), to name a few, and all of them are commonly dysregulated in the PH vasculature [114]. Moreover, the literature presents an abundance of evidence for the roles of receptor tyrosine kinase (RTK) and non-receptor tyrosine kinase (non-RTK) dysregulation in PAH [114–116]. Expression of the platelet-derived growth factor (PDGF) isoforms A and B, and platelet-derived growth factor receptor (PDGFR) subunits alpha and beta are upregulated in the lungs of iPAH patients [117], and a loss-of-function mutation of PDGFRβ abolishes vascular remodeling and chronic hypoxia-induced PH in mice [118]. Serum levels of the vascular endothelial growth factor (VEGF) correlate with increased systolic pulmonary artery pressure (sPAP) and possible PH predisposition in patients with systemic sclerosis (SSc) [119], and increased expression of the vascular endothelial growth factor receptor 2 (VEGFR2) is

found in the plexiform lesions in patients with severe PH [120]. Endothelial-derived fibroblast growth factor-2 (FGF-2) exacerbates smooth muscle hyperplasia and contributes to the progression of PH [121], whereas pharmacological inhibition of the fibroblast growth factor receptor (FGFR) signaling alleviates MCT PAH in rats [122]. Additionally, the epidermal growth factor receptor (EGFR) inhibition dramatically improves hemodynamic parameters and survival of MCT treated rats [123]. However, the extensive cross-talk between these signaling pathways complicates the development of effective kinase-targeting therapies and their clinical use. Despite a promise of using tyrosine kinase inhibitors (TKIs) as a line of defense against PH and PAH, their potential cardiotoxicity was found to potentiate disease progression, and current TKI therapies provide poor risk / benefit ratio in the context of PAH, especially in patients with underlying cardiac impairment [124].

1.1.4 In vitro models of PH

As described earlier, the process of a pathological pulmonary vascular remodeling in PH affects all three layers of the vascular wall and involves alterations in the endothelial, smooth muscle cell, fibroblast, and pericyte biology. Therefore, a number of in vitro models were developed to study each cell type in this disease. Early discovery of a differential response of pulmonary [125] and systemic [126,127] vessels to hypoxia underscored the importance of using cells from an appropriate vascular bed to improve translational applicability of the in vitro findings. As a common way to study pulmonary endothelial and smooth muscle function in disease conditions, pulmonary endothelial and smooth muscle cells obtained commercially or explanted from the lungs of PH human patients and rodents are often treated with PH-relevant stimuli, such as hypoxia (pO_2 = 1-2%) [128,129] and inflammatory cytokines (IL-1β [130], TGF-β [131], TNFα [132], and their combination [133]). An added use of gene silencing or overexpression technologies has allowed for

the elucidation of molecular pathways and mechanistic identification of specific proteins in PAH- and PH-associated phenotypes. Additionally, as vascular stiffening emerges as a possible early contributor to disease-associated vascular pathology [134], manipulations with cellular matrix and substrate stiffness are being more commonly employed [108,135]. Changes in blood flow associated with vasoocclusion and vessel narrowing can be approximated in vitro by manipulating the level of fluid flow-induced shear stress applied to a cultured cell monolayer [136,137]. Finally, because of the tight interconnection between cells comprising the pulmonary vascular wall, mechanistic in vitro studies are starting to increasingly use co-culture systems [107,138] or conditioned mediums [139] to study PH-relevant interactions between different vascular cell types (i.e., PASMCs and PAECs [107]), or between vascular and immune cells (i.e., PASMCs and macrophages [138]). Lastly, three-dimensional cell culture systems [140] and organ-on-chip technologies [141] that are being rapidly developing in the last several years, present an opportunity to improve the translational applicability of the traditional in vitro methods [142].

1.1.5 In vivo rodent models of PH

In the last five decades, our understanding of the pathology and signaling mechanisms involved in PH progression has greatly improved, largely thanks to the development of animal models approximating the disease in vivo. The development of reliable and universally accepted PH animal models allowed for a better understanding of the underlying vascular pathology and aided the identification of novel translationally relevant molecular players important for the disease progression. Currently, the three rodent models of PH utilized most often in preclinical research studies include chronic hypoxia mouse [143] and rat [144] PH models, monocrotaline (MCT) rat PAH model [145], and Sugen-Hypoxia (Su-Hx) rat PAH model [146]. In addition to the more

common PH and PAH models described above, less-established models of the disease exist. BMPR2 mutant mice [147,148], S100A4- [149,150] and IL-6 [151] overexpressing mice, neprilysin- [152] and vasoactive intestinal peptide [153] knockout mice and *Schistosoma mansoni*-infected mice [154] are predisposed to PH development and develop signs of PH at baseline or upon the secondary challenge. However, to this day, no animal model perfectly recapitulates all features of human PH, and all of the models share significant limitations in their use and translational applicability [155].

In order to identify and describe PH symptoms in the animal models and to have a reference point for disease severity, studies often combine post-mortem histological studies of pulmonary vascular remodeling and RV hypertrophy with hemodynamic measurements acquired by RV and LV (left ventricle) catheterization. Miniature pressure or pressure-volume catheters utilized in most in vivo studies have the capability to record pulmonary pressures or more comprehensive pressure-volume loops (PV loops), respectively, that can be used to calculate a variety of disease-relevant cardiovascular parameters, including mean PAP (mPAP), maximum RV pressure (max RVP), RV systolic pressure (RVSP), PAWP and pulmonary vascular resistance (PVR), among many others. The most commonly used readouts for PH severity include mPAP, max RVP, RVSP, Fulton index, or a weight ratio between RV and left ventricle plus septum (LV + S), and a ratio between the RV weight and the tibia length (RV / tibia). Elevated max RVP accompanied by an increased Fulton index or elevated RV / tibia ratio indicates that experimental animals had developed hemodynamic symptoms of PH. Most recently, owing to the rapid development of the non-invasive mouse and rat-adapted live imaging technologies, studies began to utilize rodent echocardiography and cardiac magnetic resonance imaging (CMR) to monitor the heart function in live PH animals [156–158], thus allowing to study the dynamics of the disease progression in real time.

Among the three most commonly used PH and PAH animal models, the chronic hypoxia PH model, which has been in use since at least the 1970s [144], remains widely employed to this day, despite its limitations. The chronic hypoxia PH model is generally regarded as a less severe model of PH and is mostly relevant to human Group III PH (associated with hypoxia and / or lung disease) [159]. Rats and mice placed in a hypoxic environment with a low oxygen pressure (pO_2 = 10%) tend to develop symptoms of PH as early as ten days from the beginning of treatment [143,144]. However, the severity of disease symptoms differs greatly between species and strains, where rats generally develop a more pronounced disease phenotype compared to mice [160]. Hypoxia-exposed rodents develop an increase in RVSP, accompanied by an increase in the Fulton index, but tend to reverse their symptoms upon return to normal oxygen pressure (normoxia), which stands in dramatic contrast to human PAH, symptoms of which are largely irreversible [155]. Generally, chronic hypoxia exposure induces a mild PH phenotype, with small- to medium-degree of remodeling that initially affects small non-muscular arterioles of the distal lung vascular tree and triggers their muscularization and subsequently progresses toward thickening of already muscularized proximal vessels due to the SMC proliferation and hypertrophy [155]. Inflammation appears to contribute to PH progression in chronic hypoxia-exposed rats [161], whereas in mice it quickly resolves, and irreversible fibrosis and plexiform lesions do not develop in either animal species [162]. In contrast to other commonly used models that primarily utilize rats, the chronic hypoxia model allows for the utilization of genetically engineered mice, thus expanding the possibilities to perform mechanistic studies in live animals. Additionally, the chronic hypoxia model remains to be widely used in PH research because of its reproducibility and the minimal physical and financial efforts required to produce a disease-relevant phenotype if hypoxia chambers are accessible to researchers.

Monocrotaline (MCT) rat PH model is achieved by a single subcutaneous or intraperitoneal injection of a toxic plant alkaloid monocrotaline pyrrole (MCTP) which, through a series of reactions with liver cytochrome P-450, CYP3A, gets metabolized into the active compound monocrotaline that induces vascular endothelial injury and progressive development of PH [163,164]. Unfortunately, this model is burdened with significant limitations, including high variability in the degree of response to monocrotaline across species, and significant side effects of MCT administration in the form of liver and kidney damage, as well as myocarditis [165,166]. Despite significant efforts to develop the MCT model in mice, those undertakings have been largely fruitless [167–169]. Unlike rats injected with MCTP that develop PH characterized by pulmonary vascular endothelial injury, interstitial pulmonary fibrosis, inflammation, RV remodeling and hypertrophy three- to four- weeks post-injection [145], mice do not progress past the stage of extensive pulmonary edema even when injected with doses of active MCT magnitudes higher than those used in rats [168]. The role of endothelial injury as a crucial event for MCT PH progression is also challenged by some, considering that the model is primarily characterized by medial hypertrophy, but not the intimal angioobliterative lesions characteristic of the human disease [145]. Despite the outlined limitations, the rat MCT model has greatly benefited the field of PAH research and remains one of the most widely used models of preclinical PAH [170,171].

In an effort to circumvent the problems associated with other rodent PH models and to develop a disease model driven by endothelial injury and recapitulating the plexogenic vasculopathy seen in severe human PAH, the combination of vascular endothelial growth factor receptor (VEGF) antagonist SU-5416 (Sugen) with chronic hypoxia has been used to induce severe PAH in rats since 2001 [146]. SU-5416-injected rats exposed to 3 weeks of chronic hypoxia (Su-Hx) demonstrated signs of increased endothelial apoptosis, which is thought to potentiate the selection

of apoptosis-resistant cells with a hyperproliferative phenotype that contribute to luminal obstruction and plexiform lesion formation. In addition to striking histological features that also include pulmonary vascular medial hypertrophy, Su-Hx rats develop progressive RV hypertrophy and increase in mPAP that remains elevated even upon return to normoxic conditions [172]. Remarkably, vascular effects of Sugen appear to be contained within the lung and, unlike MCT, do not affect other organ systems [146]. Because of how closely the Su-Hx rat model approximates late-stage human PAH, efforts of many research teams were spent on adapting the model for use in mice, with an ultimate goal to utilize a wide array of available genetic modifications to mechanistically study roles of any specific genes in PAH development. A modified version of Su-Hx treatment for mice, where Sugen is subcutaneously injected once a week for 3 weeks simultaneously with hypoxia exposure, has been described in 2011 [173], and, since then, successfully employed by investigators worldwide [52,133,174–177]. In general, PH symptoms of Su-Hx mice are milder than those of rats and do not include plexiform lesions formation. However, Su-Hx mice still present with muscularization of previously non-muscularized pulmonary vessels, increased interstitial collagen deposition, inflammation, and medial wall thickening [173].

1.2 Ezrin-Radixin-Moesin-Binding Phosphoprotein 50 (EBP50)

1.2.1 Structure and Function of EBP50

Ezrin-radixin-moesin-binding phosphoprotein 50 (EBP50), also known as Na+/H+ Exchanger Regulatory Factor (NHERF1) or sodium-hydrogen antiporter 3 regulator 1, is a scaffolding adaptor protein encoded by the gene *SLC9A3R1*. As the name suggests, EBP50 can

directly bind to ezrin, radixin, and moesin proteins of the ERM family and facilitates integration between transmembrane- and cytoskeletal proteins, such as F-actin. Structurally, the 358 residue-long EBP50 polypeptide consists of two N-terminal PDZ (postsynaptic density 95/disc large/zona occludens, PSD-95/DlgA/ZO-1) domains, PDZ-1 and PDZ-2, and a C-terminal ERM-binding region (EB) [178].

PDZ domains are globular 80-90 amino acid-long protein motifs usually composed of 6 β-strands (βA - βF), a short α-helix (αA), and a long α-helix (αB) and arranged into a β-sandwich, or up-and-down β-barrel, tertiary structure [179,180]. PDZ domains can form homo- or heterotypic dimers [181–183] or interact with other proteins or peptides by binding to specific recognition sequences at their extreme C-termini. Class I PDZ domains bind to the C-terminal sequence [Ser/Thr-X-Φ COOH], Class II PDZs bind to the sequence of [Φ-X-Φ-COOH] and Class III PDZs – sequence of [Asp/Glu-X-Φ-COOH] where Φ is any hydrophobic amino acid and X is any amino acid [184]. Interestingly, despite the structural similarities between EBP50 PDZ1 and PDZ2 (both belong to PDZ Class 1), the two domains differ in their binding affinity to PDZ-binding partners [185]. Via its PDZ domains, primarily through the PDZ1 domain, EBP50 can interact with various transmembrane receptors and ion channels, such as the purinergic P2Y1 receptor [186], PDGFR [187], β2-adrenergic receptor (β2-AR) [186], parathyroid hormone receptor (PTHR) [188,189], and κ-opioid receptor [190], sodium–hydrogen exchanger 3 (NHE3) [191], type II sodium-dependent phosphate co-transporter (Npt2a) [192], B1 subunit of the H^+ ATPase [193], aquaporin 9 (AQP9) [194], cystic fibrosis transmembrane conductance regulator (CFTR) [186,195,196], as well as other intracellular partners, such as G-protein-coupled receptor kinase 6 (GRK6A) [197], spleen tyrosine kinase (SYK) [198], phosphatase and tensin homolog (PTEN) [199], phospholipase C (PLC) [200], β-catenin [201], and Yes-associated protein 65 (YAP65, aka YAP1) [202]. Consequently, the interactions between EBP50 and

its binding partners can modulate a variety of cellular processes. For instance, EBP50 can modulate sodium-hydrogen exchange induced by the cyclic AMP-elevating hormones, such as epinephrine and norepinephrine, by binding to the NHE3 [191], control subcellular localization of YAP65 [202], promote Wnt signaling through associating with β-catenin and dictating is positioning between membrane association and nuclear translocation[201], and directly potentiate PDGFR activity [187].

In addition to the N-terminal PDZ domains, EBP50 can interact with other proteins via its C-terminal ERM-binding region. Eleven C-terminal residues of EBP50 were identified to interact with N-terminal four-point one ERM (FERM) domain of moesin and facilitate other EBP50-ERM protein binding [203]. Notably, ERM proteins are adaptor proteins in their own right and can directly interact with cytoskeletal actin filaments via their C-terminal actin-binding domains (ABDs). Therefore, EBP50 serves as a key adaptor molecule that integrates its PDZ-binding partners with cytoskeleton, and thus has a potential to regulate their activity and intracellular trafficking. In addition to the ERM proteins, the EB domain of EBP50 can interact with a focal adhesion kinase (FAK) and promote focal adhesion turnover and SMC migration [204].

EBP50 polypeptide possesses multiple Serine- / Threonine- phosphorylation sites, and EBP50 phosphorylation is important for regulation of its activity (**Table 1**). Despite the absence of the canonical protein kinase A (PKA) and protein kinase C (PCK) phosphorylation motifs, both kinases can phosphorylate EBP50 [205–211]. EBP50 can also be phosphorylated by GRK6A [197], Akt [212], and cyclin-dependent kinase 1, CDK1 (Cdc2) [213]. Phosphorylation of the C-terminal tail serine residues promotes EBP50 oligomerization, regulates its subcellular localization and affects PDZ2 binding capability [206,210,211,213,214]. Phosphorylation of PDZ1 Ser 77 and Thr 95 is critical for β2-AR, PDGFR, Npt2a, and CFTR binding [205,207,208,215], whereas phosphorylation state of Ser 162 of EBP50 PDZ2 domain is important for CTFR activity [209].

EBP50 is widely expressed across tissues, and particularly enriched in polarized epithelial cells, where it plays a role in organizing apical membranes [216,217]. Increased EBP50 expression was found in hepatocellular carcinoma [201] and schwannoma [218] samples, and altered EBP50 expression in breast cancer is thought to play a role in tumor progression and cancer invasiveness [219,220] . Interestingly, multiple studies reported an association between subcellular localization of EBP50 and tumor aggressiveness [221–223], and it has been suggested that EBP50 acts as a tumor suppressor when localized at the apical membrane, and as a pro-oncogenic factor when accumulated in the nuclei or cytoplasm [224]. Of particular interest is the fact that EBP50 is expressed in vascular cells, including SMCs [204,212,225–227], endothelial cells [214,228], and macrophages [229].

Table 1. EBP50 Phosphorylation Sites

Phosphorylation site	Region	Kinase	Action	Citation
Thr 71	PDZ1	undetermined	undetermined	206
Ser 77	PDZ1	PKA, PKC	β2-AR, PDGFR, Npt2a, CFTR binding	205,207,208,215
Thr 95	PDZ1	undetermined	Phosphate transport, renal proximal tubule cells	207
Thr 156	PDZ2	Akt	Proliferation, primary VSMC	212
Ser 162	PDZ2	PKC	CTFR activity	209
Ser 277	C-terminal tail	undetermined	undetermined	206
Ser 279	C-terminal tail	Cdc2	EBP50 oligomerization	213
Ser 287 / 289 / 290	C-terminal tail	GRK6a, PKA, others	EBP50 oligomerization	205,206,230–232
Ser 288	C-terminal tail	undetermined	Subcellular localization	206,214
Ser 299	C-terminal tail	undetermined	undetermined	206
Ser 301	C-terminal tail	Cdc2	EBP50 oligomerization	213
Ser 310	C-terminal tail	undetermined	Subcellular localization	214
Ser 337 / 338	C-terminal tail	PKC	EBP50 oligomerization	206
Ser 339 / 340	C-terminal tail	PKC	Regulation of PDZ2 binding capacity	210
Ser 347 / 348	C-terminal tail	PCK	Subcellular localization, regulation of Rac1 function	211

1.2.2 Role of EBP50 in vascular biology

Historically, most studies investigating the role of EBP50 in vascular biology have been focused on systemic circulation. In vitro experiments on aortic SMCs isolated from global EBP50 KO mice indicated that EBP50 promotes EGF-induced vascular SMC migration and focal adhesion turnover [204], and potentiates SMC proliferation through activation of S-phase kinase-associated protein-2 (Skp2) and degradation of cell cycle inhibitor p21(cip1) [212,225]. EBP50 was also found to promote NF-κB signaling and adhesion molecule expression, where SMCs isolated from EBP50 KO mice had reduced sensitivity to lipopolysaccharide (LPS) and TNFα and did not respond with dramatic upregulation of adhesion molecules intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) [229]. Interestingly, unlike mouse SMCs, rat aortic SMCs transfected with EBP50 siRNA demonstrated increased migration and impaired cytokinesis mediated through the uncoupling of the cytoskeletal microtubule–myosin IIa network and activation of a small GTPase Ras-related C3 botulinum toxin substrate 1 (Rac1) [227]. EBP50 was also found to mediate the Ang II-induced SMC hypertrophy and reactive oxygen species production by directly associating with NADPH oxidase (Nox) organizing subunit $p47^{phox}$ [226]. In systemic vascular endothelial cells, like systemic SMCs from EBP50 KO mice, human umbilical vein endothelial cells (HUVECs) transfected with EBP50 siRNA had reduced ICAM-1 and VCAM-1 expression in response to TNFα treatment [229]. In bovine PAECs, EBP50 phosphorylation was found to promote wound healing [214].

Overall, our understanding of the role of EBP50 in human vascular diseases remains limited. However, studies of EBP50 in the context of cancer biology point us in new directions connecting EBP50 to cell reprograming relevant to cardiovascular diseases including PAH and PH.

For nearly two decades, EBP50 has been known to directly associate and interact with β-catenin, and to regulate epithelial-to-mesenchymal transition (EMT) in various cancers [201,224], a process associated with increased invasiveness, metastasis and chemoresistance [233]. Notably, the Wnt / β-catenin pathway can promote EMT by activating transcriptional repressors of E-cadherin Snail [234] and Slug [235], and by indirectly activating Zeb1 via other Wnt target genes, such as cyclooxygenase 2 (COX2) [236] and IGF1 [237]. Loss of EBP50 in biliary cancer cells was found to stimulate EGFR signaling and EMT, manifested in upregulation of Slug, reduction of epithelial E-cadherin and loss of cell polarity [238]. In colorectal cancer, loss of apical plasma membrane EBP50 expression induced nuclear translocation of β-catenin and decreased expression of E-cadherin, simultaneous with increased expression of mesenchymal marker fibronectin [239]. Strikingly, overexpression of the wild-type EBP50 in EBP50-depleted Caco-2 cell line of human epithelial colorectal adenocarcinoma cells did not rescue its apical distribution, but rather resulted in cytoplasmic and nuclear overexpression and failed to recover E-cadherin expression. However, consistent with the previous reports, overexpression of a membrane-targeted myristoylated EBP50 was sufficient to restore E-cadherin expression, suggesting that apical localization of EBP50 is critical for EMT inhibition [224,239].

It is interesting, then, that the endothelial-to-mesenchymal transition (EndMT), a process similar to EMT, is being increasingly recognized as playing important role in vascular pathologies associated with PAH and PH. Strong associations between EBP50 and EMT make it a promising candidate for studying its role in the context of EndMT and PAH.

1.3 Endothelial-to-Mesenchymal Transition (EndMT) in PH

1.3.1 Definition of EndMT

In the efforts to identify triggering events for the pulmonary vascular remodeling, the attention of many research groups has been focused on the role of endothelium in PH [37,39]. Interestingly, in recent years, an event termed "endothelial-to-mesenchymal" transition (EndMT), also sometimes referred to as transdifferentiation, has emerged as one such potentiating mechanism involved in endothelial dysregulation and PH progression [240].

While EndMT primarily occurs during embryonic development and was first described in the context of endocardial cell differentiation during heart cushion morphogenesis [241], it is also present during wound healing [242,243] and inflammation [244,245] in adult organisms and contributes to a variety of cardiovascular diseases, including atherosclerosis [246,247], valvular disease [248–250], fibroelastosis [251], and, most recently, pulmonary hypertension [133,252]. A highly dynamic process controlled by a set of developmental signaling pathways, EndMT is somewhat loosely defined as a process by which endothelial cells undergo a shift towards a mesenchymal-like cellular state [253]. Nonetheless, EndMT is a complex process with a broad range of intermediate phenotypes and multiple end-points, and the sequence of signaling events occurring during the EndMT progression has been henceforth difficult to fully elucidate and concisely define. Akin to the epithelial-to-mesenchymal transition (EMT) [254,255], induction of EndMT invokes an activation of multiple transcription factors, most notably Snail (SNAI1) [256], Slug (SNAI2) [257], Twist-1 [89], Zeb1 [258] and Zeb2 [259], which were first identified as transcriptional repressors of E-cadherin during EMT [260–264]. Acting in concert, these transcription factors function as repressors and / or activators of endothelial and mesenchymal gene expression, commanding the loss of expression of

characteristic endothelial marker proteins such as Von Willebrand Factor (vWF, also known as Factor VIII-related antigen), platelet endothelial cell adhesion molecule (PECAM, also known as cluster of differentiation 31, CD31), vascular endothelial cadherin (VE-cadherin, aka type 2 cadherin 5 and CD144), vascular endothelial growth factor receptor (VEGFR), and angiopoietin receptor (Tie-2), and the gain of expression of mesenchymal proteins including S100 calcium binding protein A4 (S100A4, also known as fibroblast-specific protein 1, FSP-1), alpha-smooth muscle actin (α-SMA), transgelin (SM22α), fibronectin (FBN), and vimentin [265] **(Figure 1)**. Ultimately, genetic cell reprogramming and phenotypic switching seen in EndMT results in a change in cell morphology from compact cobblestone to elongated and spindle-like due to cytoskeletal remodeling and alterations in extracellular matrix composition [266,267]. The range of EndMT progression is thought to vary from partial (pEndMT, co-expressing both endothelial and smooth muscle markers) to complete, cEndMT, where endothelial cells lose the expression of key endothelial markers, such as PECAM, vWF, VE-cadherin, eNOS, and BMPR2, and gain phenotypic and functional characteristics of mesenchymal cells (upregulation of Collagen 1a1 and 1a2, S100A4, α-SMA, and myosin-11, increased migration and proliferation) [174]. Furthermore, cEndMT cells explanted from Su-Hx mice can fully transform into smooth-muscle-like cells [174], suggesting that they can directly or indirectly contribute to PH-associated vascular pathology. As such, experiments using the conditioned media from cEndMT cells suggest that resident cEndMT vascular cells can promote proliferation and migration of non-endothelial mesenchymal cells and stimulate the angiogenesis in non-transitioned ECs in a paracrine manner [174].

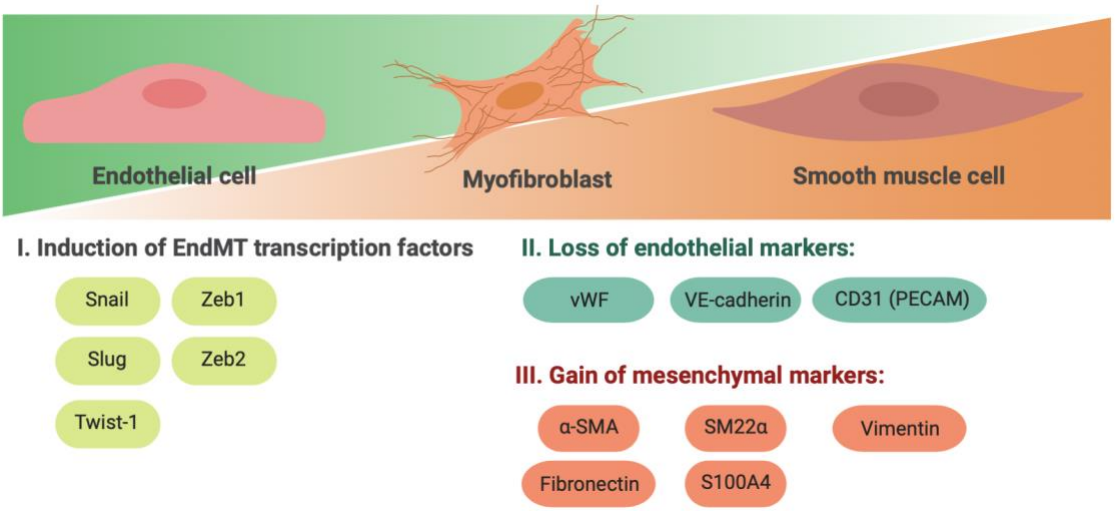


Figure 1. The sequence of events in the course of Endothelial-to-Mesenchymal transition (EndMT) progression.
Induction of EndMT-associated transcription factors Snail (Snai1), Slug (Snai2), Twist-1, Zeb1, and Zeb2 results in progressive loss of endothelial markers PECAM and VE-cadherin, and gain of mesenchymal markers vimentin, fibronectin, transgelin (SM22α) and alpha-smooth muscle actin (α-SMA).

1.3.2 EndMT in PH: groundwork discoveries and seminal studies

Despite an early discovery of EndMT in the late 1970s in the settings of embryonic heart development [268], the relevance of this process to adult physiology and pathophysiology in the vasculature was uncovered much later [266,269], and the notion of EndMT contributing to PAH development only began to emerge in the first decade of the 21st century [133,252,270–272] **(Figure 2)**.

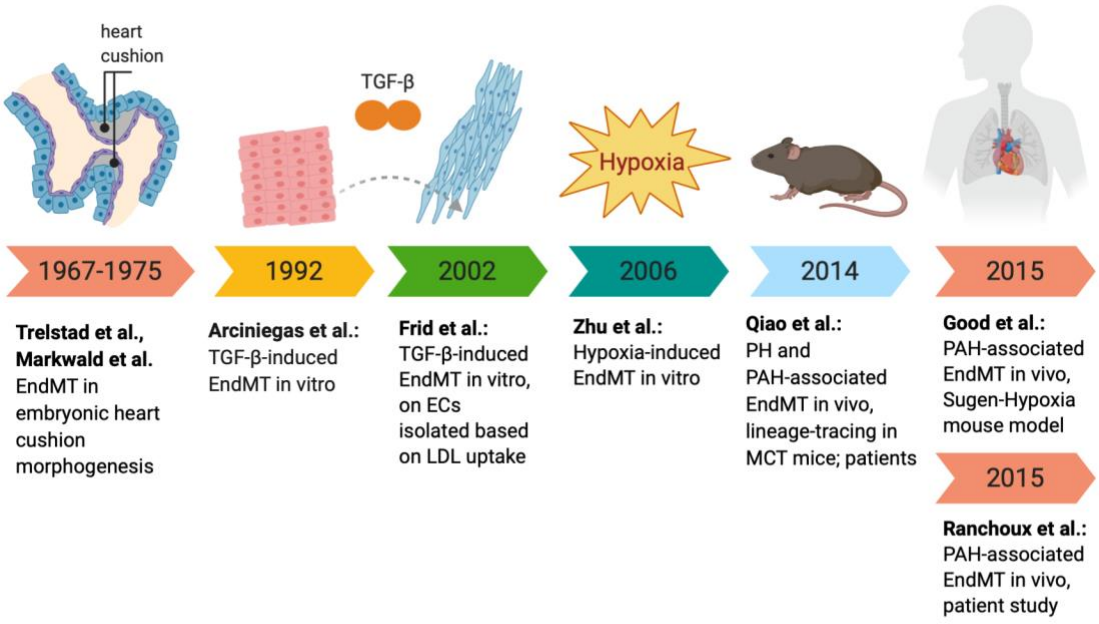


Figure 2. Timeline of seminal discoveries of EndMT with relevance to PH research.
Following the early studies of EndMT in cardiac development (Trelstad, Markwald) and isolated endothelial cells in vitro responding to TGF-β (Arciniegas, Frid) or hypoxia (Zhu) treatment, relevance of EndMT to PH was highlighted in the works by Qiao, Ranchoux and Good.

Identified as a critical process by which endocardial cells of embryonic heart atrioventricular cushions undergo structural and functional changes to give rise to cardiac valves and septa[241,268], EndMT was initially thought to occur exclusively during cardiac and pulmonary vascular [273] development. However, between the early 1990s and 2000s, EndMT was observed in isolated mature pulmonary and systemic ECs responding to the proinflammatory cytokine TGF-β1 [266,269]. Setting the stage for studying EndMT beyond embryogenesis and further in the context of cardiovascular disease, mature bovine aortic ECs were found to reversibly transform into mesenchymal-like cells in vitro, manifested in a gain of expression of α-SMA paired with a loss of vWF expression [266]. In a later study aiding to alleviate concerns of a potential contamination of primary ECs with mesenchymal cells upon isolation [274], ECs were selectively isolated from bovine

aortas and pulmonary arteries based on PECAM expression and low-density lipoproteins (LDL) uptake via fluorescent-activated cell sorting (FACS) [269]. Even in these extensively purified endothelial cultures, TGF-β1 challenge could drive EndMT, as assessed by the emergence of elongated mesenchymal-like cells among the compact cobblestone monolayer and a progressive loss of the cellular VE-cadherin and vWF, paired with a simultaneous gain of the smooth muscle α-SMA, SM22α, and calponin expression. Furthermore, the loss of LDL uptake capability, indicating a potential impairment of the circulating cholesterol clearance and a consequent predisposition to cardiovascular pathologies associated with high blood cholesterol levels, was correlated to a change in cell shape and a progressive gain of smooth muscle markers, indicating a connection between EndMT and a loss of healthy endothelial function. Despite the number of important insights gleaned from this study, it is important to recognize that the LDL uptake capability is not exclusive to endothelial cells, but also highly utilized by macrophages [275] and smooth muscle cells [276]. Therefore, effects of a non-endothelial cell contamination in these studies cannot be entirely ruled out.

Building on these findings, the first report attempting to link EndMT to PH-associated vascular remodeling identified an upregulation of myocardin, a transcription factor critical for the smooth muscle cell differentiation [270,277,278], as a common denominator between a hypoxia-induced EndMT in porcine PAECs in vitro, and a chronic hypoxia-induced vascular remodeling and increased α-SMA expression in rats in vivo [270]. However, the direct evidence of the connection between EndMT and PH-associated vascular remodeling did not come until nearly a decade later, perhaps spurred by the advances in genetic recombination-based techniques for cell lineage tracing in living animals [279].

Vascular remodeling and neointimal thickening of pulmonary vessels in PH are complex pathophysiological adaptations that involve vascular and non-vascular cell types. Proliferation and migration of adventitial fibroblasts and medial SMCs, dedifferentiation of SMCs from contractile to synthetic state [280], and a generalized proinflammatory and metabolically impaired state of the intimal ECs have all been identified in PH lung vasculature [30]. With the emergence of EndMT as an additional potential contributor, a mouse-adapted experimental MCT PH model was used to uncover the cellular origins of pathological neointimal remodeling in fate-mapping studies for cells of endothelial origins [271]. As mentioned previously, while the MCT administration is widely used in rats to induce severe pulmonary vascular remodeling, inflammation, and PH [164,281], mice are largely resistant to MCT, and do not develop severe PH symptoms even after the chronic MCT administration [169]. To adapt this model to mice and achieve severe PH and pulmonary vascular remodeling resulting in the neointima formation, the authors subjected EC-reporter mice generated by crossing the dual fluorescent mTomato/mGFP flox mice with the endothelial VE-cadherin Cre recombinase mice to left pneumonectomy, followed by an injection with the active metabolite MCTP [271]. Immunostaining for α-SMA showed colocalization with a small percentage of GFP-labeled cells of endothelial lineage, suggesting that cells of pulmonary vascular endothelial origin can undergo EndMT and potentially contribute to the neointima formation under stresses associated with PH in vivo [271]. The colocalization of endothelial antigens PECAM and vWF with α-SMA within neointimal cells was also reported in human PAH patients, highlighting the translational relevance of EndMT to human disease. This finding was later corroborated in another report focused on PAH patients, where immunofluorescent labeling of ECs within intimal and plexiform pulmonary vascular lesions showed colocalization of endothelial marker PECAM with a smooth muscle marker α-SMA [252]. In addition, in a BMPR2-deficient rat line that exhibits

hypertrophy of muscular pulmonary arteries characteristic of PH, EndMT was linked to the impairment in BMPR2 signaling [252], as evidenced by an upregulation of EndMT transcription factor Twist-1 and mesenchymal marker phospho-vimentin in the whole lung tissue of BMPR2-mutant rats [252].

Strengthening the experimental evidence for the role of EndMT in the animal models of PH, classic characteristics of EndMT were demonstrated in the Su-Hx mouse PH model [133]. Colocalization of vWF and α-SMA was seen in 6% of lung vessels from the Su-Hx mice compared to 1% in control animals [133], whereas, supportive of the relevance to human disease, the colocalization between vWF and α-SMA was seen in 4% of the pulmonary arterioles from patients with SSc-PAH and was absent in non-PAH controls [133]. These double-labeled cells likely depict the transitioning ECs that have not completed the transdifferentiation to SMCs, which may explain the low percentage, even in disease. Despite the small percentage of transitioning cells, this evidence clearly implicates EndMT as a process at play in PH progression.

Increased expression of endothelin-1 (ET-1) and subsequent activation of the endothelial nitric oxide synthase (eNOS) is linked to EndMT. ET-1 is a vasoreactive compound that regulates the production of the endothelial-derived vasodilator nitric oxide (NO, discussed in detail in **Section 1.3.5**) [282] by modulating the activity of eNOS [283]. However, ET-1 is also a potent vasoconstrictor [284], with the excessive production of ET-1 observed in pathological conditions, including PH [285]. Indeed, the ET-1 pathway is a target of the current PAH standard therapy [286]. Whereas the expression of VE-cadherin in the total lung homogenates of the MCT-treated rats was downregulated, the EndMT transcriptional regulators Snail and Slug, which act as the repressors of VE-cadherin transcription, were upregulated. These changes are associated with an increased expression of ET-1 and a phosphorylation of eNOS. Supportive of these in vivo associations,

cultured rat ECs treated with a synthetic ET-1 responded with an induction of Snail and a decrease in VE-cadherin expression, along with an upregulation of the mesenchymal markers vimentin and α-SMA. Notably, a cotreatment with the eNOS inhibitor N_{ω}-nitro-l-arginine methyl ester (L-NAME) blocked the pro-EndMT actions of ET-1, indicating that eNOS and ET-1 are mechanistically linked in driving EndMT under PH-related conditions.

At this juncture, it is important to note that quantifying EndMT in vivo by purely relying on counting the instances of perivascular endothelial and smooth muscle marker colocalization or measuring their total lung expression or expression of EndMT transcription factors, suffers from significant limitations. Firstly, expression of many classic endothelial and smooth muscle markers is not exclusive to endothelial or smooth muscle cells. For example, PECAM is present on the surface of immune cells, including T- and B- lymphocytes [287]; vWF is expressed by cancer cells of non-endothelial origins [288] as well as megakaryocytes [289]; and vimentin can also be found in the focal adhesions of endothelial cells in addition to its presence on mesenchymal cells [290]. Thus, the co-staining can sometimes be misleading, and relying on the total lung quantification of the endothelial and smooth muscle marker expression can present similar problems. Secondly, the EndMT transcription markers are also involved in the epithelial-to-mesenchymal transition, EMT, and, given the high prevalence of airway epithelial cells vs. vascular endothelial cells, quantifying the total lung expression of these markers in relation to PH needs to be interpreted with caution. Studies, therefore, need to follow the call made by prominent researchers in the field to standardize and unify the methods of EndMT assessment for both in vitro and in vivo studies [291], and employ multiple methods for the EndMT quantification in combination with robust mechanistic approaches on pure primary cell cultures in vitro.

EndMT is functionally linked to PH-associated endothelial dysfunction and reprogramming, such as increased migration [90,133,174,292], dysregulated proliferation [90,133,174], and impaired barrier function [133,293]. PAECs undergoing EndMT driven by a combination of tumor necrosis factor (TNFα), TGF-β, and IL-1β exhibit a significant increase in motility to a level approximating the rate of migration of human lung fibroblasts isolated from patients with scleroderma-associated PAH [133]. Consistent with these observations, increased migration of smooth muscle-like cells of endothelial origins is observed in response to hypoxia [90], in transitioning ECs driven by the silencing of bone-morphogenetic protein-7 [292], as well as in completely transitioned ECs isolated from lungs of mice exposed to Sugen-hypoxia [174]. Links between EndMT and cell proliferation, on the other hand, are not as obvious. While some studies link EndMT to increased cell proliferation [90,174], others report contrasting findings [133]. For example, arguing for the negative connection between EndMT and proliferation, unstimulated human PAECs exhibit a greater increase in cell number compared to PAECs treated with pro-EndMT cytokines [133]. However, PMVEC-derived smooth muscle-like cells display an EndMT-associated increase in cell proliferation compared to controls PMVECs [90], and transitioning ECs isolated from the lungs of the endothelial lineage-tracing Su-Hx mice proliferate faster than mesenchymal cells of non-endothelial origins [174]. Inconsistencies in proliferation studies' findings possibly highlight the complexities of a potential EndMT-proliferation pathways crosstalk, but also may have a technical explanation. For example, the absence of the unified methods for quantifying cellular proliferation poses a challenge to contrast observations made by different experimental groups utilizing different assays (e.g., cell count vs. cell metabolism).

In addition, the impairment of endothelial barrier function in response to pro-EndMT stimuli in cultured HPAECs connects EndMT to increased transendothelial leakage and the loss of the endothelial monolayer integrity [133,293].

Despite the limitations outlined above, many of the early studies of PAH-associated EndMT established a strong connection between the disease development, EndMT, and the processes contributing to the endothelial dysfunction in PAH. Because of the importance of endothelial injury as a priming event in PAH development [146], studies of EndMT in PAH are instrumental to our understanding of the disease pathobiology and invaluable for the future development of the therapeutic interventions. A substantial body of data collected over the past four decades supports the association between EndMT and PH in animal models [133,271,272] and in human patients with PAH [133,252]. Functionally, EndMT is linked to endothelial dysfunction, manifesting in changes in LDL uptake [269], migration [90,133,174,292], proliferation [90,133,174], and impaired barrier function [133,293]. To further qualify these relationships, it is crucial to recognize that, within the pulmonary vascular milieu, a number of organ- and systems-level pathophysiological responses are associated with the disease state and can contribute to the disease progression. These include hypoxia-induced vascular remodeling [294], a general proinflammatory state [295], impairment of the BMP pathways homeostasis [296], and an overall elevation in the oxidative stress and redox pathways [297]. Interestingly, there are evidence of links between EndMT and these disease-related states, and these associations are detailed in the sections to follow.

1.3.3 The Role of Hypoxia in PH-Associated EndMT

As discussed above, an external hypoxic environment can induce PH in rodents [143,144], as well as in humans [55]. However, a generalized hypoxic microenvironment often exists in forms of

the disease involving severe vasoocclusion and pulmonary remodeling (primarily Group III and Group IV PH) [4,55]. As such, the presence of hypoxia under any condition can be an important stimulus for potentiation of pulmonary vascular pathophysiology and has been linked to the induction of EndMT in pulmonary arteriolar [270], microvascular [90], and arterial [292] endothelial cells, and smooth muscle phenotype switching [298]. Importantly, several recent studies established a causal connection between the hypoxic exposure, induction of individual HIF isoforms, and EndMT [69,89,90,109,110,299] **(Figure 3)**.

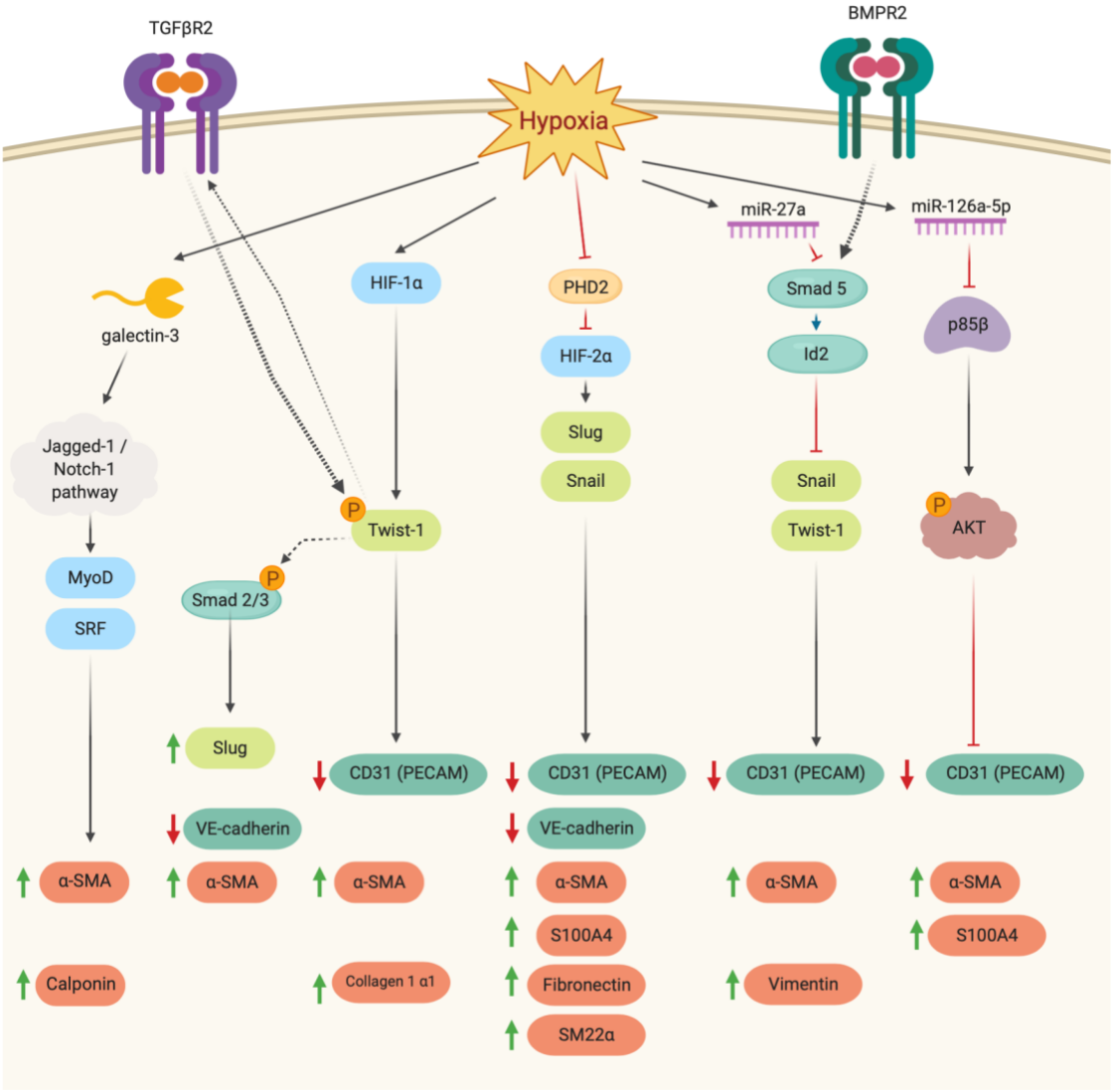


Figure 3. EndMT signaling cascades activated in response to hypoxia.
Hypoxia exposure stimulates a variety of signaling pathways converging on EndMT. TGFβR2 – transforming growth factor beta receptor 2, BMPR2 – bone morphogenetic protein receptor 2, miR – microRNA, PHD2 – hypoxia-inducible factor prolyl hydroxylase 2, HIF-1α –hypoxia-induced factor 1 alpha, HIF-2α – hypoxia-induced factor 2 alpha, α-SMA – alpha-smooth muscle actin, MyoD – myoblast determination protein 1, SRF – serum response factor. Light green depicts EndMT TFs, dark green endothelial markers, orange mesenchymal markers.

Illustrative of its role in the upregulation of the EndMT transcription factor Twist-1, HIF-1α can directly bind to the Twist-1 promoter in rat PMVECs, and the HIF-1α knockdown is able to partially reverse the hypoxia-induced loss of PECAM, and upregulation of α-SMA and collagen type 1 alpha 1 (Col1a1) expression in PMVECs [90]. Importantly, in PMVECs, whereas the HIF-1α knockdown reverses hypoxia-induced upregulation of Twist-1, the effects of HIF-1α knockdown are recapitulated by Twist-1 silencing, supportive of a PH-relevant mechanistic link between HIF-1α signaling and EndMT. Additionally, a high expression of HIF-2α isoform is observed in the pulmonary vascular endothelial cells (PVECs, a mixed population of PAECs and PMVECs) isolated from the lungs of patients with iPAH [69]. Indicative of the EndMT progression in iPAH PVECs compared to non-PAH control PVECs, mRNA expression profiles of genes differentially expressed during the transition show an increase in the EndMT transcription factors Snail and Slug, mesenchymal markers α-SMA, S100A4, and fibronectin, as well as a decrease in the endothelial markers PECAM and VE-cadherin. Interestingly, arguing for the mechanistic link between HIF-2α and EndMT, the silencing of HIF-2α, but not HIF-1α, in isolated iPAH PVECs decreases Snail and Slug expression. Moreover, the endothelial-specific deletion of HIF-2α prevents hypoxia-induced PH in mice, a response that was not observed in endothelial-specific HIF-1α deletion mice. It still remains to be determined whether the HIF-2α deletion is sufficient to rescue hypoxia-induced EndMT in vivo and whether the reported contradictions on the role of endothelial HIF-1α in PH-associated EndMT are indicative of complex regulatory mechanisms (with HIF-1α control over Twist-1 and HIF-2α control over Snail and Slug) or reflect the technical, or species-related differences between the studies.

Even though Twist-1 upregulation was implicated in EndMT [90,300] and detected in PH and PAH lung samples [91,133,252,271], the mechanism of Twist-1 control over EndMT progression in PH

remain largely unknown. A recent study shows that hypoxia-induced upregulation of Twist-1 and Twist-1 phosphorylation on Ser42 residue increases expression of TGFβR2 and potentiated its signaling [89]. In vitro, the overexpression of the full-length Twist-1 in human PAECs recapitulates the effects of hypoxia, leading to an increase in Slug expression, a loss of VE-cadherin, and a gain of α-SMA, along with an increased phosphorylation (indicative of activation) of the molecular transducers of TGFβR signaling Smads 2/3. Importantly, the overexpression of a dominant-negative mutant of Twist-1 (Ser-42-Ala) is unable to induce similar changes, and prevents hypoxia-induced downregulation of VE-cadherin and upregulation of α-SMA, compared to cells transfected with a full-length Twist-1. Highlighting the role of Twist-1 in hypoxia-induced PH in vivo, the endothelial-specific conditional knockout of Twist-1 partially alleviates the vascular remodeling and the hemodynamic manifestations of the disease in mice subjected to chronic hypoxia. Consequently, by mechanistically connecting Twist-1 signaling to the TGFβR2 pathway, these observations point to an important connection between hypoxia, EndMT, and the critical pathways in PH pathobiology (extensively discussed in **Section 1.3.4**).

Several reports suggest that, in addition to modulating the EndMT-related transcription factors, hypoxia may control EndMT progression via micro RNAs (miRNAs) [109,110]. miRNAs, the highly conserved non-coding short RNA fragments, can regulate gene expression at the posttranscriptional level by binding to nucleotide sequences on the 3'-untranslated regions (3'-UTRs) of the target gene mRNA transcripts [301]. Recently, miR-27a was found to contribute to the hypoxic PH-associated EndMT in the pulmonary vasculature through the transcriptional effector Inhibitor of DNA Binding 2 (Id2) [109]. In addition to its upregulation in pulmonary arteries of chronic hypoxia exposed rats, miR-27a is upregulated in human PAECs subjected to hypoxia in vitro. Interestingly, the treatment with the miR-27a inhibitor rescues the hypoxia-induced EndMT

in culture, and, conversely, the miR-27a mimic induces EndMT in normoxia, evidenced by changes in the expression of PECAM, α-SMA, and vimentin. In addition, miR-27a suppresses Smad5, a mediator of BMP signaling, leading the study authors to suggest that the miR-27a-induced downregulation of Smad5 can potentially downregulate Id2, a suppressor of Snail and Twist-1, and thereby induce EndMT. Additionally, an unbiased microarray screen identified the upregulation of the miR-126a-5p in the lungs of PH neonatal rats and in plasma of human PH neonates (persistent PH of the newborn, Group I PH) compared to healthy neonates [110]. The hypoxia-induced upregulation of miR-126a-5p in newborn rats in vivo and rat PMVECs in vitro contributes to EndMT likely through an inhibition of p85-β / Akt pathway, a signaling cascade previously demonstrated to inhibit EndMT in the endothelial progenitors cells [302].

Finally, the most recent evidence on hypoxia control of EndMT implicates yet another set of processes involving β-galactoside-binding lectins (galectins), a family of secretory proteins with intracellular and extracellular functions [299]. Galectins have been increasingly linked to chronic and acute inflammatory responses in cardiovascular pathologies [303], and used clinically as serological prognostic markers in patients with chronic heart failure [304]. A member of this family, galectin-3, mediates the PDGF signaling in PAH [305] and promotes the pulmonary vascular remodeling in PH [306]. Moreover, the global galectin-3 gene deletion is protective against hypoxia-induced PH in mice [307]. Hypoxia-induced upregulation of galectin-3 contributes to the gain of the α-SMA expression by pulmonary endothelial cells via the Jagged-1 / Notch-1 pathway [299], an evolutionarily conserved pathway critical for the embryonic development and previously implicated in EMT [308]. Galectin-3 expression is increased in the intima and adventitia of the chronic hypoxia-exposed rats in vivo, and the treatment of PAECs with the recombinant galectin-3 contributes to the gain of α-SMA expression. This effect is abolished in cells preincubated with the Notch signaling pathway

inhibitor 3,5-difluorophenylacety-lL-alanyl-L-2-phenylglycinetert-butyl ester (DAPT), and the hypoxia-induced upregulation of α-SMA in PAECs is reversed upon the galectin-3 gene silencing in vitro.

Collectively, the studies described above shed mechanistic insight onto the hypoxia-mediated control of EndMT in the context of PH, highlighting the central role of HIF signaling with implications for the potential involvement of miRNAs and galectins. EndMT transcription factors Twist-1 [89,90], Snail [69,109], and Slug [69,89] appear to be the downstream targets. However, the precise underlying molecular pathways controlling the hypoxia-induced EndMT in the context of PH-associated endothelial pathologies remain to be delineated. In addition to galectin-3, a growing body of work implicates galectin-8 in the vascular angiogenesis [309], and cell adhesion and growth [310], warranting its further study in the context of PH pathologies and EndMT. In addition, while the reports causally linking miRNAs to PAH and PH-associated EndMT are still scarce [109,110], they persuasively highlight the importance of this relationship and warrant further exploration.

1.3.4 The Impact of Inflammation and Dysregulated TGF-β-BMP Signaling on PH-associated EndMT

The first reports hinting at the role of inflammation in PAH described an infiltration of T- and B- cells and macrophages to the plexiform lesions [34], and a dramatic increase in the concentration of the inflammatory cytokines IL-1β and IL-6 in the serum of patients with severe PAH [311]. Currently, the evidence for the inflammatory disease component is well-established in human PAH [295] as well as the various animal models of PH, including MCT [312], Su-Hx [313], chronic hypoxia [314], and Schistosomiasis-associated PH [315].

Endothelial cells can be critically affected by the proinflammatory environment, and the impact of inflammation on the endothelial function is extensively described [316,317]. Inflammatory cytokines, such as the IL-1β, TNFα and TGF-β1, activate widespread signaling networks in endothelial cells, increasing the endothelial monolayer permeability [318] and upregulating the expression of the adhesion molecules aiding immune cell recruitment and extravasation [319,320]. Of note, a large body of data collectively reveals the connection between inflammation, EndMT and endothelial dysfunction in the systemic circulation [244,265]. Inhibition of the inflammatory NF-κB signaling pathway in a MCT mouse model of PH can alleviate disease manifestations, indirectly suggesting the presence of a link between inflammation, PH pathology, and EndMT reversal [321]. Compared to wild-type mice, mice constitutively expressing the inactive mutated IκBα driven by the Club (Clara) cell-10 (CC-10) promoter are protected from the MCT-induced rise in RV pressure, the upregulation of the total lung expression of α-SMA, and the downregulation of CD31 and VE-cadherin. Despite being instrumental for drawing parallels between the inflammatory signaling and EndMT in PH, the abovementioned study is mostly associative and subject to other interpretations, and suffers from significant limitations in its choice of the animal model, including significant side effects of MCT administration in the form of myocarditis and liver and kidney damage [165,166] (See **Section 1.1.5**).

As mentioned in the **Section 1.1.2**, dysregulated BMPR2 signaling, which is strongly linked to inflammation, has strong connections to PH development. The family of TGFβ-BMP receptors is comprised of the two subtypes: type 1, which includes Activin receptor-like kinases (ALK) 1/2 and 3/6, and type 2, which includes BMPR2, Activin A Receptor Type 2A (ACVR2A), and Activin A Receptor Type 2B (ACVR2B) [322]. The BMPR ligands, BMPs, activate the downstream mediators SMADs 1/5/8, while the TGF-β primarily signals through the recruitment

of SMADs 2 and 3 [323]. The cross-talk on the level of common signaling mediators, including SMADs and others, adds extra layers of complexity and interdependency between the BMP and TGF-β signaling cascades [324], which can play a role in a differential regulation of various cellular responses, including EndMT, and can have relevance to the vascular remodeling in PH.

The dysregulation of the BMPR signaling has been long associated with a predisposition to PAH [325,326], and the expression of the BMP signaling antagonists, such as gremlin-1, was found to be upregulated in PAH patients and animals models of PH [327–329]. Additional in-depth insights from the animal studies demonstrated that the loss of BMPR2 in the endothelium, as well as the overexpression of the mutated BMPR2 in vascular smooth muscle cells, predispose mice to a spontaneous development of PAH [330,331]. Moreover, the BMPR2 deficiency augments the inflammatory cell infiltration to the pulmonary vasculature [148], corroborating reports of the anti-inflammatory effects of the BMPR2 signaling in the pulmonary endothelium, as opposed to in the systemic endothelial cells [332]. The reduced BMPR2 signaling facilitates the pro-inflammatory state of PAECs by inducing the expression of the pro-inflammatory cytokines IL-6 and IL-8 [333], further connecting the BMP signaling deficiency to the development of the inflammatory environment in PAH. Collectively, and considering the evidence for the links between inflammation and EndMT in multiple pathologies including PH, these observations support the notion of the existence of a link between the PH-associated inflammation, dysregulation of BMPR2 signaling, and EndMT.

Some of the early reports on the link between BMPR2 signaling restoration in pulmonary endothelial cells and improvement of PH-associated hemodynamic impairment directly [292] and indirectly [131] also implicate inhibition of EndMT as a potential mechanism. Targeted delivery of the *BMPR2* gene to the pulmonary endothelium using vector-harboring adenovirus conjugated to an antibody against angiotensin-converting enzyme (ACE; a membrane-bound protease highly

expressed on pulmonary endothelial cells) restored the BMPR2 signaling and reversed an elevation in RVSP, PVR, and RV hypertrophy and restored cardiac output in MCT-treated rats. Moreover, *BMPR2* gene delivery reversed the MCT-induced TGF-β1 expression in the lungs. Supportive of a causal role for impaired BMPR2 signaling in promoting PH-associated EndMT, the characteristic change in cell shape of human PMVECs from cobblestone to spindle-like, along with the upregulation of the mesenchymal markers fibronectin and S100A in response to TGF-β1 were partially reversed in cells treated with the recombinant human BMP-2 or BMP-7.

These findings were corroborated by a more recent report [292]. The expression and endothelial staining of BMP-7 is decreased in the lungs of rats subjected to chronic hypoxia and in PAECs exposed to hypoxia [292]. Treatment with recombinant BMP-7 (rhBMP-7) improved the hemodynamic parameters in experimental animals, and partially reduced the colocalization of endothelial VE-cadherin and mesenchymal vimentin in the lining of small pulmonary vessels, suggestive of EndMT reversal. The hypoxia-induced upregulation of EndMT-associated transcription factors Snail, Slug, and Twist-1, and downregulation of α-SMA, vimentin, and fibronectin are reversed in total lung tissue of animals treated with rhBMP-7. Echoing and extending the earlier findings [131], another study showed that rhBMP-7 treatment reduced an upregulation of EndMT transcription factors and mesenchymal markers, and colocalization of α-SMA with VE-cadherin following hypoxia exposure in vivo. The effects of BMP7 appear be mediated through a mechanism that involves the mammalian target of rapamycin (mTOR) pathway via mTORC1 signaling. Functionally, addition of the exogenous BMP-7 abolishes the hypoxia-induced migration, a phenotype associated with EndMT [90,133,174], while BMP-7 siRNA by itself is sufficient to increase the PAECs migration.

Connection between TGF-β1, PDGF and membrane-bound zinc metallopeptidase neprilysin (also named neutral endopeptidase, NEP) is also relevant to PH-associated EndMT [267] (**Figure 4**). NEP is a negative regulator of the hypoxia-induced pulmonary vascular remodeling [152] with a known connection to the PDGF signaling [334]. In hypoxia-treated rats, while the total lung expression of NEP is downregulated, PDGF and PDGFR are upregulated, and those animals exhibit signs of EndMT in the PA endothelium manifested in the upregulation of mesenchymal markers fibronectin and vimentin. In vitro, the treatment of calf PAECs with recombinant PDGF-AA and PDGF-BB dimers induces EndMT, promoting the loss of the endothelial VE-cadherin and the gain of mesenchymal markers fibronectin, vimentin and α-SMA. At the same time, the siRNA-driven PDGF-B silencing prevents hypoxia-induced EndMT. Indicative of a mechanistic connection between NEP and EndMT, the transfection of PAECs with NEP siRNA downregulates VE-cadherin and upregulates fibronectin and vimentin, whereas an addition of the exogenous recombinant NEP reverses the hypoxia-induced EndMT. Interestingly, the levels of TGF-β1 and PDGF-B are increased by siNEP, while the hypoxia-induced upregulation of PDGF-B and TGF-β1 are ablated by exogenous NEP, thus tying together the connection between the TGFβ and BMPR pathways in controlling PH-mediated EndMT.

Molecular links between the loss of BMPR2 in endothelial cells and an increase in EndMT in PH reveal a role for the chromatin architecture factor High Mobility Group AT-hook 1 (HMGA1) [91] as outlined in **Figure 4**. HMGA1 is a protein that can bind to the AT-rich DNA motifs and alter the chromatin structure, thereby influencing the gene transcription [335]. While the expression of HMGA1 is nearly undetectable in mature tissues, it is upregulated in a subset of cancers [336] and has been linked to EMT [337]. Intriguingly, HMGA1 expression is increased in the isolated pulmonary endothelial cells and neointima of the pulmonary vessels from PAH patients

91. HMGA1-positive cells within the neointima co-express the endothelial marker vWF and the smooth muscle marker SM22α, suggesting an association between HMGA1 and EndMT. Mechanistic studies in cell culture demonstrate that the BMPR2 knockdown upregulate the HMGA1 expression and induce EndMT, where the EndMT-associated transcription factors Snail and Slug and the smooth muscle markers α-SMA and phospho-vimentin are upregulated, and the endothelial marker PECAM is downregulated. The HMGA1 gene silencing alone and in combination with the silencing of BMPR2 gene demonstrates that the upregulation of Slug and α-SMA driven by BMPR2 silencing is abrogated in HMGA1 siRNA-transfected PAECs, thus suggesting that HMGA1 acts as an effector of the dysregulated BMPR2 signaling.

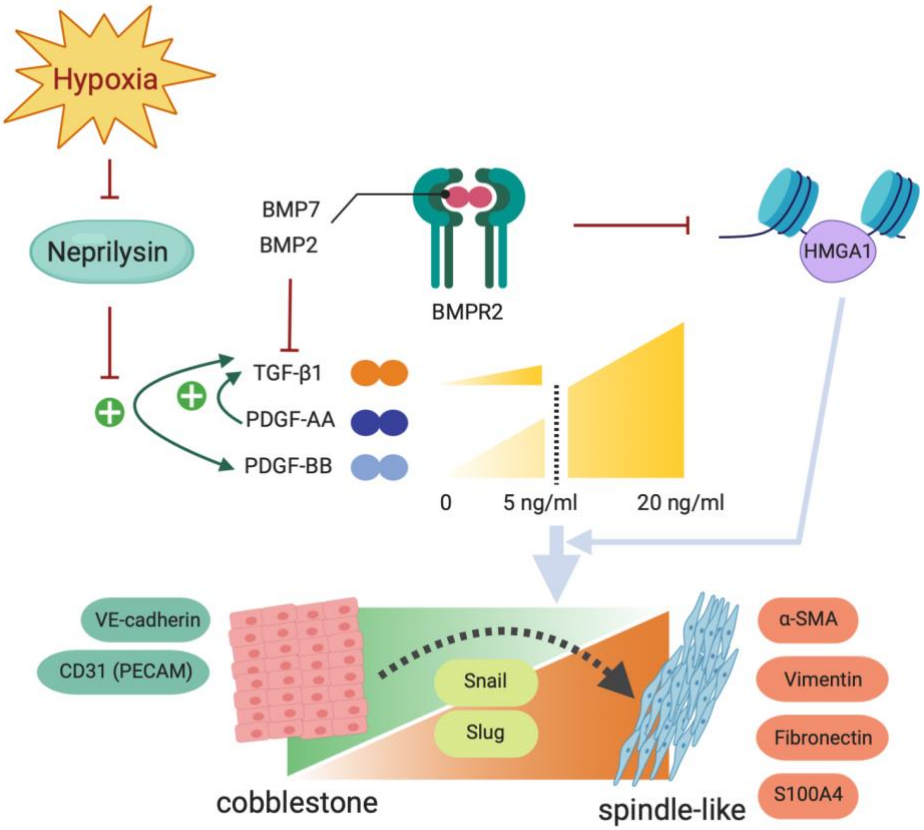


Figure 4. Cross-talk between BMP / TGF-β and PDGF signaling in EndMT progression.
Transforming growth factor-beta 1 (TGF-β1), platelet-derived growth factor-AA (PDGF-AA) and platelet-derived growth factor-BB (PDGF-BB) induced EndMT in a dose-dependent manner. Transitioning cells exhibited the loss of VE-cadherin and PECAM, the induction of EndMT transcription factors Snail and Slug, and the gain of alpha smooth muscle actin (α-SMA), vimentin, fibronectin and S100A4 expression, along with a shift of cell morphology from cobblestone to spindle-like. Bone morphogenetic proteins BMP2 and BMP7 inhibit the pro-EndMT effects of TGF-β1, and dysregulated bone morphogenetic protein receptor 2 (BMPR2) releases inhibitory effects on the high mobility group AT-hook 1 (HMGA1), potentiating EndMT. The "+" signs indicate the potentiating effect of PDGF-AA and PDGF-BB on TGF-β1 and vice versa.

Building on the earlier work indirectly pointing towards a link between inflammation, PH, and EndMT [321] and providing a further mechanistic insight into the role of the BMPR2 dysregulation for PH-associated EndMT, the PH-associated activation of the pro-inflammatory NF-κB signaling was connected to the upregulation of miR-130a, the loss of BMPR2 and the TGF-β1-induced EndMT [338]. In vitro, in addition to a decrease of BMPR2 expression and the miR-130a

upregulation, which is predicted to directly bind to the 3'-UTR of BMPR2 mRNA, the treatment of the isolated pulmonary microvascular ECs with TGF-β1 induces the gain of α-SMA and the loss of PECAM expression, suggestive of the EndMT progression. Moreover, the changes in the endothelial and mesenchymal gene expression can be recapitulated by the BMPR2 siRNA transfection, and the treatment with the miR-130a inhibitor restores the TGF-β1-induced downregulation of BMPR2 and protects the ECs from the EndMT-associated loss of PECAM and gain of α-SMA.

Together, the studies highlighted in this section emphasize the complex interconnections between hypoxia, dysregulated BMPR2 signaling, inflammation, and EndMT in PAH. Presenting compelling data arguing for the utilization of BMP signaling restoration strategies targeting EndMT in the treatment of PH [131,292], and offering the mechanistic insights into the BMPR-mediated control over EndMT progression [91,267,292,338], these reports make a persuasive argument for the development of the therapeutics aimed at the reversal of the PAH-associated EndMT and pave a way for the future explorations.

1.3.5 The Impact of Oxidative Stress on PH-associated EndMT

In addition to the hypoxic and proinflammatory environments discussed above, the pulmonary vasculature undergoing PH-associated remodeling also experiences an upregulation of the reactive oxygen and nitrogen species and an increased susceptibility to the oxidative stress. Oxidative stress is implicated in PH either as a consequence of the excessive production of the reactive oxygen species (ROS) and/or reactive nitrogen species (RNS), imbalance in the antioxidant capacity [297,339], or both. Some of the major enzymes involved in the ROS and RNS production in physiological and pathophysiological conditions include the iron and iron-

derivatives (such as heme or iron-sulfur clusters), NADPH oxidases, mitochondrial electron transport chain complexes I, II and III, NO synthases (uncoupled) and xanthine oxidase [340]. Antioxidant systems include catalase, superoxide dismutase, thioredoxin system, glutathione peroxidases and peroxiredoxins, as well as the antioxidant molecules such as Vitamin C and E [340]. The parent ROS compound, superoxide anion radical (O_2-•) and its dismutation product hydrogen peroxide (H_2O_2) increase in various vascular cells of the pulmonary circulation and the right ventricle under PH conditions and related stimuli, and contribute to the observed pathophysiology [329,341]. Similarly, the products of NO oxidation, such as the RNS peroxynitrite (ONOO-) have also been implicated in PH [339,342], whereas the levels of the vasodilatory NO and its reaction products NO_2-, NO_3-, and S-nitrosothiols, are decreased in the lungs of PAH patients [343].

While the oxidative stress remains one of the hallmark events in the PH vasculature [344] and the upregulation of ROS has been causally connected to the PAH development [345], the relevance of the oxidative stress to PH-associated EndMT has not been sufficiently explored until recently. Even though the endothelial dysfunction has long been connected to an excessive ROS production [346] and ROS were known to induce EMT via the stimulation of the TGF-β1 signaling [347], the mechanistic insights into the role of the oxidative stress in mediating EndMT were first explored only in the mid-2010s [348]. Exposure of endothelial cells to the exogenous H_2O_2 induces EndMT in a mechanism dependent on the synthesis and secretion of TGF-β1 and -2, as well as the activation of a TGF-β receptor subunit ALK5 and its downstream signal transduction modulator Smad3. In addition to inducing the expression of TGF-β1 and -2, the potent activators of EMT in keratinocytes and renal tubular epithelial cells [347,349] as well as of EndMT as discussed above [249,256,266,267,269,338], H_2O_2 concentrations as low as 0.1 μM and as high as 10 μM induce the loss of the endothelial markers VE-cadherin and PECAM, the gain in expression of the mesenchymal

markers α-SMA and S100 Calcium Binding Protein A4 fibroblast-specific protein 1 (S100A4, also known as Fibroblast-Specific Porein-1, FSP1), the loss of cell-cell contacts and a change in cellular morphology from cobblestone-like to the elongated spindle-like shape characteristic of transitioning ECs. This indicative shift from the endothelial to the mesenchymal-like cellular phenotype is associated with the H_2O_2-depedent increase in the biosynthesis of collagen III and fibronectin, two extracellular matrix proteins associated with fibrotic responses.

Additional evidence for this link identifies a role of tetrahydrobiopterin (BH4), an indispensable cofactor for NO production by nitric oxide synthases, in the pulmonary vascular remodeling associated with idiopathic pulmonary fibrosis (IPF) [350]. BH4 and sepiapterin reductase, an enzyme that transforms the exogenous sepiapterin into BH4 in an alternative "salvage pathway", have the protective antioxidant and antifibrotic properties in the myocardial infarction and PH [351,352], and are critical for the maintenance of the healthy vascular homeostasis [353,354]. IPF is a disease of the lung parenchyma characterized by an excessive scarring and myofibroblast activation which can often be complicated by the development of the associated pulmonary hypertension (Group III PH) [355]. The rescue of the BH4 levels by stimulation of its de novo biosynthesis using sepiapterin administration reduces the pathological vascular remodeling in the bleomycin animal model of IPF [356]. Sepiapterin also alleviates the bleomycin-induced upregulation of iNOS and rescues expression of eNOS, as well as reduces the number of α-SMA and VE-cadherin co-expressing cells in the pulmonary arteries [356]. In vitro, the treatment of HPAECs with the exogenous sepiapterin reverses TGF-β1- and ET-1-induced an upregulation of Snail and Slug, and of α-SMA and SM22α, and a downregulation of VE-cadherin and VEGFR. Importantly, these sepiapterin effects are attributed to the downstream downregulation of ROS and phosphorylation of Smad3. These results strengthen the evidence of the role of BH4 in alleviating the bleomycin-

induced EndMT, and aligns with the previous findings on the impact of the chronic inhibition of eNOS and neuronal nNOS on the induction of EndMT in the kidney [357]. On the other hand, and in contrast with a decrease in eNOS expression in the IPF-associated PH observed in this study [356], other studies reported increases in the levels of eNOS expression and phosphorylation in the plexiform lesions of PAH patients, and associated the dysfunctional eNOS activity with the enzyme uncoupling and increased ROS production [358,359]. Indeed, in a report discussed earlier [272], the study authors observed an increase in the eNOS phosphorylation as causal of EndMT. Moreover, the increased eNOS activity in Cav-1 deficient mice induces PH through the increased nitration of cGMP-dependent protein kinase or protein kinase G (PKG) [360]. These discrepancies may be related to the degree of disease severity, the PH subtypes (i.e. Group 1 vs. Group 3), and/or the upstream signal activation cascade controlling the observed eNOS pathophysiology (e.g. uncoupling vs. increased expression and/or phosphorylation). Despite these discrepancies, the convergence on ROS appears to play a central part, and further studies of the therapeutic potential of modulating eNOS or iNOS expression and restoration of the enzyme uncoupling, especially in its relation to EndMT and pulmonary vascular remodeling, are warranted.

Highlighting a potentially protective role for ROS as signaling mediators, the oxidation of a protein kinase G Iα (PKGIα) was identified as an adaptive mechanism limiting PH, likely acting through a disruption of EndMT [361]. PKGIα, an isoform of PKGI mainly expressed in the heart and lungs, is a redox-sensitive Serine/Threonine kinase whose oxidation leads to a formation of an active disulfide homodimer associated with the endothelium-derived hyperpolarizing factor (EDHF)-dependent vasodilation [362]. Previous studies have demonstrated an upregulation of the pulmonary PKGI expression following the chronic hypoxia exposure and the hypoxia-associated increased ROS production [363], and reported that the PKGI knockout mice develop spontaneous

PAH in normoxia [364]. However, the functional significance of the posttranslational modifications of PKGI in PAH remained largely unexplored, and its connection to the EndMT signaling was unknown. Transcriptomic analysis of the whole lung tissue of the Cys42Ser PKGIα knock-in (KI) mice resistant to PKGIα oxidation demonstrated an upregulation in the expression of the EndMT-related genes compared to the wild-type (WT) mice after three days of hypoxia exposure (i.e. prior to the elevation of the RVSP or the structural remodeling in the pulmonary vasculature of WTs, but at the beginning of the remodeling in KIs). Hypoxic KI mice also develop increased vascular muscularization in the lung, as well as the higher number of cells co-expressing PECAM and α-SMA compared to WTs. Consistent with the EndMT paradigm, the expression of Twist-1 is upregulated in the whole lung tissue, along with increased α-SMA, desmin and phospho-vimentin. In must be noted, however, that gleaning insight into EndMT using the whole lung tissue expression rather than the specific pulmonary vascular endothelial expression suffers from significant limitations and needs to be interpreted with caution, as outlined earlier in the **Section 1.3.2**. Therefore, building on the current findings, additional mechanistic studies implementing the robust experimental techniques are needed to disentangle the role of oxidative stress and PKGIα oxidation in the development of PAH-associated EndMT in vitro and in vivo.

As can be collectively gleaned from this literature, over the last five years, several reports implicated a direct link between the oxidative stress, TGF-β, and EndMT signaling in PH-relevant context. Following the proof-of-concept in vitro study [348], the roles of the specific ROS- and RNS-producing enzymes and cofactors, oxidation-sensitive signaling mediators, and their connection to PH-associated EndMT were later explored in the translational animal studies [356,361]. However, despite these advances, additional work is needed to determine the mechanisms by which redox

signaling mediators may control EndMT in PH, and identify the links to PH-associated hypoxia, inflammation and dysregulated BMPR signaling.

1.3.6 Emerging Therapeutic Strategies Targeting EndMT in the Treatment of PAH

Given the urgent need for the development of novel therapeutic approaches for PH, the accumulating evidence for the role of EndMT in PH-associated pulmonary vascular remodeling positions the strategies targeting the EndMT reversal as a viable option for the disease management. While the evidence for this remains sparse, a few recent in vivo studies demonstrate the potential of the drug-targeting of EndMT to offer protection against PH. The strategies to target EndMT include, for example, the usage of the mesenchymal stem cells [365], inhibitors of dipeptidyl peptidase-4 [366], and the drugs targeting the adhesion molecule CD44 [367]. Related to the former, the latest developments of the cell-based regenerative therapies galvanized the research into the utility of stem cells as a treatment strategy for PH [368]. Promising early evidence of the efficacy of mesenchymal stem cells (MSCs) for PH reversal shows that the intravenous injection of MSCs can reduce the RV systolic pressure, RV hypertrophy, and pulmonary vascular muscularization, attenuate increases in the collagen deposition, and normalize the MMP2 and MMP9 tissue expression in Su-Hx rats [365]. Pointing to the potential effect of the MSC therapy on the PH-associated EndMT reversal, increased colocalization of α-SMA and vWF in cells within the pulmonary vascular wall of Su-Hx rats was attenuated in the MSC-injected animals. These findings are supported by the in vitro studies indicating that the hypoxia-induced accumulation of HIF-2α, whose association to EndMT was discussed in the **Section 1.3.3**, and EndMT progression in PMECs are reversed in PMECs exposed to the MSCs-conditioned cell culture medium. However, despite the number of promising findings, the abovementioned study assesses the effects

of MSC therapy on EndMT as an association, rather than a causative mechanistic connection, and suffers from the limitations in the experimental design, lacking the appropriate non-MSC-injected control animals.

The inhibition of the dipeptidyl peptidase-4 (DPP-4, CD26), a serine protease that regulates the activity of secreted polypeptides such as chemokines, cytokines and vasoactive peptides, is another potential strategy for the PH treatment via EndMT inhibition [366]. DPP-4 is widely expressed on the plasma membrane of a variety of cell types including endothelial cells of pulmonary capillaries [369]. At present, the DPP-4 inhibitors are used in clinic for the management of the type 2 diabetes mellitus, as they lower the plasma glucose levels by inhibiting the breakdown of the incretin hormone glucagon-like peptide-1 (GLP-1) [370]. Interestingly, in addition to their antihyperglycemic activity, the DPP-4 inhibitors confer protective effects on the cardiovascular system [371,372] and, importantly, reverse the pulmonary EndMT following endotoxin-induced acute lung injury [373]. To examine the role of DPP-4 in PH and pulmonary vascular remodeling, the DPP-4 inhibitor, sitagliptin, was utilized in the rat monocrotaline model of PH [366]. MCT treatment increases the serum levels of DPP-4, whereas, alluding to the association between DPP-4 and EndMT [373], the number of α-SMA/PECAM double-positive cells in pulmonary artery sections of the MCT rats is reduced with sitagliptin. In addition, the total lung protein expression of α-SMA, vimentin and fibronectin are reduced, while the expression of VE-cadherin and vWF are restored to normal with sitagliptin treatment in MCT rats.

A follow-up study providing the additional mechanistic insights into the protective effects of DPP-4 inhibition on EndMT in PH demonstrates that the DPP-4 inhibition using the orally administered drug sitagliptin restores the glucagon-like peptide 1 (GLP-1) signaling in the lung [374]. Administration of the GLP-1 analog, liraglutide, also decreases the number of α-SMA /

PECAM double-positive cells in the pulmonary vasculature of MCT rats. In addition, liraglutide partially reverses the MCT-induced downregulation of the total lung mRNA expression of VE-cadherin and vWF, and the upregulation of vimentin and fibronectin. Beyond the associative in vivo observations, liraglutide treatment dose-dependently rescues the TGF-β-IL-1β-induced loss of VE-cadherin and gain of α-SMA and vimentin expression in cultured systemic ECs. This observation supports the hypothesis that the therapeutic action of GLP-1 on PH reversal is at least partially due to its role as an EndMT inhibitor, especially since the therapeutic effect of liraglutide on EndMT phenotype is blocked in cells treated with the GLP-1R antagonist.

Lastly, a recently published study exploring the parallels between the PH pathophysiology and cancer connected the upregulation of the cancer-associated isoform of adhesion molecule variant CD44v8-10 to EndMT and PH [375]. Similar to cancer cells, endothelial cells in remodeled pulmonary vessels exhibit the increased proliferation and resistance to apoptosis [376], as well as a metabolic shift from the oxidative phosphorylation to glycolysis [377]. Beyond its functionality in normal physiological conditions, CD44 was shown to promote cancer metastasis [378], and increased expression of v8-10 isoform of CD44 protein, which results from the alternative splicing of the CD44 gene, is present on the surface of cancer stem cells [379]. Interestingly, expression CD44v8-10 is detected in cells undergoing EndMT in the neointimal lesions in the lung vasculature of patients with PAH as well as in lungs of mice subjected to the Su-Hx treatment [367]. Supporting the involvement of CD44 in EndMT, the stimulation of EndMT in cultured HPAECs with a combination of TGF-β, TNFα and IL-1β results in an increased expression of the preprocessed CD44 and its isoform CD44v8-10, along with an induction of the EndMT transcriptional factors Snail and Slug, the loss of endothelial PECAM, and the gain of mesenchymal markers α-SMA and SM22α, and matrix metalloproteinases (MMPs) 2 and 9, which promote the basal lamina

degradation and thus potentiate EndMT [380]. In addition, a small subset of transitioned CD44v8-10-positive HPAECs exhibit a high expression of the cysteine-glutamate antiporter subunit xCT, which, in cancer cells, serves as a protective mechanism against oxidative stress by modulating the levels of the intracellular antioxidant GSH. Additionally, the treatment of Su-Hx mice with the xCT inhibitor sulfasalazine, an FDA-approved drug used for the management of rheumatoid arthritis, not only reduces the expression of CD44v8-10 in the lungs of PH mice and decreases the percentage of vessels containing the α-SMA and vWF co-expressing cells, but also alleviates the extent of RV hypertrophy and reduces RVSP. Therefore, owing to the evidence of the involvement of CD44v8-10 in PH-associated EndMT, CD44 presents as a promising therapeutic target for the reversal of PH with a realistic timeline of development.

New advances in our understanding of the role of EndMT in PH and PAH pathobiology offer rich material for targeting this process in the development of early disease interventions. In addition to the inhibition of DPP-4 [366] and CD44 [367], and utilizing the EndMT-targeting MSC therapies [365] described in this section, targeting other mediators of PH-associated EndMT detailed above, such as microRNAs [109,110,338], galectin-3 [299] and HMGA1 [91], as well as aiding the restoration of the BMPR signaling [131,292] and the redox balance [356] in the pulmonary vasculature, present new promising avenues to reverse EndMT and alleviate PH-induced vascular remodeling. Indeed, accumulating evidence for EndMT in PH and increasing understanding of its underlying mechanisms in disease pathophysiology make it an appealing target in the development of future treatments. However, the dearth of information on its upstream regulators and an incomplete knowledge on its precise roles in PH development and progression necessitate further studies and call for a deeper investigation into the novel upstream players. For example, scaffolding proteins, research on which in the PH field has been limited, might present particularly promising

therapeutic targets, owing to their connections with many signaling pathways implicated in PH progression, including EndMT.

2.0 Expression of EBP50 is Downregulated in PH Lungs

2.1 Introduction

As detailed in **Chapter 1**, the continually evolving field of PH research had uncovered numerous insights into the molecular pathways and proteins with potential roles in the pulmonary vascular remodeling and disease progression. However, to date, there are no therapies that can reverse the PH-associated vascular remodeling and cure affected patients. Since about a decade ago, a handful of studies identified mutations of the cytosolic and mitochondrial scaffolding proteins such as Cav-1 [18], NFU1 iron-sulfur cluster scaffold [381,382] in PH, and connected them to the PH-associated vascular remodeling and disease progression. Other studies connected the alterations of expression of other such proteins, including PDZ And LIM Domain Protein 5, PDLIM5 [383,384], and Shroom [385], to PH. A most notable example, as mentioned above in **Sections 1.1.2 and 1.3.5**, is Cav-1, a scaffolding protein and an essential component of the plasma membrane caveolae which can interact with a variety of proteins, including eNOS [272], and regulate their function. Additionally, Cav-1 negatively regulates production of NADPH-oxidase-derived ROS, and loss of Cav-1 expression in the pulmonary endothelium and adventitia leads to upregulation of Nox2 and Nox4 expression, increased ROS production and heightened oxidative stress in PH [386]. Furthermore, the NADPH oxidase isoform Nox1 was found to colocalize with Cav-1 at the plasma membrane of vascular SMCs, linking Noxs to growth factor and integrin signaling in the vessel media [387]. Cav-1 mutations have been reported in patients with hPAH [18], and the downregulation of the Cav-1 expression was identified in the whole lungs [388,389] and pulmonary endothelial cells of iPAH patients [389], as well as the endothelial cells and the total lung homogenates of MCT [390] and Su-Hx PAH rats [388].

While interest in other scaffolding proteins in PH research remains limited, in general those proteins may still offer valuable knowledge of this devastating disease as they are essential orchestrators of the signaling pathways in health and disease, and their dysregulation can consequently affect a wide range of cellular processes [391]. Moreover, owing to their ability to have multiple binding partners and varying subcellular localizations, these proteins can exhibit cell type-specific functions and serve as cellular "rheostats" to precisely regulate the distinct molecular cascades. Therefore, scaffolding proteins can offer a unique opportunity to home-in on the disease-relevant intercellular signaling pathways by way of targeted interruption of the particular protein-protein interactions, while leaving others intact [391].

Despite the fact that PDZ scaffolding proteins are abundantly expressed in all mammals, and that between 200 and 300 PDZ proteins are encoded in the human genome [392], the number of studies concerning the investigation of PDZ proteins in PH is woefully low. PDZ proteins regulate a wide array of cellular functions, controlling the receptor trafficking in synapses [393], and regulating drug transporters [394] and ion exchangers [395]. Yet, to our knowledge, only a single study has reported a SMC-specific downregulation of the PDZ protein Shroom in PAH [385]. Likewise, a role for EBP50, which, as detailed above in **Sections 1.1.2-3**, is a member of this family implicated in the in the systemic vasculature [204,212,225], has not been studied in PH. However, this protein was demonstrated to play a role in the regulation of several essential functions that are affected in PAH and PH, such as the smooth muscle cell migration [204] and proliferation [212,225], inflammation [229], and ROS production [226], and was found to play an important role in the angiogenesis and neovascularization [396,397], and to regulate the cell division in PAECs [214].

Owing to the connections between the endothelial phenotypes in PAH and cancer [31,37,398,399] on one hand, and EBP50 and cancer on the other [201,224], in the first part of our study, we aimed to determine whether EBP50 is altered in PH.

2.2 Methods

Human lung tissue samples: Human tissue samples were obtained through the University of Pittsburgh Tissue Processing Core, Boston Children's Hospital, Brigham and Women's Hospital, and University of California, Los Angeles, and approved by the University of Pittsburgh, Boston Children's Hospital, Brigham and Women's Hospital, and University of California, Los Angeles Institutional Review Boards. Human pulmonary artery endothelial cells from de-identified PAH patients and non-diseased subjects were obtained from the University of Pittsburgh VMI Cell Processing Core and Pulmonary Hypertension Breakthrough Initiative (PHBI) in agreement with the University of Pittsburgh and PHBI protocols.

Animals: All animal experiments were approved by and conducted in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee. To model the monocrotaline (MCT)-induced severe PAH, 10-14 weeks old adult male Sprague-Dawley rats (Charles River Laboratories, Hudson, NY) were injected once with 60 mg/kg of monocrotaline and euthanized after 3 weeks [400]. For the Sugen-Hypoxia model of severe PAH, 10-14 weeks old adult male Sprague-Dawley rats received a subcutaneous injection of 20 mg/kg vascular endothelial growth factor receptor inhibitor SU5416 (Sigma-Aldrich, St. Louis, MO) followed by three weeks of exposure to normobaric hypoxia (10% O_2) in a temperature and humidity-controlled chamber (BioSpherix, Parish, NY) and two weeks of normoxia. Control animals were maintained

under normoxia and injected with vehicle. 10-12 weeks old adult male C57BL/6 wild type mice (Taconic Biosciences, Germantown, NY) were used as model animals for PH associated with *Schistosoma mansoni* infection. *S. mansoni* ova infection was performed as previously described [400]. To model the chronic hypoxia-induced PH, 10-12 weeks old adult male C57BL/6 wild type mice (Taconic Biosciences, Germantown, NY and Jackson Laboratory, Bar Harbor, ME) were subjected to continuous normobaric hypoxia (10% O_2) for three to four weeks (21 – 28 days). Control animals were maintained under normoxia. At the termination of all animal studies, lung samples were snap frozen in liquid nitrogen and stored for the future analysis.

Human pulmonary endothelial cells isolation: First and second order pulmonary arteries from PAH patients and non-PAH controls were extensively cleaned from fat and adventitia and perfused with 0.5% Trypsin (Gibco, Carlsbad, CA) and 2.5 mg/ml Collagenase I (Worthington Biochemical Corporation, Lakewood, NJ). Resulting endothelial cell-rich perfusate was collected and plated on cell culture-treated plastic dishes. The purity of the resulting culture maintained in VascuLife VEGF Endothelial Medium (Lifeline Cell Technology, Frederick, MD) was confirmed by a combination of vWF immunofluorescent staining (vWF antibody, Abcam, United Kingdom) and visual identification.

Mouse pulmonary endothelial cells isolation: Mouse lung tissue was collected and stored in ice-cold PBS (Gibco, Carlsbad, CA) and used the same day for the PECAM+ cell isolation. PECAM+ cells were isolated using MACS Cell Separation Technology (Milteniy Biotec, Germany) and mouse CD31 magnetic microbeads (Milteniy Biotec, Germany) as previously described [400] .

Cell culture: Human pulmonary artery endothelial cells (HPAECs) were obtained from PromoCell GmbH, Germany [Catalog #: C-12241, Lot # 432Z010.2 (donor info: Caucasian 50

y.o. female, non-smoker). HPAECs were grown in the heparin-free Endothelial Cell Growth Medium 2 (PromoCell, Germany).

Cell treatments: Recombinant human IL-1β was obtained from Peprotech, Inc. (Cat #: 200-01B); lyophilized powder was reconstituted in mQ H_2O with an addition of 0.1% bovine serum albumin (Sigma Aldrich, St.Louis, MO) and stored in aliquots in -80$_o$ C for no longer than one year since the date of preparation. Immediately prior to cell treatment, IL-1β was diluted in cell culture medium to be used at a concentration of 10 ng/ml. For hypoxia exposure, cells were cultured in temperature-humidity controlled chamber under pO_2 = 1%, pCO_2 = 5%, with N_2 balance at 37$_o$C.

Western blot: Western blot experiments were performed as described previously [329]. Total protein (20 μg) from tissue homogenates or cell lysates were added to Tris-glycine SDS sample buffer, boiled, resolved with SDS / PAGE, and transferred onto Trans-Blot nitrocellulose membranes (Bio–Rad, Hercules, CA). Membranes were blocked with the Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) and incubated with rabbit anti-EBP50 (1:1000 dilution, Invitrogen, Carlsbad, CA), mouse anti-vinculin (1:1000, Santa Cruz Biotechnology, Dallas, TX), or mouse anti-β-actin (1:1000, Santa Cruz Biotechnology, Dallas, TX). Membranes were probed with fluorescence-tagged (680 or 800 nm) anti-rabbit or anti-mouse secondary antibodies (1:10,000 dilution, LI-COR Biosciences, Lincoln, NE). Digital membrane scans were obtained using the Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE). Optical density (OD) of protein-of-interest bands were quantitated using ImageJ software (NIH, USA) and normalized to vinculin or β-actin.

Tissue immunofluorescence: Human and rat lung tissue immunofluorescence staining was performed as previously described [400]. Briefly, samples were blocked in 10% donkey serum and incubated with anti-EBP50 (rabbit, 1:100, Invitrogen, Carlsbad, CA), anti-CD31 (rat, 1:200,

Abcam, United Kingdom), anti-α-SMA (mouse, 1:200 dilution, Sigma Aldrich, St. Louis, MO) over night at 4$_{o}$C, followed by 488-, 594-, and 633- fluorophore-conjugated secondary antibodies (1:1000) for 1 hr at room temperature, and DAPI. Images were captured using ZeissLSM780 confocal microscope. Small pulmonary vessels (<100 μm diameter) present in a given tissue section (>10 vessels / section) not associated with bronchial airways were selected for analysis. The vessel wall was defined based on α-SMA staining, and intensity of EBP50 in the vessel wall was quantified using ImageJ software (NIH).

Statistics: All results are reported as mean ± SEM. Unpaired two-tailed t-test was used for comparison between two groups. For all analyses, $p < 0.05$ was deemed statistically significant. GraphPad Prism software was used for data analyses (GraphPad Software v7).

2.3 Results

2.3.1 EBP50 Expression is Downregulated in the Lungs of PAH Patients.

As explained above, historically, most studies of EBP50 in vascular biology were focused on the systemic circulation, and whether EBP50 plays a role in the pulmonary circulation remained unclear [204,226,229]. To test whether the expression of EBP50 is altered in the pulmonary vasculature of PAH patients and determine whether dysregulation of EBP50 can offer additional insights into our understanding of the human disease, we utilized the lung tissue specimens from PAH patients, as well as from mouse and rat PH and PAH models.

To first determine whether the EBP50 expression is altered in the pulmonary vasculature of PAH patients, we performed the immunofluorescent staining of the lung tissue specimens obtained from patients with PAH, as well as from matched no-PAH control subjects **(Figure 5)**.

PAH patient demographic is detailed in the **Appendix Table 1**. Using the laser scanning confocal microscopy to detect EBP50 expression, we observed a decrease in the EBP50 fluorescence in and surrounding resistance pulmonary arteries < 100 μm in diameter in PAH patients compared to the control group (red pseudocolor; 0.55 ± 0.07-fold from no-PAH, $p < 0.01$, n = 4 no-PAH, n = 8 PAH) indicative of vascular and perivascular attenuation. In addition, we observed an increase in the PAH vessel muscularization evidenced by an increased medial alpha-SMA staining (white pseudocolor), and a loss of the continuous PECAM staining in the intima of PAH vessels (green pseudocolor), supporting a dysregulation of the endothelial layer integrity and, potentially, early EndMT. Collectively, this finding indicates a prospective translational importance of the EBP50 downregulation to PAH and supports the investigation into its role in disease progression.

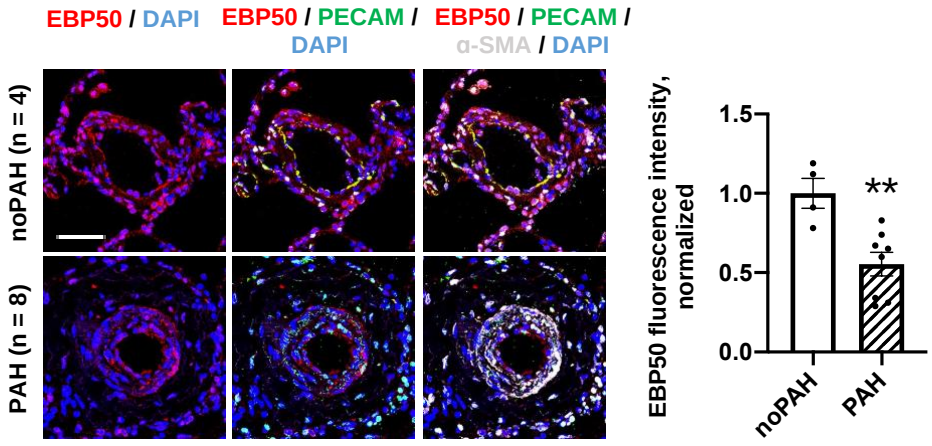


Figure 5. EBP50 expression is decreased in the lungs of PAH patients.
Immunofluorescent staining against EBP50 (red) is decreased in PAH lung tissue vessels compared to non-PAH controls (noPAH). Staining for endothelial cell marker PECAM (green) and smooth muscle marker alpha-smooth muscle actin (α-SMA, white) reveals a loss of endothelial cell layer integrity and an increase in muscularization in PAH lung vessels (Scale bar = 50 μm). Quantification of the immunofluorescent signal of EBP50 per organ donor per vessel demonstrates a decrease in EBP50 signal in PAH patients normalized to non-PAH controls (n = 4, control; n = 8, PAH. ** - $p < 0.01$).

2.3.2 EBP50 Expression is Downregulated in Mouse and Rat Models of PH and PAH.

Next, in order to understand whether the downregulation of the pulmonary vascular EBP50 is a universal feature of PH, we tested whether our human findings are replicated in the three well-established preclinical rodent models of PAH and PH (**Figures 6-7**). Immunostaining against EBP50 in the PAH MCT-injected rats revealed a decrease in the pulmonary perivascular EBP50 expression (**Fig.6**; 0.43 ± 0.09-fold from Vehicle, $p < 0.01$, $n = 6$), accompanied by a loss of the intimal PECAM staining and an increase in the medial alpha-SMA, similar to changes seen in human PAH patients.

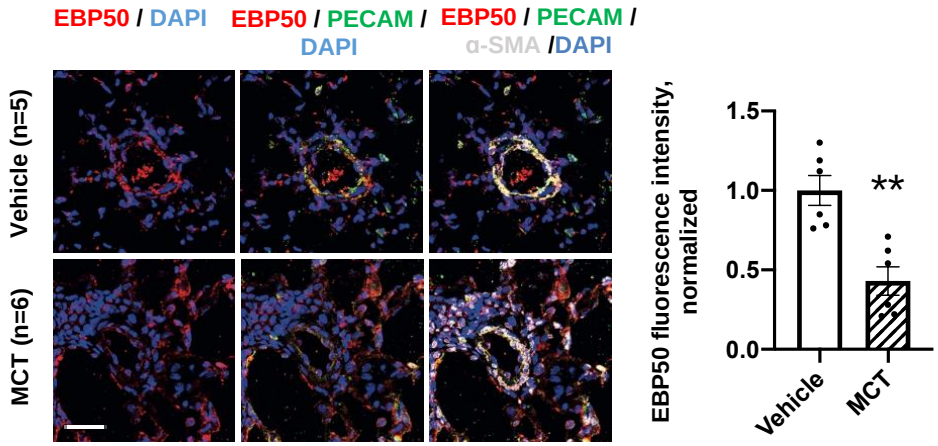


Figure 6. EBP50 expression is decreased in the lungs of MCT PAH rats.
Immunofluorescent staining against EBP50 (red) is decreased in PAH lung tissue vessels of monocrotaline-treated rats (MCT) compared to Vehicle control group. Staining for PECAM (green) and α-SMA (white) reveals a decrease in endothelial marker expression in PAH vessels and an increase in vascular muscularization (Scale bar = 50 μm). Quantification of the immunofluorescent signal of EBP50 per animal per vessel demonstrates a decrease in EBP50 signal in MCT rats compared to vehicle-treated controls (n = 5, vehicle; n = 6, MCT. ** - $p < 0.01$).

In addition, EBP50 mRNA and protein expression in the total lung homogenates from the MCT animals were decreased compared to vehicle-injected controls (**Fig. 7A**; mRNA: 0.78 ± 0.04-fold, $p < 0.05$, $n = 4$; protein: 0.52 ± 0.12-fold, $p < 0.01$, $n = 4$). In addition, MCT samples displayed two distinct bands, both of which were less pronounced compared to vehicle samples, suggestive of a potential modulation of EBP50 phosphorylation states in addition to protein expression. This

observation will be intriguing to follow up on in future studies. PH animal models showed comparable results. Pulmonary EBP50 mRNA level was decreased in PH mice subjected to chronic hypoxia (**Fig. 7B**; mRNA: 0.73 ± 0.07-fold from Normoxia, $p < 0.01$; $n = 6$), and in *Schistosoma mansoni*-infected mice, which develop the symptoms of PH associated with pulmonary vascular remodeling and heightened proinflammatory response [154] (**Fig. 7C**; mRNA: 0.61 ± 0.05-fold from non-infected, $p < 0.05$, $n = 4$). Consistent downregulation of EBP50 across the various disease models and animal species corroborated our patient findings and indicated a potential universal involvement of EBP50 in the development or progression of the PH WHO Groups I and III.

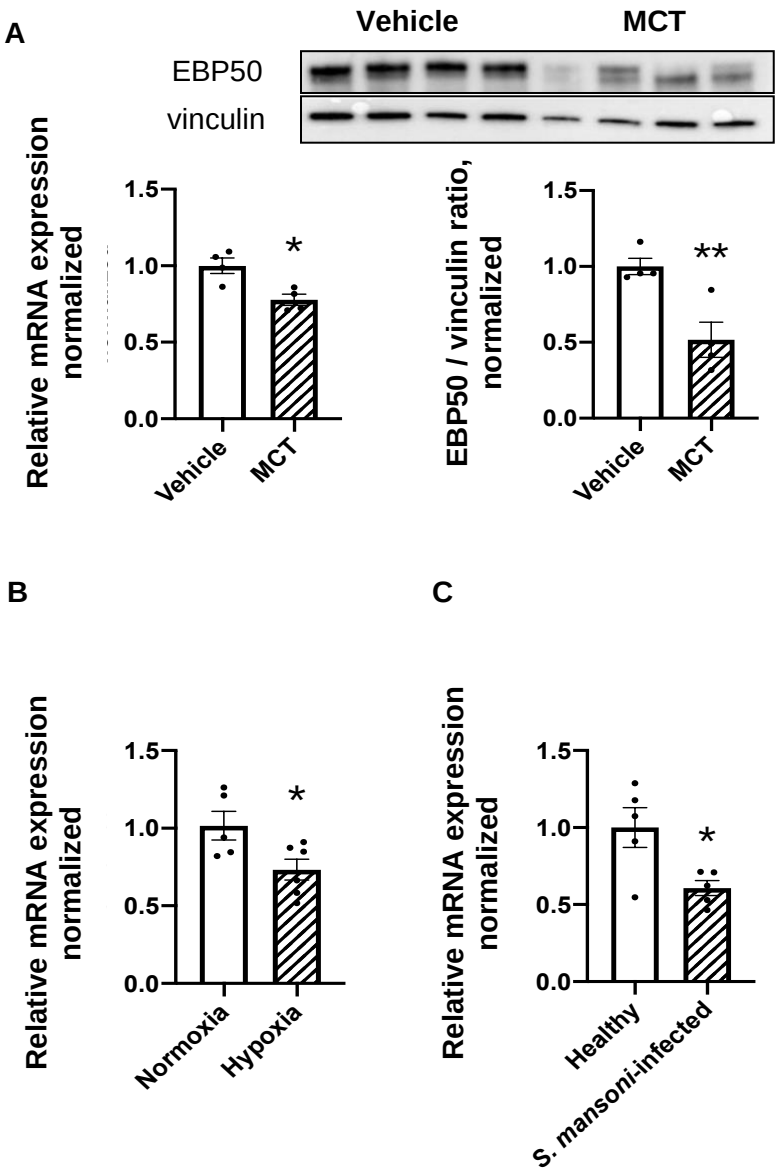


Figure 7. EBP50 expression is decreased in the lungs of PAH and PH rodents.

A. EBP50 mRNA expression is decreased in the total lung homogenates from the MCT-treated PAH rats compared to Vehicle-injected controls (n = 4. * - $p < 0.05$). Representative Western immunoblot (top) and densitometric quantification (bottom) demonstrates a decrease in EBP50 protein expression in the total lung homogenates from the MCT-treated rats normalized to Vehicle controls (n = 4. ** - $p<0.01$).

B. EBP50 mRNA expression is decreased in the total lung homogenates from the chronic hypoxia-exposed PH mice compared to normoxia controls (n = 11-14, * - $p < 0.05$).

C. EBP50 mRNA expression is decreased in the total lung homogenates from the *Schistosoma mansoni*-infected PH mice compared to healthy controls (n = 5, * - $p < 0.05$).

Relative EBP50 mRNA expression normalized to 18s was quantified using ΔΔCt rt-PCR method. Relative EBP50 protein expression was quantified as a ratio to vinculin (B) or β-actin (C) level in a respective sample.

2.3.3 EBP50 Expression is Downregulated in Pulmonary Endothelial Cells in PH.

Despite the studies of EBP50 in the systemic vascular SMCs, its role in the pulmonary endothelium remains underexplored, and whether pulmonary endothelial EBP50 expression is altered in PAH and PH was unknown. Considering the importance of endothelial cells for the maintenance of vascular homeostasis and a key role of the pulmonary endothelial dysfunction in the PH-associated vascular remodeling, we assessed whether the expression of EBP50 was altered in the pulmonary ECs of PAH patients and PH mice (**Figures 8-9**). EBP50 protein levels were analyzed in the endothelial cells harvested from the first and second order pulmonary arteries of human PAH patients via immunoblotting. We used cells isolated from 6 PAH and 6 age- and sex-matched non-PAH control patients as detailed in **Appendix Table 2**. In each PAH and non-PAH cohorts there were 6 female and 2 male patients (2:1 female : male), consistent with the established observation of a higher incidence of PAH in females [24,25]. To ensure the antibody specificity, the commercially obtained human pulmonary artery endothelial cells (HPAECs) were transfected with EBP50 siRNA for 72 hrs (knockdown efficiency at mRNA level ~ 80%, **Appendix Figure S1**), and used as a negative control. EBP50 expression was markedly downregulated in the PAH endothelial cells compared with cells from the matched non-PAH controls (**Fig. 8**; 0.33±0.05-fold from non-PAH, $p<0.0001$, $n=6$).

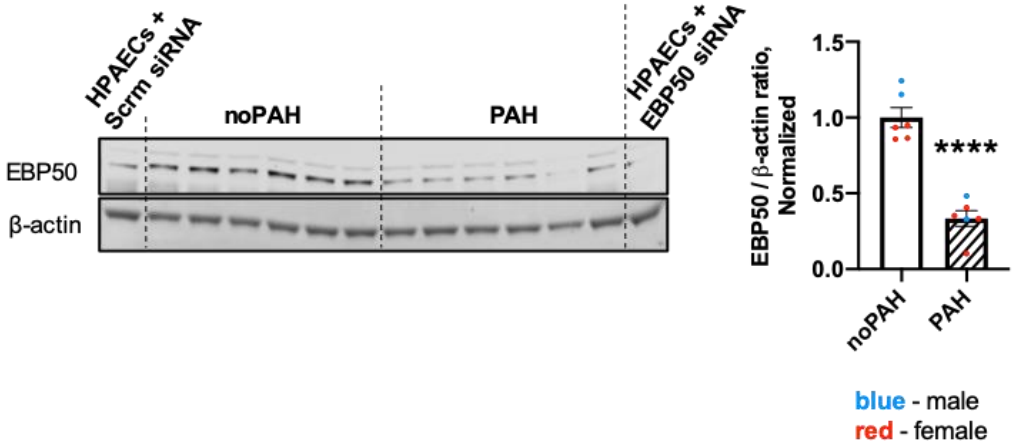


Figure 8. EBP50 expression is decreased in pulmonary arterial endothelial cells of PAH patients.
Endothelial von Willebrand Factor positive (vWF+) cells isolated from pulmonary arteries of PAH patients (n=6) display decreased relative EBP50 protein expression compared to non-PAH donor controls (n=6) in a protein expression analysis using Western immunoblot (**** - $p < 0.0001$). Relative EBP50 expression was quantified as a ratio to β-actin level in the respective sample. Commercially obtained HPAECs transfected with EBP50 siRNA for 72hrs were used as a negative control for the antibody specificity (see Appendix, Fig. S1).

These data were supported by the finding from the animal studies where EBP50 mRNA expression was reduced in the purified PECAM+ pulmonary endothelial cells isolated from mice exposed to 3 - 4 weeks of hypoxia compared to the normoxia controls (**Fig. 9**; 0.75 ± 0.04-fold, $p < 0.01$; n = 11-14).

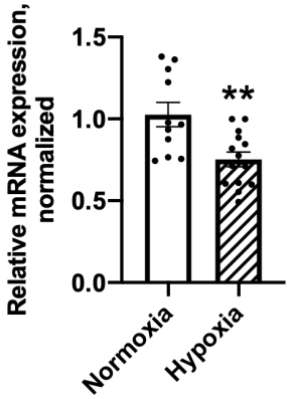


Figure 9. EBP50 expression is decreased in pulmonary endothelial cells of PH chronic-hypoxia mice.
Pulmonary endothelial PECAM positive cells isolated from chronic hypoxia-exposed mice (n = 14) display decreased relative EBP50 mRNA expression compared to normoxia controls (n=11) (** - $p < 0.01$). Relative EBP50 mRNA expression normalized to 18S was quantified using ΔΔCt rt-PCR method.

Together these data indicate that EBP50 downregulation observed in PH is, at least in part, localized to the endothelial layer of the pulmonary vessels, and suggest an endothelium-specific role for the EBP50 dysregulation in the disease initiation and/or propagation.

2.3.4 EBP50 Expression is Time-Dependently Downregulated in Response to PH-related Stimuli in HPAECs.

As detailed in **Chapter 1**, chronic hypoxia exposure [55] and sustained hypoxic [4] and proinflammatory [295] environment all exacerbate and accompany the PH-associated pulmonary vasoocclusion and vascular remodeling. To investigate the functional relevance of the EBP50 downregulation observed in the pulmonary endothelial cells of PAH patients and animal PH and PAH models in vivo, we first set to determine whether the PH-related stimuli induce the downregulation of EBP50 in the HPAECs in vitro.

To model the environment experienced by the pulmonary ECs in PH in vitro, we used the hypoxic challenge and the IL-1β treatment. Both stimuli time-dependently downregulated the EBP50 expression, albeit to a different extent. Whereas hypoxia (pO_2 = 1%) did not induce the EBP50 downregulation following 48 hrs of exposure (**Figure 10, A**), it was sufficient to produce a ~ 40% decrease in the EBP50 mRNA expression after 72hrs of exposure (**Fig. 10B**; 0.58±0.07 from normoxia, $p<0.01$, n=3). On the other hand, the treatment of HPAECs with 10 ng/ml of IL-1β resulted in a marked decrease in the EBP50 gene (**Fig. 11A**; 0.51±0.02 from untreated, $p<0.0001$, n=3) and protein (**Fig. 11B**; 0.54±0.04 from untreated, $p<0.001$, n=3) expression as early as 48 hrs, and sustained 72 hrs post initial treatment (**Fig. 11C, D**; mRNA: 0.51±0.07 from untreated, $p<0.01$, n=3; protein: 0.61±0.02 from untreated, $p<0.0001$, n=3;).

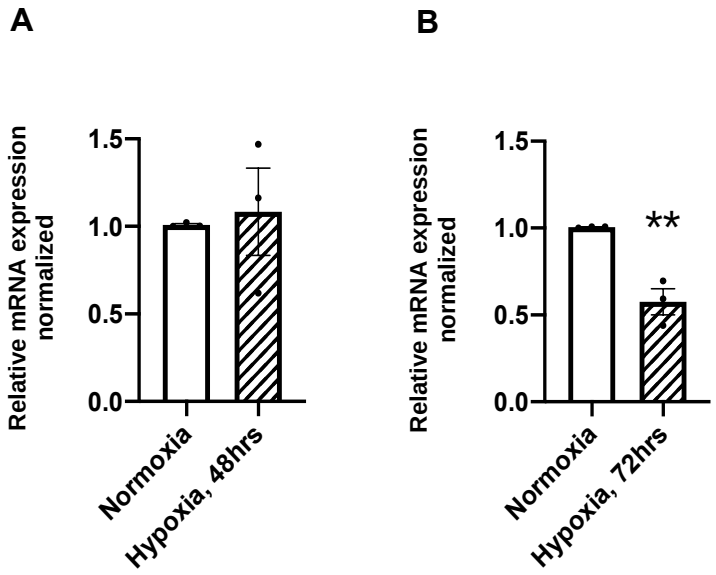


Figure 10. EBP50 is time-dependently downregulated by hypoxia.
A. 48 hrs of hypoxia exposure (pO_2 = 1%) is not sufficient to induce stable changes in EBP50 mRNA expression.
B. EBP50 mRNA is downregulated following 72 hrs of hypoxia exposure (** - $p < 0.01$, n = 3).
Relative mRNA expression was normalized to 18s and quantified using ΔΔCt rt-PCR method.

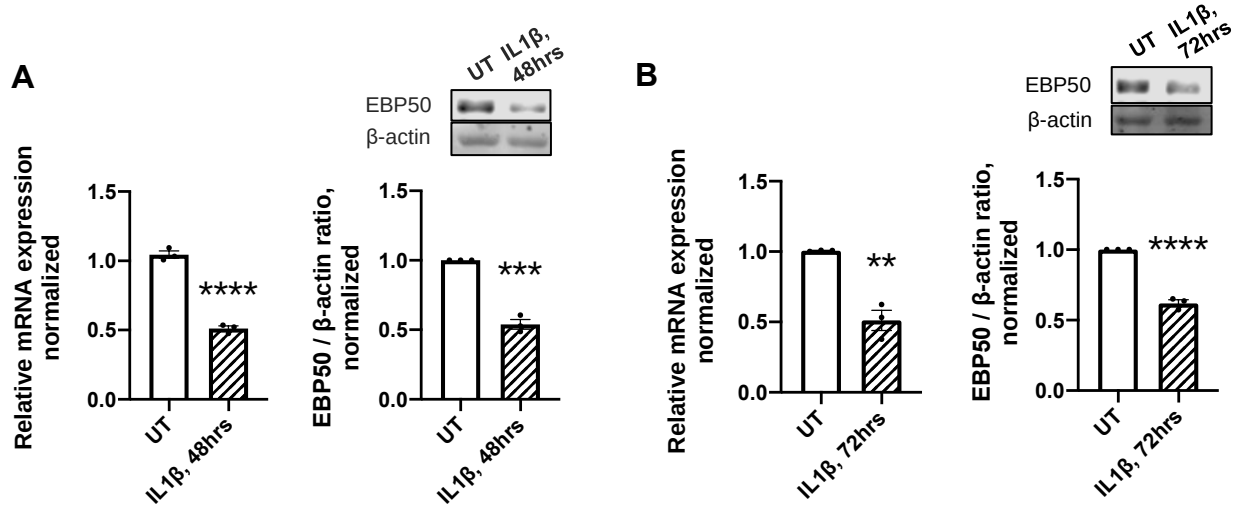


Figure 11. EBP50 is time-dependently downregulated by the IL-1β treatment.
A. mRNA and protein expression of EBP50 is downregulated following 48 hrs of 10 ng/ml IL-1β treatment (*** - $p < 0.001$, **** - $p < 0.0001$, n = 3).
B. mRNA and protein expression of EBP50 is downregulated following 72hrs of 10 ng/ml IL-1β treatment (** - $p < 0.01$, **** - $p < 0.0001$, n = 3).
Relative mRNA expression was normalized to 18S and quantified using ΔΔCt rt-PCR method. Relative protein expression was calculated as a ratio to β-actin level in a respective sample.

2.4 Discussion

Despite significant progress in our understanding of molecular mechanisms driving PH-associated vascular remodeling, and the importance of endothelial homeostasis in the maintenance of a normal vascular function, little is known about molecular players that regulate endothelial phenotypes associated with PH. In this study, we for the first time, identified the downregulation of a scaffolding PDZ protein EBP50 in the pulmonary vasculature and pulmonary endothelial cells as a common feature of PH in human patients and animal models of the disease.

Indicative of its significance, the EBP50 expression was downregulated in all animal models of PH and PAH used in the study, including a mild chronic-hypoxia PH and a severe MCT PAH. Critically, we demonstrate that the downregulation of EBP50 is also featured in the pulmonary vasculature of PAH patients, which underscores the translational importance of this finding and hints at the potential of the EBP50 expression restoration for the treatment of PH, such as by using in vivo adenoviral gene delivery. Studies on isolated pulmonary endothelial cells from PAH patients and PH mice confirmed that the EBP50 downregulation occurs in endothelial cells, arguing for the importance of exploring the functional relevance of this event in relation to PH-associated endothelial dysfunction. Moreover, the potential relevance of the EBP50 downregulation to the propagators of the human disease is highlighted by in vitro findings from primary human pulmonary endothelial cells in which PH-relevant challenges of hypoxia and IL-1β recapitulated the in vivo downregulation of EBP50. Our group had previously identified the role of EBP50 as a regulator of the agonist-induced Nox1-derived superoxide production and oxidative stress in systemic vascular SMCs [226]. It is interesting, therefore, to explore a connection between the loss of EBP50 and a possible inactivation of endothelial Nox isoforms, including a vasoprotective hydrogen-peroxide-generating Nox4 [401], in the propagation of PH-associated

EndMT. Furthermore, our observation opens doors for studying a functional link between the hypoxia-induced loss of EBP50 and an upregulation of the endothelial Nox1 expression, known to drive the transactivation of the BMP antagonist Gremlin-1 and migration and proliferation of PAECs in PH settings [85].

Building on these findings we next sought to determine whether the downregulation of EBP50 affects the signaling pathways associated with endothelial activation and dysfunction, such as seen in the PH pulmonary vasculature. The next chapter details our efforts to identify the functional relevance of the EBP50 downregulation to EndMT, a process increasingly implicated in the PH pathogenesis, and determine whether EBP50 downregulation is a driver, rather than a consequence, of PH-associated endothelial dysfunction.

3.0 Knockdown of EBP50 Promotes EndMT in HPAECs and Impairs Proliferation and Barrier Function

3.1 Introduction

As detailed in **Chapter 1**, at the tissue level, patients with severe PH, including PAH, present with signs of extensive pulmonary vascular remodeling, perivascular inflammation, vascular wall thickening, and vessel narrowing, occlusion, rarefaction and stiffening [29–31]. Remodeling of pulmonary vessels is also characterized by the distal muscularization of the precapillary arterioles and the emergence of cells expressing the smooth muscle markers within the intimal layer of those vessels, resulting from the inward migration of the medial SMCs into the intima and EndMT [30]. Studies have shown that endothelial cells isolated from the small pulmonary arteries explanted from PAH patients and PAH animal models exhibit signs of EndMT, including a loss of endothelial markers and a gain of smooth muscle markers expression [174,252]. Ultimately, genetic cell reprogramming and phenotypic switching seen in EndMT results in a change in cell shape from a compact cobblestone to an elongated and spindle-like due to the cytoskeletal remodeling and alterations in the extracellular matrix composition [402], and potentiates an increase in the endothelial monolayer permeability [133,293], impairing the endothelial barrier function essential for the maintenance of the vascular wall integrity [37]. However, the underlying mechanisms triggering the process of EndMT in PAH vessels remain unknown.

In recent years, as more attention has been brought to the role of pulmonary endothelium in PAH pathogenesis, many prominent researchers in the field point out significant similarities between the endothelial phenotype in PAH and cancer [31,37,398,399]. Interestingly, the aberrant expression and subcellular localization of EBP50 is increasingly studied in the context of cancers

403, and several studies highlighted the role of EBP50 in regulating the activity of β-catenin 201,404–406, an effector of the Wnt signaling pathway with known roles in carcinogenesis, tumor progression, and EndMT, process that has also been implicated in PAH pathogenesis by multiple research groups 91,133,402,407–409 .

As we have identified the downregulation of EBP50 as a common denominator between the human PAH and the rodent models of PH and PAH, and localized the EBP50 downregulation to the pulmonary endothelial cells, we sought to determine whether EBP50 regulates P(A)H-relevant endothelial phenotypes. Given our findings of modulation of EBP50 expression in PH detailed in **Sections 2.3.1 – 2.3.3** and **Figs. 5-9**, and considering the implication of EndMT in PH and the known role of EBP50 as a regulator of EMT 238 and its association with β-catenin, one of the key players of the EndMT pathway, we sought to determine whether this PH-related downregulation of EBP50 has a potentiating effect on EndMT in HPAECs.

3.2 Methods

EBP50 knockdown: To induce EBP50 gene knockdown, HPAECs were transiently transfected with Silencer Select EBP50 siRNA (Cat #: 4390824, ID: s17920, Life Technologies, Carlsbad, CA) using the Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Silencer Select Negative Control No. 1 siRNA (Invitrogen, Carlsbad, CA) was used as a control for potential off-target effects of the transfection procedure.

Quantitative real-time PCR: Levels of mRNA transcripts were quantified as previously described 329. Briefly, total RNAs were extracted using the RNeasy Plus Mini Kit (Qiagen,

Germany). Total RNA (0.5 μg) was reverse transcribed to cDNA using Superscript III reverse transcriptase (Life Technologies, Carlsbad, CA) following the manufacturer's protocol. Taqman probe and Universal PCR Master Mix and SLC9A3R1, SNAI1, SNAI2, Zeb1, Zeb2, CTNNB, PECAM1, CDH5, CDH2, TAGLN, S100A4, FN1 or 18S primers (Life Technologies, Carlsbad, CA) were used for qPCR in a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol for 40 cycles. Relative quantitation to corresponding control was obtained using the threshold cycle (Ct) method with 18S as the housekeeping gene and relative expression calculated as $2^{-\Delta\Delta Ct}$.

Western blot: Western blot experiments were performed as previously described [329]. Total protein (20 μg) from cell lysates were added to Tris-glycine SDS sample buffer, boiled, resolved with SDS / PAGE, and transferred onto Trans-Blot nitrocellulose membranes (Bio–Rad, Hercules, CA). Membranes were blocked with the Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) and incubated with rabbit anti-Snail (1:1000 dilution, Cell Signaling Technology, Danvers, MA), rabbit anti-Zeb1 (1:1000 dilution, Cell Signaling Technology, Danvers, MA), mouse anti-PECAM (1:1000 dilution, Cell Signaling Technology, Danvers, MA), rabbit anti-VE-cadherin (1:1000 dilution, Cell Signaling Technology, Danvers, MA), rabbit anti-N-cadherin (1:1000 dilution, Cell Signaling Technology, Danvers, MA), rabbit anti-SM22 (1:1000 dilution, Abcam, United Kingdom), or mouse anti-β-actin (1:1000, Santa Cruz Biotechnology, Dallas, TX). Membranes were probed with anti-rabbit or anti-mouse secondary antibodies (1:10,000 dilution, LI-COR Biosciences, Lincoln, NE). Digital images were obtained using the Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE). Optical density (OD) of protein-of-interest bands were quantified and normalized to β-actin using ImageJ software (NIH, USA).

Immunofluorescence: Immunofluorescence staining was performed on HPAECs grown on glass coverslips precoated with rat tail collagen I (Gibco, Carlsbad, CA). Samples were fixed in 2% parafolmadehyde, permeabilized with 0.1% Triton X-100 and washed with PBS. Samples were blocked in 2% goat serum / PBS solution for 45 mins at room temperature. Samples were then incubated with anti-Snail (goat, 1:100, R&D Systems, Minneapolis, MN), anti-Zeb1(rabbit, 1:100, Invitrogen, Carlsbad, CA), anti-PECAM (mouse, 1:100, Cell Signaling Technology, Danvers, MA), or anti-SM22 (rabbit, 1:100, Abcam, United Kingdom) for 1 hr at room temperature, followed by goat anti-rabbit Cy3- and goat anti-mouse Cy5-conjugated secondary antibodies (1:1000) for 1 hr at room temperature. Samples were then stained for nuclei (DAPI) and affixed on slides using gelvatol mounting media (polyvinylalcohol, glycerol, H_2O, sodium azide and Tris pH = 8.5). Primary delete was used as a negative control. Confocal images (15 images per treatment group) were captured using a Nikon A1 spectral confocal microscope. Intensity of staining was quantified using NIS-Elements software (Melville, NY).

3.3 Results

3.3.1 EBP50 Knockdown Upregulates the EndMT Transcription Factors in HPAECs.

To phenocopy PH-relevant downregulation of EBP50 in vitro, we used siRNA gene silencing technology to transiently downregulate EBP50 expression in HPAECs. In phenocopy experiments, as early as 24 hrs post-transfection, EBP50 knockdown (~ 60% efficiency, **Appendix A, Appendix Figure 1**) upregulated mRNA expression of the EndMT regulator β-catenin (1.43±0.12-fold, $p<0.05$), and EndMT transcription factors and repressors of endothelial

gene expression Snail (1.53±0.16-fold, $p<0.05$), Zeb1 (1.58±0.05-fold, $p<0.001$), and Zeb2 (1.71±0.18-fold, $p<0.05$) (from Scramble siRNA, n=3; **Figure 12**).

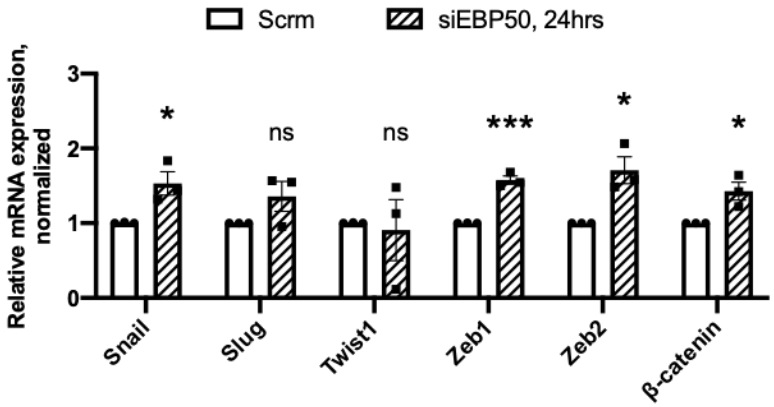


Figure 12. EBP50 knockdown for 24 hrs induces expression of EndoMT transcription factors.
mRNA levels of EndoMT transcription factors Snail, Zeb1, Zeb2 and β-catenin are upregulated following 24hrs of EBP50 knockdown (two-tailed Student' t-test, * - $p<0.05$, ** - $p<0.01$ vs Scrambled siRNA, n=3).

Following the 48hrs of EBP50 knockdown (**Figure 13**), mRNA expression of Snail and Zeb1 remained upregulated (Snail: 1.74±0.19-fold, $p<0.05$; Zeb1: 1.55±0.15-fold, $p<0.05$ from Scramble siRNA; n=3), and expression of Snail homolog Slug was significantly increased (2.05±0.14-fold from Scramble siRNA, $p<0.01$; n=3). Interestingly, while we have not directly compared expression levels at 24 vs 48 hrs, our data is suggestive that expression of Snail and Slug continued to increase in magnitude between 24 and 48 hrs post-transfection, but that increases in Zeb1 and Zeb2 expression appeared to subside at 48 hrs. We speculate that this may be attributed to the dynamic nature of the EndMT process, where expression of EndMT transcription factors is expected to be activated at different time-points.

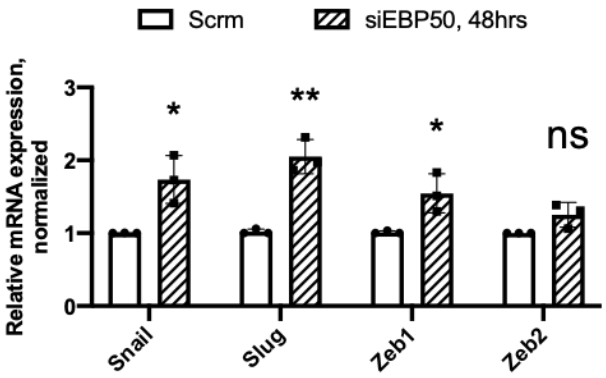


Figure 13. EBP50 knockdown-induced expression of EndoMT transcription factors is sustained 48 hr post-intervention.
mRNA levels of EndoMT transcription factors Snail, Zeb1, Zeb2 and β-catenin are upregulated following 24hrs of EBP50 knockdown (two-tailed Student' t-test, * - $p<0.05$, ** - $p<0.01$ vs Scrambled siRNA, n=3).

Changes in mRNA expression of Snail and Zeb1 were mirrored by the increases in their respective nuclear abundance and protein level. Nuclear abundance of Snail was increased in HPAECs transfected with EBP50 siRNA for 24 hrs (**Figure 14**, 1.44±0.16-fold from Scramble siRNA, $p<0.05$, n=5), and the nuclear abundance (**Figure 15B**) and protein level (**Fig. 15A**) of Zeb1 were increased in HPAECs transfected with EBP50 siRNA for 48 hrs (nuclear abundance: 1.37±0.07-fold; protein: 1.19±0.02-fold from Scramble siRNA, $p<0.01$, n=3), suggesting that EBP50 downregulation can drive the activation of EndMT transcription factors and regulate Snail- and Zeb1-driven transcriptional changes.

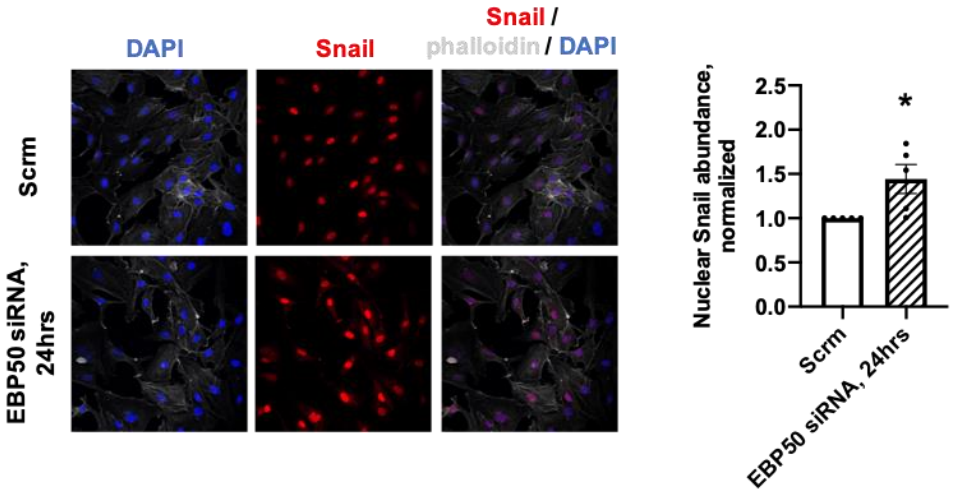


Figure 14. EBP50 knockdown for 24 hrs increases nuclear abundance of EndMT transcription factor Snail. As visualized by an immunofluorescent staining (representative image, left; Snail, red; nuclei, blue), nuclear abundance of Snail is increased following 24 hrs of EBP50 knockdown (bar graph, right; * - $p<0.05$ vs Scrambled siRNA, n=3).

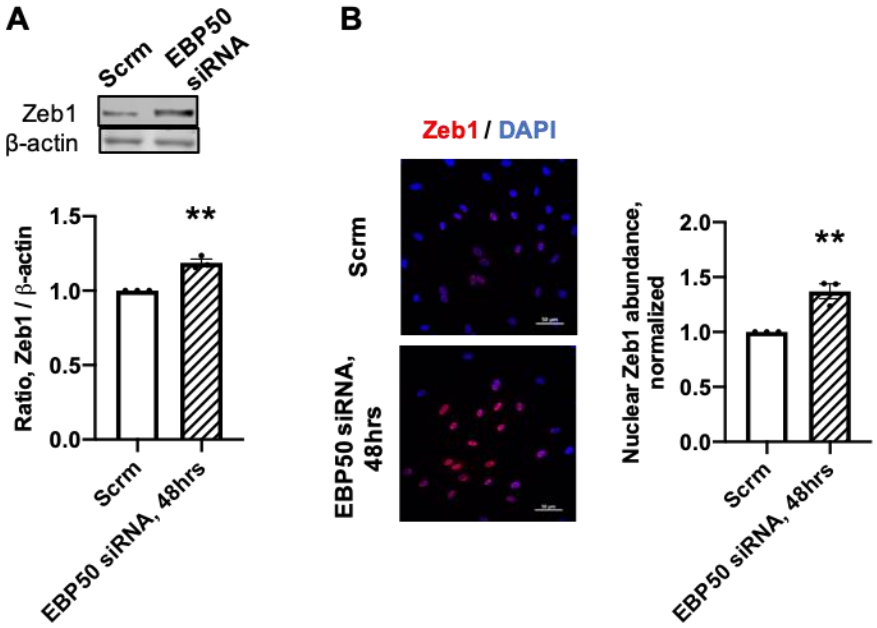


Figure 15. EBP50 knockdown for 48 hrs induces expression and the nuclear abundance of EndMT transcription factor Zeb1.

A. Representative Western immunoblot (top) and densitometric quantification (bottom) of EndMT transcription factor Zeb1 demonstrate an increase in protein expression following 48 hrs of EBP50 knockdown (** - $p<0.01$ from Scrambled siRNA, n = 3). Relative protein expression was calculated as a ratio to β-actin level in a respective sample.

B. As visualized by immunofluorescent staining (representative image, left; Zeb1, red; nuclei, blue), nuclear abundance of Zeb1 is increased following 48 hrs of EBP50 knockdown (** - $p<0.01$ from Scrambled siRNA, n = 3).

Importantly, changes in the expression profiles of the EndMT transcription factors driven by the EBP50 knockdown were similar to changes induced by a known EndMT stimulus IL-1β [245,410,411] (**Appendix A, Figure 2**). Expression of Snail and Slug changed in the same direction and to a similar extent between the HPAECs treated with IL-1β and transfected with EBP50 siRNA, compared to their respective controls. However, unlike the EBP50 knockdown that upregulated expression and nuclear abundance of Zeb1, IL-1β treatment resulted in a downregulation of Zeb1 mRNA and protein expression. Similarities between the effects of IL-1β and EBP50 knockdown on the expression of a subset of EndMT transcription factors suggest that EBP50 can play a role in regulating EndMT progression in HPAECs. At the same time, the inconsistencies between the effects of IL-1β and EBP50 siRNA on Zeb1 expression most likely reflect the broad nature of the signaling pathways induced by IL-1β.

After observing that EBP50 downregulation can drive the activation of EndMT transcription factors in the absence of an additional stimulus, we sought to determine whether changes in expression driven by the PH-relevant stimuli hypoxia and IL-1β are exacerbated by the dysregulated EBP50 signaling. Interestingly, while EBP50 knockdown did not have an additive effect on the expression of EndMT transcription factors stimulated by IL-1β (**Appendix A, Figure 4**), a combination of EBP50 knockdown and hypoxia resulted in a greater increase of Slug mRNA level following 48 hrs compared EBP50 silencing in normoxia and hypoxia in the absence of EBP50 gene silencing (**Figure 16,** $p < 0.05$, $n = 4$). In addition to changes in Slug expression, we also observed trends indicating that expression of Zeb1 and Zeb2 are differentially altered by the decreased level of EBP50 expression in hypoxia, compared to normoxia ($p = 0.056$ and $p = 0.051$, respectively; $n = 4$); these, however, were not statistically significant. Effect of hypoxia on Snail expression was not significantly further increased by hypoxia ($p = 0.91$, $n = 4$).

Together, observed changes in expression and intracellular localization of EndMT transcription factors in response to EBP50 downregulation are indicative of the role of EBP50 as a regulator of EndMT, and support the possibility of observing phenotypic changes in endothelial cells in which EBP50 expression is reduced.

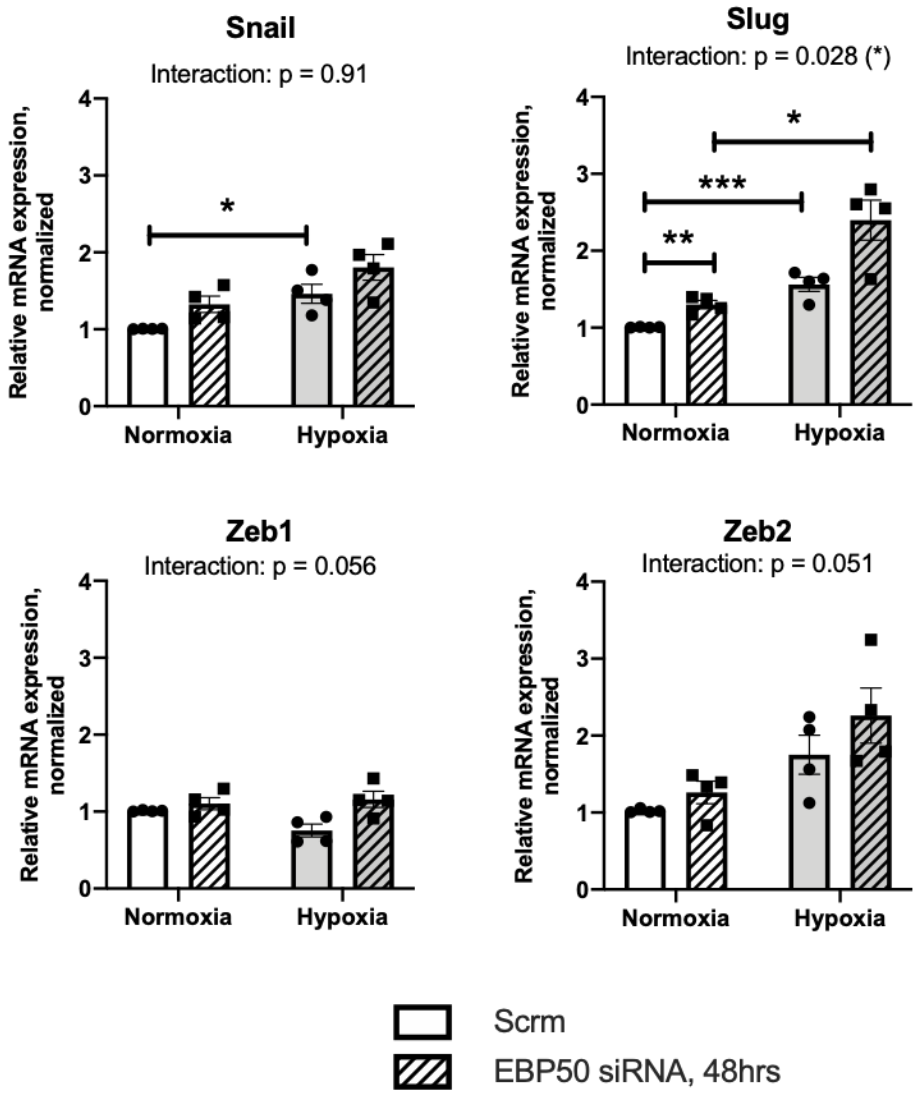


Figure 16. EBP50 knockdown exacerbated hypoxia-induced upregulation of Slug expression 48hrs post-procedure.

mRNA expression of EndMT transcription factor Slug, upregulated following 48 hrs of hypoxia exposure, is increased further in HPAECs transfected with EBP50 siRNA. EBP50 knockdown did not have an additional effect on the expression of Snail, Zeb1, and Zeb2. Exacerbation of the effect of hypoxia on Slug expression by EBP50 knockdown was analyzed using a two-way ANOVA interaction analysis with bootstrapping (* - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, $n = 4$). mRNA normalized to 18s was quantified using ΔΔCt rt-PCR method.

3.3.2 EBP50 Knockdown Induces the Gain of Mesenchymal Markers in HPAECs

Considering the effects of EBP50 silencing on expression and cellular localization of the EndMT transcription factors, we sought to determine whether the EBP50 knockdown is sufficient to induce downstream changes in endothelial and mesenchymal markers in HPAECs (**Figure 17**). Sustained EBP50 silencing (~ 80% efficiency, 72 hrs of knockdown, **Appendix A, Figure 1**) upregulated mesenchymal markers N-cadherin and SM22 (N-cadherin: mRNA, 1.77±0.20-fold, $p<0.05$; protein, 1.37±0.15-fold, $p<0.05$; SM22: mRNA, 1.89±0.10-fold, $p<0.01$; protein, 2.55±0.37-fold, $p<0.01$; compared to Scramble-transfected controls, n=3-5), and induced expression of fibrotic mesenchymal markers S100A4 (mRNA, 1.66±0.10-fold and 1.46±0.18-fold from Scramble-transfected controls, respectively; $p<0.05$; n=3). However, EBP50 knockdown did not affect endothelial markers PECAM (mRNA, 1.53±0.41-fold; protein, 1.06±0.11-fold; n=3) and VE-cadherin (mRNA, 1.35±0.32-fold; protein, 0.86±0.07-fold; n=3).

Notably, the EBP50 knockdown closely recapitulated effects of IL-1β on the expression of mesenchymal markers (**Appendix A, Figure 3**). In HPAEC treated with IL-1β for 72 hrs, mRNA and protein levels of N-cadherin and SM22 were both upregulated (N-cadherin: mRNA, 1.97±0.36-fold, $p<0.05$; protein, 1.60±0.20-fold, $p<0.05$; SM22: mRNA, 1.46±0.18-fold, $p<0.01$; protein, 1.90±0.20-fold, $p<0.05$; compared to untreated controls, n=3-4). However, unlike EBP50 siRNA, IL-1β also downregulated expression of endothelial markers PECAM and VE-cadherin (PECAM: mRNA, 0.65±0.08-fold, $p<0.01$; protein, 0.64±0.05-fold, $p<0.01$; VE-cadherin: mRNA, 0.81±0.02-fold, $p<0.0001$; protein, 0.65±0.07-fold, $p<0.01$; compared to untreated controls, n=3-4), thus indicating that EBP50 may likely function as a regulator of adoption of mesenchymal characteristics of late-stage EndMT, while having minimal effect on downregulation of endothelial markers.

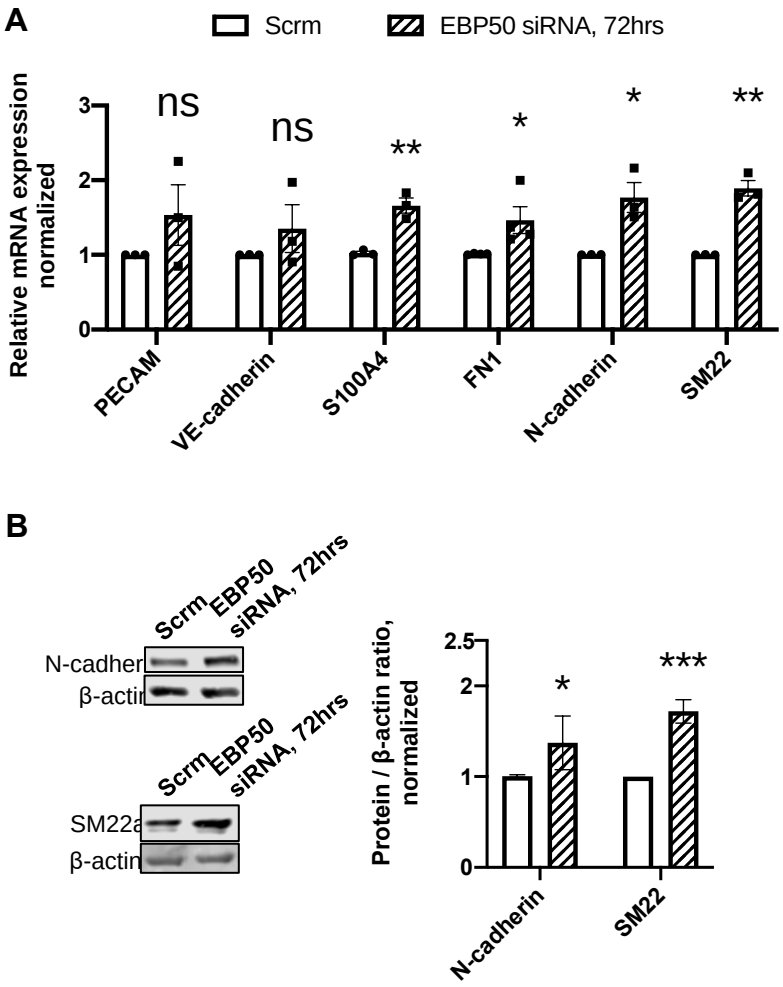


Figure 17. EBP50 knockdown promotes the gain of mesenchymal markers by the pulmonary endothelial cells.

A. mRNA expression of endothelial markers PECAM and VE-cadherin remains unchanged, while mesenchymal markers S100A4, fibronectin, N-cadherin and SM22 are upregulated following 72 hrs of EBP50 knockdown (* - $p < 0.05$, ** - $p < 0.01$ from Scrambled siRNA, n = 3). mRNA normalized to 18s was quantified using ΔΔCt rt-PCR method.

B. Representative Western immunoblots (left) and densitometric quantification (right) of N-cadherin and SM22 demonstrate an increase in protein expression following 72 hrs of EBP50 knockdown (* - $p<0.05$, ** - $p<0.01$ from Scrambled siRNA, n = 3). Relative protein expression was calculated as a ratio to β-actin level in a respective sample.

3.3.3 EBP50 Knockdown Impairs Endothelial Proliferation and Barrier Function

While increased endothelial proliferation is recognized as a feature of PAH-associated vascular remodeling [329,412], studies of the impact of EndMT on proliferation are not in universal agreement. While many studies link EndMT to increased cell proliferation [90,174], others report

contrasting findings, arguing that the unstimulated human PAECs exhibit a greater increase in cell number compared to PAECs treated with pro-EndMT cytokines, such as TNFα, TGF-β, and IL-1β [133]. On the other hand, impaired endothelial barrier function and increased endothelial monolayer permeability, have been previously associated with both EndMT [133,293] and PAH [175,413,414]. Together, a combination of increased vascular permeability and disordered endothelial proliferation impairs the restoration of endothelial monolayer integrity and barrier function in response to damage and contributes to the exacerbated vascular remodeling.

To test whether the PH-relevant downregulation of EBP50 is associated with alterations in endothelial proliferation in HPAECs transfected with EBP50 siRNA, we quantified HPAECs proliferation using the fluorescence-activated cell sorting (FACS) analysis and DNA replication and repair by monitoring protein expression of DNA polymerase processivity factor proliferating cell nuclear antigen (PCNA; **Figure 18**). Extent of *de novo* DNA biosynthesis preceding cell division was decreased in EBP50 siRNA-transfected HPAECs by ~30% (**Fig. 18A**; % proliferating cells: 19.16±0.49 vs. 13.21±1.13 in Scrambled siRNA-transfected vs. EBP50 siRNA-transfected HPAECs, $p<0.01$, n=3), as indicated by a lower rate of thymidine analog EdU incorporation during the S phase of the cell cycle 24hrs post-transfection. Moreover, the EBP50 knockdown produced a stable downregulation of PCNA at 48 and 72hrs of gene silencing, suggesting the DNA replication impairment or increased DNA damage (**Fig. 18B, C**; 0.71±0.07-fold, $p<0.05$ and 0.57±0.02-fold, $p<0.0001$ from Scrambled siRNA, respectively; n=3). EBP50 knockdown did not appear to affect apoptotic cell death, as indicated by the lack of detection of a difference in caspase 3/7 cleavage between the Scrambled and EBP50 siRNA-transfected HPAECs (**Appendix A, Figure 6**). However, a more detailed analysis of other forms of cell death, such as necrosis and

extrinsic apoptosis pathways, is required to make a conclusive statement about the lack of effect of the EBP50 silencing on cell death.

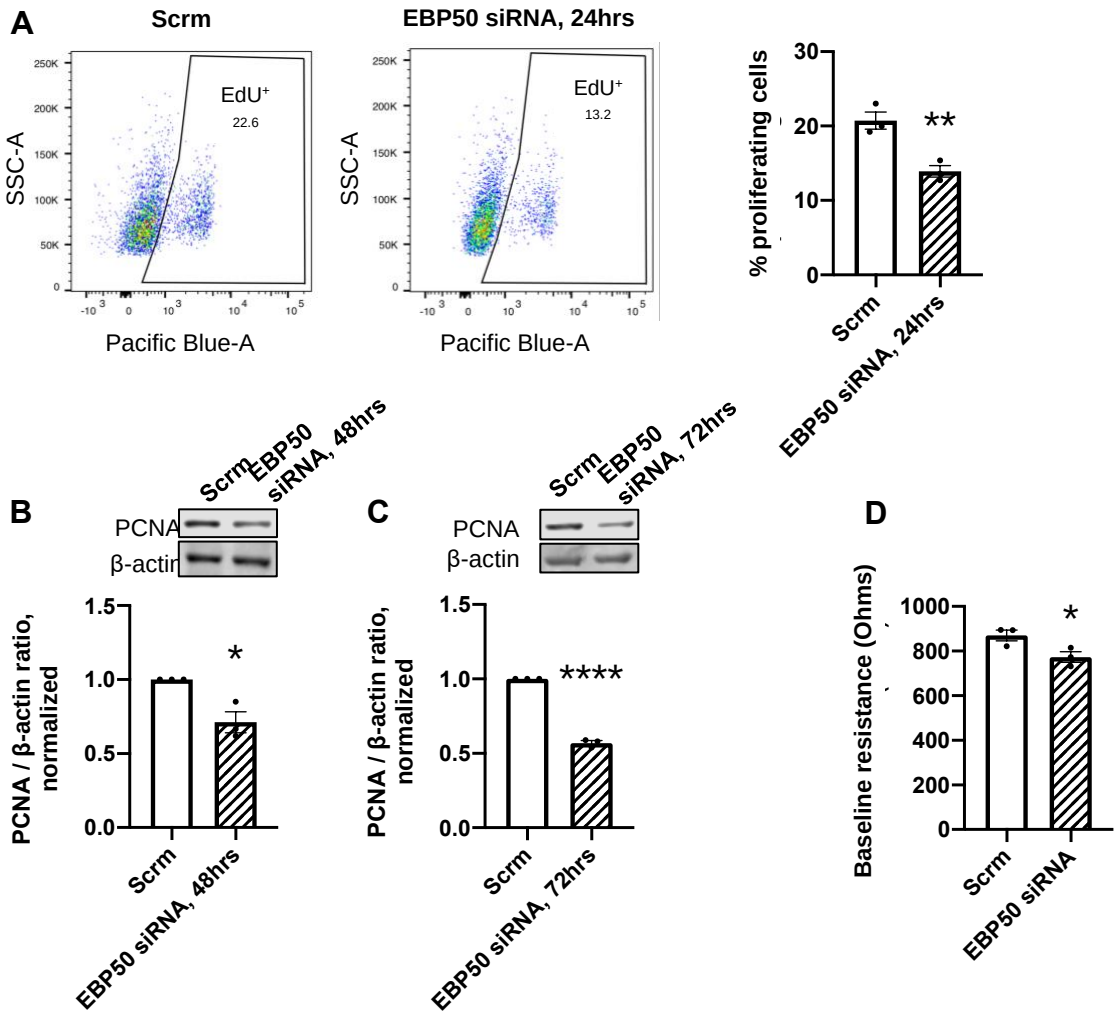


Figure 18. EBP50 knockdown decreases pulmonary endothelial cell proliferation and decreases baseline resistance of endothelial monolayer.

A. DNA incorporation of a thymidine analog EdU measured by flow cytometry is decreased in cells transfected with EBP50 siRNA for 24 hrs (** - $p < 0.01$ from Scrambled siRNA, n = 3).

B,C. Representative Western immunoblots (top) and densitometric quantifications (bottom) of proliferating cell nuclear antigen (PCNA) demonstrate a decrease in protein expression following 48 and 72 hrs of EBP50 knockdown compared to Scrambled control (* - $p < 0.05$, **** - $p < 0.0001$, n = 3).

D. Baseline resistance of the HPAECs monolayer transfected with EBP50 siRNA is decreased compared to Scramble-transfected, indicating increased endothelial permeability (* - $p < 0.05$, n = 3).

To determine whether EBP50 regulates endothelial permeability and drives vascular manifestations of EndMT in PH, the transendothelial electrical resistance (TEER) of EBP50 siRNA-transfected HPAEC monolayer was assessed real-time using the Electric Cell-substrate Impedance Sensing (ECIS) technology [415]. In response to the low frequency applied current (4000 Hz), the baseline resistance of EBP50 siRNA-transfected cells was lower than of Scrambled-transfected HPAECs (**Fig. 18D**, 870.1±24.1 ohm vs. 773.2±24.16 ohm for Scrambled- vs. EBP50 siRNA-transfected cells, $p<0.05$, $n=3$), indicating that the disruption of EBP50 has a negative effect on endothelial barrier function. However, co-stimulation of transfected cells with IL-1β did not result in a further impairment of the endothelial barrier function, corroborating our earlier findings that EBP50 knockdown does not have an additive effect on IL-1β-induced upregulation of EndMT transcription factors (**Appendix A, Figure 5**).

3.4 Discussion

Building on our early observations of the downregulation of EBP50 expression in the lungs and pulmonary endothelial cells of PAH patients and PH and PAH animal models (**Chapter 2**), our follow-up studies were intended to investigate the potential role of EBP50 in PH-associated signaling pathways implicated in endothelial dysfunction. By way of the in vitro phenocopy experiments utilizing the EBP50 siRNA, we identified a disruption of EBP50 as a regulator of EndMT in cultured HPAECs and demonstrated that EBP50 plays an important role in regulating cell proliferation and maintenance of the endothelial barrier function.

Whereas our understanding of the signaling mechanisms contributing to PAH- and PH-associated vascular remodeling is expanding continuously, much remains to be discovered about

the role of endothelial reprograming and EndMT in the disease progression. Knowledge amassed in the last decade and discussed in **Chapter 1** points at the importance of this process in the early events leading to a dysregulation of the vascular homeostasis and impaired intercellular cross-talk within the vascular wall [174,252]. Additionally, a number of recent studies highlighted the commonalities between the dysregulation of proteins previously implicated in cancer development and metastasis and PH-associated EndMT. As recently as last year, the elevation of the cancer-linked adhesion molecule CD44v8-10 was localized to the neointimal lesions in patients with PAH and was linked to EndMT in in vitro studies on HPAECs [367], as detailed in **Section 1.3.6.** Consistent with these findings and the known association between the dysregulated expression and nuclear localization of EBP50 in cancers and EMT, we identified the functional importance of the PH-associated downregulation of EBP50 in driving EndMT in HPAECs, demonstrated by a time-dependent upregulation of EndMT transcription factors, Snail, Slug and Zeb1, and a gain of mesenchymal markers N-cadherin, SM22, S100A4 and FN1 in HPAECs transfected with EBP50 siRNA construct. In our hands, the effects of EBP50 knockdown on expression of EndMT transcription factors and mesenchymal markers partially recapitulated actions of a known EndMT inducer IL-1β, prompting us to conclude that EBP50 dysregulation culminates, at least partially, in the induction of EndMT. Notably, the EBP50 knockdown had a potentiating effect on the hypoxia-induced activation of EndMT signaling, further arguing for the key role of EBP50 as a regulator of PH-associated EndMT. Consistent with the earlier reports linking EndMT to impaired barrier function [133,293], we have demonstrated that the knockdown of EBP50 increases the HPAEC monolayer permeability. However, the magnitude of the effect of EBP50 knockdown alone on HPAECs permeability appeared to be less pronounced than the combination of PH-relevant pro-EndMT and pro-PH cytokines IL-1β, TNFα and TGFβ used in a similar study. This discrepancy

is not surprising, given the cytokine's multifactorial nature, and argues that baseline EBP50 downregulation can exacerbate or predispose to the loss of endothelial barrier function driven by circulating proinflammatory molecules or hypoxic environment in PH. This observation, therefore, warrants deeper exploration. Moreover, combined with an identified role of the EBP50 downregulation as an inhibitor of endothelial proliferation, these results further argue that a decreased level of EBP50, such as one seen in the PH vasculature, contributes to the exacerbated vascular remodeling by impairing the restoration of endothelial monolayer integrity and barrier function in response to damage. It is worth exploring whether the negative regulatory effect of the EBP50 loss on ECs proliferation is important during the early stages of PH development, where ECs proliferation has a compensatory mechanism to protect against the vascular damage, rather than in terminal stages where uncontrolled endothelial proliferation contributes to the vascular pathology.

To further understand whether EBP50 downregulation seen in PAH and PH (**Chapter 2**) may drive PH development, we modeled hypoxia-induced PH in genetically altered global EBP50 heterozygous mice (EBP50$^{+/-}$, EBP50 Het). The study detailed in the **Chapter 4** is aimed to test whether the observations made in **Chapters 2 and 3** translate to in vivo phenotypes of consequence to dysregulation of EBP50 in PH.

4.0 Downregulation of EBP50 Expression Exacerbates Hypoxia-induced PH in Mice

4.1 Introduction

As a next step to deepen our understanding of the role of EBP50 downregulation in the progression of PH, we sought to determine whether mice with a partial deletion of EBP50 are predisposed to PH at baseline or when subjected to a chronic hypoxia exposure. In the last several decades, efforts of many research groups studying the molecular mechanisms behind PH-associated vascular remodeling have been aimed towards the search for molecular regulators of the vascular dysfunction, which can be targeted by the therapeutic interventions or gene therapies. In order to elucidate the hierarchy among the signaling pathways implicated in PH, genetically modified mice are used most commonly. For our studies, since the pulmonary EBP50 expression in human PAH tissue and different in vivo preclinical PAH and PH models is downregulated by approximately 50% but not entirely abolished compared to non-PH controls (**Fig. 1-2**), we chose to utilize EBP50 heterozygous (EBP50 Het, $EBP50^{+/-}$) mice instead of complete EBP50 knockouts (**Appendix A, Figures 8-9**).

4.2 Methods

All animal experiments were approved by and conducted in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee.

EBP50 Heterozygous mice: 9-16 weeks old adult male and female EBP50 Heterozygous mice ($EBP50^{+/-}$, EBP50 Het) bred on C57BL/6 background were subjected to continuous normobaric hypoxia (10% O_2) in a temperature-humidity controlled chamber (BioSpherix, Parish, NY) for

four weeks. At the termination of the study, hemodynamic data was obtained using appropriate size pressure-volume PV catheters (Transonic Systems Inc., Ithaca, NY) inserted directly through the right then left ventricle. After the hemodynamic data was obtained, heart and lung samples were collected and snap frozen in liquid nitrogen and stored for the future analysis. Hearts were separated into RV and left ventricle (LV) + septum, and Fulton index was calculated as an RV / (LV + septum) ratio.

4.3 Results

4.3.1 EBP50 Heterozygous Mice Subjected to Hypoxia Exhibit an Exacerbated PH Phenotype

Following the in vitro studies indicating that EBP50 plays a role in regulating endothelial cell reprogramming and function, we sought to determine whether the genetic downregulation of EBP50 exacerbates chronic hypoxia-induced PH in vivo (**Figure 19**). Genetic interruption of EBP50 in female EBP50 Het mice did not alter the baseline RV maximum pressure (RVmaxP; WT Normoxia, 26.18 ± 2.29 mmHg vs. Het Normoxia, 25.59 ± 1.34 mmHg, n = 5-9) or RV hypertrophy (Fulton Index: WT Normoxia, 0.25 ± 0.02 vs. Het Normoxia, 0.25 ± 0.01, n = 5-9). In contrast, there was a further rise in hypoxia-induced RVmaxP in EBP50 Het female mice subjected to hypoxia compared to hypoxia-exposed WT controls (WT Hypoxia: 35.56 ± 1.30 mmHg, vs. Het Hypoxia: 42.14 ± 0.92 mmHg, $p < 0.05$, n = 5-9). This increase was surprisingly not associated with an exacerbation of the hypoxia-induced RV hypertrophy (WT Hypoxia: 35.56 ± 1.30 mg/mm, vs. Het Hypoxia: 42.14 ± 0.92 mg/mm, n = 5-9), suggesting that, in the hypoxia mouse PH model, the functional effect of EBP50 loss may be initially manifested in the vasculature rather than the heart, which appeared to be well-adapted to the ~ 7mmHg additional pressure

overload. This conclusion is supported by analyzing the RV contractile index (max d*P*/d*t* normalized for RVmaxP pressure, 1 / sec), which remained stable among the four experimental groups. Interestingly, hypoxia-exposed male EBP50 Het mice did not demonstrate an exacerbated hypoxia-induced RVmaxP (WT Hypoxia, 35.94 ± 1.78 mmHg vs. Het Hypoxia, 35.54 ± 2.55 mmHg, n = 6), nor RV hypertrophy (WT Hypoxia, 0.34 ± 0.02 vs Het Hypoxia, 0.36 ± 0.01, n = 6) compared to WT controls, supporting a sex-specific effect of the EBP50 interruption on the pulmonary vasculature (**Appendix A, Figure 7**). This observation suggests that the effects of EBP50 may be closely tied to sex hormone signaling, consistent with the long-known role of estrogen receptor in regulating EBP50 in cancer cells [416] and linking the estrogen response in cancer to various cytoskeletal and cell signaling pathways [417], offering an insight to the prevalence of the human disease in females compared to males. Collectively, the in vivo results closely align with our in vitro data showing that the downregulation of EBP50 potentiates effects of hypoxia-driven signaling processes implicated in PH, such as EndMT (**Appendix A, Fig. 4**). Unlike the EBP50 Het mice, a total global knockout of EBP50 did not result in a greater increase in RVmaxP or Fulton index, or RV Contractile index in either female or male mice following the chronic hypoxia exposure compared to WT controls (**Appendix A, Figures 8-9**).

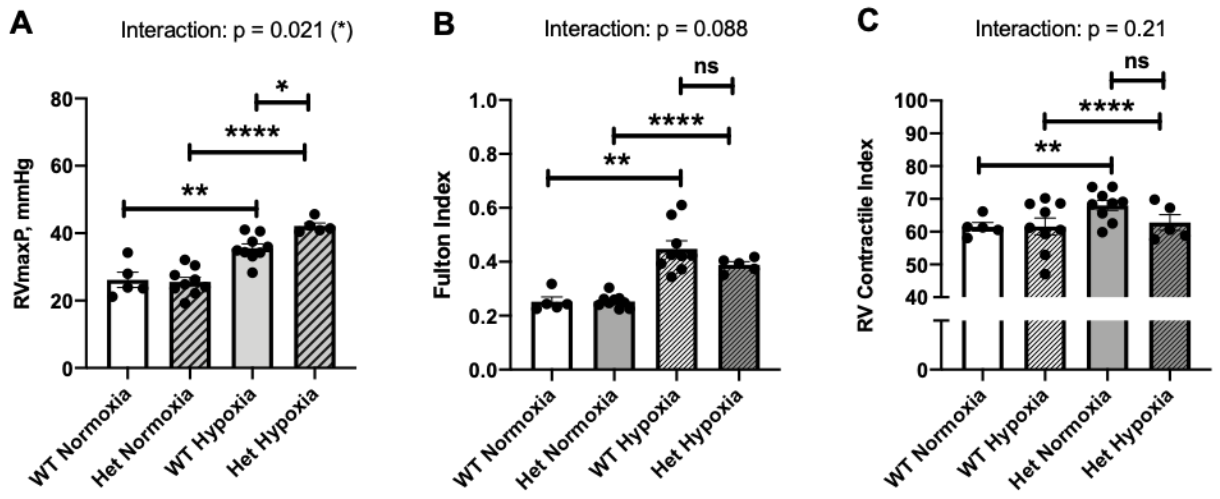


Figure 19. Female EBP50 Heterozygous mice subjected to chronic hypoxia exposure exhibit worsened hemodynamic outcomes compared to WT controls.
Experimental animals were placed in hypoxia (pO_2=10%) for 4 weeks. At the end of the treatment period, pressure-volume loop measurements were acquired through high-fidelity admittance catheter and hemodynamic parameters were calculated.

A. Hypoxia-induced elevation of the RVmaxPressure was more pronounced in EBP50 heterozygous female mice compared to wild-type controls.
B. Het Hypoxia mice did not exhibit an exacerbated RV hypertrophy (elevated Fulton Index) compared to WT Hypoxia group.
C. Het Hypoxia mice did not exhibit an altered RV Contractility Index compared to other experimental groups.

** - p<0.01, *** - p<0.001, **** - p<0.0001, n=5-9.
Data is represented as mean ± SEM.

4.4 Discussion

The data presented in this chapter identifies functional importance of the EBP50 downregulation for the exacerbation of hemodynamic manifestations of PH in response to chronic hypoxia exposure compared to wild-type animals. Moreover, the hemodynamic analysis described here supports our findings of the importance of EBP50 in the pulmonary vasculature (**Chapters 2 and 3**). Interestingly, despite an exacerbated RVmaxP, female EBP50 Het mice in hypoxia did not develop an additional increase in RV hypertrophy or a change in the contractility index, indicating

that the effects of EBP50 dysregulation in the early and somewhat moderate-severity disease stages may primarily affect the pulmonary vasculature, rather than the heart, which may be well-adapted in our mouse hypoxia model. Curiously, we have also found that the genetic downregulation of EBP50 in male mice had no effect on the hypoxia-induced increases in the RVmaxP and RV hypertrophy. This observation suggested to us that the effects of EBP50 may be closely tied to sex hormone signaling, which is not entirely surprising, considering the long-known role of the estrogen receptor in regulating EBP50 and linking the estrogen response to various cytoskeletal and cell signaling pathways [416,417]. Namely, mRNA and protein levels of EBP50 can be rapidly and directly upregulated by estrogen, but not other steroid hormones, in estrogen receptor-containing breast cancer cells [416]. Transcriptional activation of EBP50 is mediated through numerous half-estrogen response elements at the 5'-regulatory region and the first exon of EBP50 gene [417]. Knowing of the effects of estrogen on cAMP-mediated cellular pathways [418] and the formation of microvilli on the surface of breast cancer cells [419], effects of estrogen on breast tissue are postulated to be, at least in part, mediated by transient changes involving EBP50 and its interactions with NHE3 [191] and PKA [205]. Therefore, further studies warrant exploration into a potential sex-differential effect of EBP50 downregulation in the settings of cardiovascular disease and PH in particular, which predominantly affects females [24,25].

The lack of a phenotype under hypoxic exposure in both female and male KO mice was not expected but not surprising. Scaffolding proteins are known to have numerous associations and binding partners as discussed above and can therefore be involved in modulating various opposing functions in the cell. Complete knockout of EBP50 may have resulted in an overall abrogation of both pro- and anti-PH-associated EC responses. This discrepancy in response

between Het and KO mice however, remains open for interpretation and is the focus of future studies.

Collectively, further investigation is needed to tease out these relationships further, and this is an area of ongoing focused research in our laboratory.

5.0 Conclusions and Future Directions

5.1 Conclusions

In my thesis work, we identified the downregulation of a scaffolding PDZ protein EBP50 in the pulmonary vasculature and pulmonary endothelial cells as a common feature of PH and PAH in human patients and animal models of the disease. We determined that disruption of EBP50 is a driver of EndMT in cultured HPAECs and demonstrated that EBP50 plays an important role in regulating cell proliferation and maintenance of endothelial barrier function. Functional importance of EBP50 downregulation was established in the animal study utilizing genetically modified female EBP50 heterozygous mice, which demonstrated an exacerbation of the hemodynamic manifestations of PH in response to chronic hypoxia exposure compared to wild-type animals. These data identify a novel player in the pathogenesis of PH and offer mechanistic insights into the regulation of EndMT, a process increasingly recognized in this disease.

To our knowledge, we are the first to demonstrate a role for EBP50 in PH and its functional significance for the disease progression, which, our data indicate, manifests through an induction of EndMT. The highly dynamic nature of this process, characterized by hard-to-define initiation and progression, poses significant challenges for studying EndMT in vivo. There are multiple stages with considerable overlap and a broad range of intermediate phenotypes by which endothelial cells undergo a shift to mesenchymal-like cellular state [420]. To this date, little is known about where along the spectrum of PH initiation and progression EndMT most prominently manifests itself. Multiple studies have found associations between PAH and a whole lung expression of EndMT transcription factors, such as Snail and Slug [69,91,272], and Twist-1 [252]. However, those changes can also reflect the non-EndMT processes, such as EMT. It is also likely

that the process may be active to varying degrees between different forms PH, different patients within a PH WHO group, and even between different locations in the pulmonary vasculature from the same patient.

Our in vitro data indicate that, unlike other known EndMT inducers, such as IL-1β, reduction in EBP50 alone was not sufficient to fully drive complete EndMT by itself, evidenced by a lack of added effect on levels of endothelial markers, but instead had a potentiating effect on hypoxia-induced activation of EndMT transcription factors. This observation deserves further investigation and can potentially explain our in vivo findings in female EBP50$^{+/-}$ mice, which develop a more severe PH phenotype in response to chronic hypoxia compared to WT mice.

Hemodynamic analysis supports our findings of the importance of EBP50 in the pulmonary vasculature. Despite an exacerbated RVmaxP, female EBP50 Het mice in hypoxia did not develop an additional increase in RV hypertrophy or a change in the RV contractility index, indicating that the effects of EBP50 dysregulation may, in the early and somewhat moderate severity disease stages, primarily affect pulmonary arteries, rather than the heart, which may be well-adapted in the mouse hypoxia model. Interestingly, male EBP50 Het mice did not display either an exacerbated RVmaxP or RV hypertrophy after a chronic hypoxia exposure. This observation suggests that the effects of EBP50 may be closely tied to sex hormone signaling, which is consistent with the long-known role of estrogen receptor in regulating EBP50 in cancer cells and linking the estrogen response in cancer to various cytoskeletal and cell signaling pathways [416,417]. Further investigation is needed to tease out this relationship, which is an area of ongoing focused research in our laboratory. In addition, to test for a specific role of EBP50 downregulation in the endothelium we are planning to utilize EC-specific EBP50 Het mice.

It is important to recognize the limitations of the study stemming from technical challenges associated with detecting EndMT in vivo. The dynamic nature of the process, discussed earlier, complicates detection of transitioning cells. In our work, we were not able to detect a stable EndMT signature (upregulation of EndMT transcription markers, downregulation of endothelial markers and upregulation of smooth muscle markers) in isolated PECAM-positive endothelial cells of human patients and hypoxia-treated mice, or in whole lung homogenates of hypoxia-treated mice (*data not shown*). Isolating endothelial cells based on expression of endothelial markers for the study of EndMT is inherently flawed and undermines our ability to select cells that are primed for transitioning or misses cells that have undergone the transitioning and have already lost their endothelial markers. To circumvent this problem, as will be discussed in **Section 5.2**, we are planning to utilize endothelial lineage-tracing in vivo mouse models. Secondly, the low percentage of transitioning cells in the pulmonary vasculature (around 6% in Su-Hx mice and 4% in humans [133]) is making the detection of EndMT in whole lung homogenates by conventional techniques, such as rt-PCR and Western blot, highly ineffective. As such, we are planning to utilize more sophisticated whole lung imaging techniques to better detect and quantify the transition, in addition to using single-cell RNA sequencing (discussed in **Section 5.2**), that will allow us to simultaneously profile expression of thousands of genes within a single cell in the whole lung homogenates.

Our findings consistently demonstrate that the knockdown of EBP50 negatively affects cell proliferation. Impaired endothelial proliferation, however, might reflect not only EndMT-associated changes, but also indicate a connection between the loss of EBP50 and other processes, such as cell senescence, and needs to be further explored. However, because the functional connection between EndMT and PH-associated changes in endothelial proliferation is still debated

[90,133,174,293], one can speculate that such inconsistencies may reflect the disease stage and extent of EndMT progression, and could be precipitated by varying degrees of the EBP50 loss.

Together, this work enhances our understanding of the role of ERM-binding proteins in vascular biology in general and PAH / PH in particular, and maps out potential avenues for the development of therapeutics specifically targeting endothelial cells in PH. Here we present data that support the development of pharmacological interventions or gene therapies aiding the restoration of normal EBP50 expression in pulmonary endothelial cells and open doors to a more in-depth investigation of the link between EBP50 loss, EndMT and PAH progression in vivo. Indeed, whereas the precise role of EndMT in PH and the mechanisms underlying its regulation remains inadequately understood, our work sheds some light on these mechanisms and introduces EBP50 as a potential upstream regulator. Future studies are expected to elucidate the mechanistic connection between EBP50 downregulation and vascular remodeling in PH in more detail and will provide a road map for the novel therapeutic strategies for this devastating disease.

5.2 Future Directions

As discussed in **Section 5.1** and **Chapter 1**, the study of EndMT in PH is associated with numerous limitations and challenges, some of which we are hoping to circumvent and address in our future studies. As discussed earlier, the detection of EndMT in the vasculature is extremely challenging, specifically owing to the dynamic nature of this process. New technologies of the tissue-specific animal labeling allow for the tracing of cells of specific lineages along their lifespan. Namely, the development of the endothelial lineage tracing mice, already employed by some researchers [174,271], will be instrumental to further our understanding of the EndMT dynamics

throughout the disease progression. By genetically labeling cells of endothelial lineage, we will be able to trace the fate of endothelial cells and more precisely detect EndMT even in cells that have fully lost their endothelial markers and transformed into mesenchymal-like cells. In our lab, we are currently developing the endothelial lineage-tracing mice and hoping to enrich our studies by also utilizing endothelial-specific EBP50 Het mice (we are currently generating the LoxP EBP50 mice). As opposed to global EBP50 knockout and Het mice, which suffer from potential off-target effects of the EBP50 knockdown, including the alterations in the peripheral nervous system and the brain [216,421], inducible cell type-specific mice will allow us to draw more definitive conclusions between EBP50 and endothelial phenotypes associated with PH progression. Crossing these mice with endothelial lineage-tracing mice will also allow us to test the EndMT EBP50 connection in vivo.

Furthermore, the precise mechanism by which EBP50 controls EndMT progression remains to be elucidated. Our current work suggests a connection between the loss of EBP50, EndMT and an upregulation of β-catenin (**Fig. 12**), however, it is still unknown whether EBP50 induces expression and activation of EndMT transcription factors Snail, Slug and Zeb1 directly, or via an intermediate binding partner, such as β-catenin. We will expand on this finding in the future work by utilizing EBP50 WT and mutant constructs lacking PDZ or EB domains in the in vitro protein-protein interaction analyses to specifically identify EBP50-binding proteins in pulmonary endothelial cells under PH-related stimuli and map out the relevant binding sites. These experiments will be instrumental to our understanding of how EBP50 controls expression of EndMT transcription factors. By using pull-down assays of protein-protein interactions, we might also be able to identify novel binding partners of EBP50 that might offer a critical look into the regulation of EndMT process in PH settings.

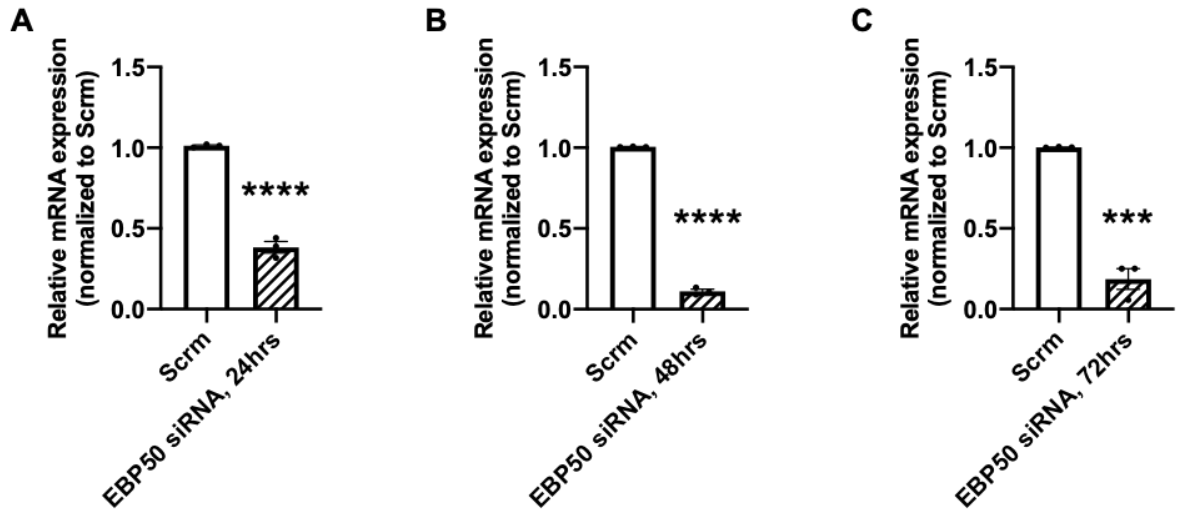
Considering a wide range of its binding partners, spanning from the receptor kinases to ion channels and exchangers [186,187,196–202,188–195], it is highly likely that EBP50 affects more than one inter- and intra-cellular signaling pathway. In our future studies, we are interested in exploring some of those connections further. For example, EBP50 has been known to interact with a member of the aquaporin channel family, AQP9 [194]. Curiously, mutations in its homolog, AQP1 were found in a subset of patients with hPAH [422], thus making a compelling argument of studying a connection between EBP50 and members of the AQP family in PH settings. In addition to being a water channel, AQP1 can also facilitate the diffusion of H_2O_2 into vascular SMCs, where it can activate Nox1 and induce ROS production and SMC hypertrophy [423]. Considering a known connection between EBP50 and Nox1 [226], it is interesting to explore a potential link between the loss of EBP50 and AQP1 activity in endothelial and smooth muscle cells in PH settings. Our preliminary data suggest that expression of AQP1 is dramatically suppressed by IL-1β treatment (*data not shown*). Considering the effects of IL-1β on EBP50 expression (**Fig. 11**), we are interested to determine whether the downregulation of EBP50, at least in part, drives the loss of AQP1 and affects the paracrine regulation of the intracellular oxidative balance.

Likewise, we are interested in exploring the link between EBP50 downregulation and endothelial senescence and metabolic reprogramming, two phenotypes strongly associated with endothelial dysfunction in the lung vasculature. Certainly, our data on a reduction in proliferation with no effect on apoptosis of EBP50 knockdown also support this notion.

Finally, owing to our observation of the sex differences between the response of EBP50 Het mice to hypoxia (**Chapter 4**), it will be interesting to dissect this relationship further and utilize the RNA sequencing techniques to determine whether the level of expression of EBP50 is a sex-specific determinant of disease severity in human patients by assessing co-association of

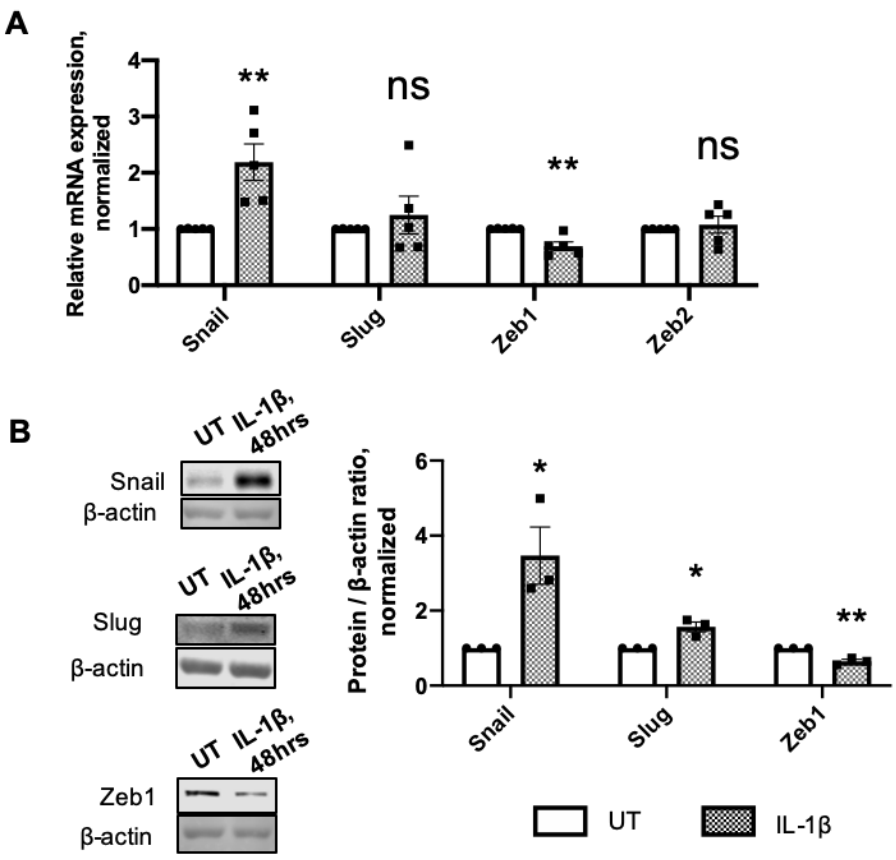
transcriptomic data on sex-hormone receptor activation of transcriptional programs with levels of EBP50 and EBP50-binding partners and downstream targets as well as indices of EndMT. These can then be followed by further interventional studies to establish causality. Furthermore, utilizing single-cell RNA sequencing will allow us to analyze the co-associations between expression of EndMT markers (such as the relative level of expression of EndMT transcription factors and the degree of the loss of endothelial markers and gain of mesenchymal markers) in relation to the level of EBP50 in the same cell. Those studies, while highly laborious, will provide invaluable insights into the mechanism of action of EBP50 in the pulmonary vasculature and have the potential to establish a human PH in vivo precedent to our in vitro findings.

Appendix A Supplementary Figures



Appendix Figure 1. EBP50 knockdown efficiency in HPAECs.

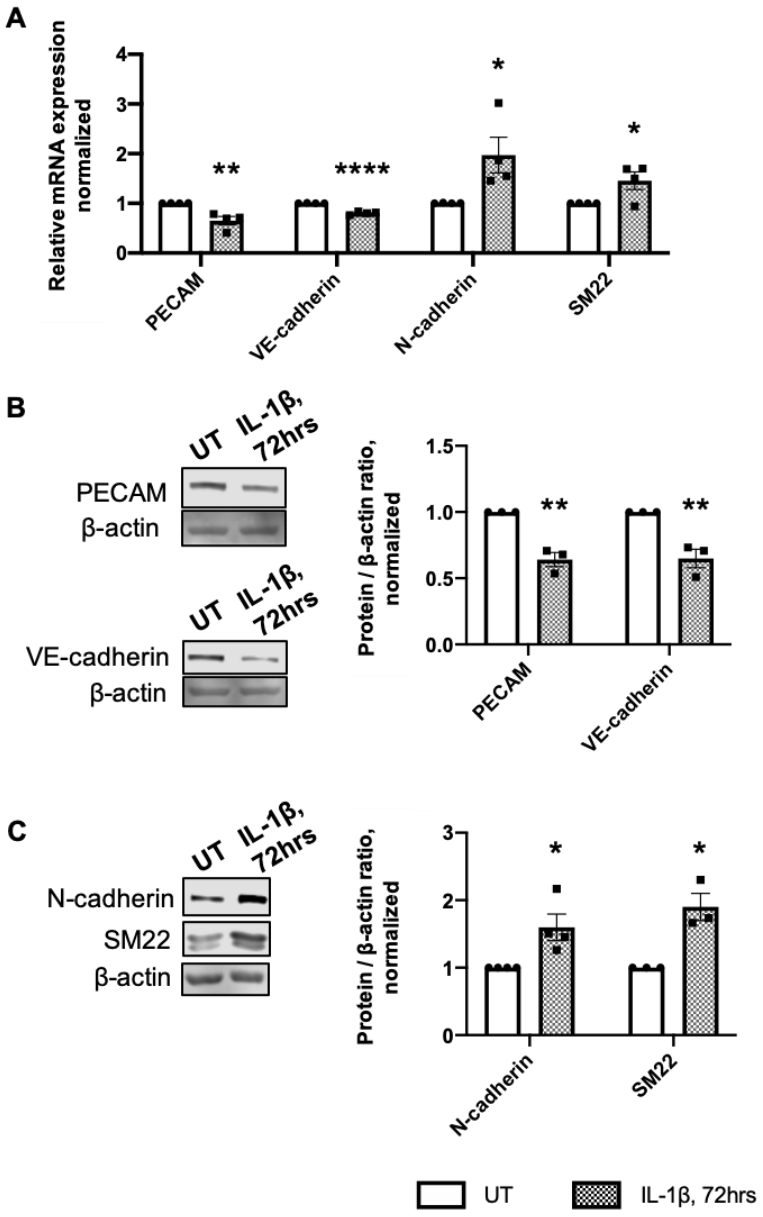
A. EBP50 siRNA transfection efficiency 24 hours post-transfection as determined by rt-qPCR: 0.38±0.06-fold from Scrambled siRNA (Scrm) [n=3, $p < 0.0001$].
B. EBP50 siRNA transfection efficiency 48 hours post-transfection as determined by rt-qPCR: 0.11±0.02-fold from Scrm [n=3, $p < 0.0001$].
C. EBP50 siRNA transfection efficiency 72 hours post-transfection as determined by rt-qPCR: 0.19±0.06-fold from Scrm [n=3, $p < 0.0001$].



Appendix Figure 2. IL-1β alters the expression of EndMT transcription factors Snail, Slug and Zeb1.

A. mRNA level of EndMT transcription factor Snail is upregulated following 48 hrs of 10 ng/ml IL-1β treatment, while Zeb1 mRNA is downregulated (** - $p < 0.01$ from Scrambled siRNA, n = 5).

B. Protein expression of EndMT transcription factors Snail and Slug is upregulated following 48 hrs of 10 ng/ml IL-1β treatment, while Zeb1 is downregulated (* - $p < 0.05$, ** - $p < 0.01$ from Scrambled siRNA, n = 3).

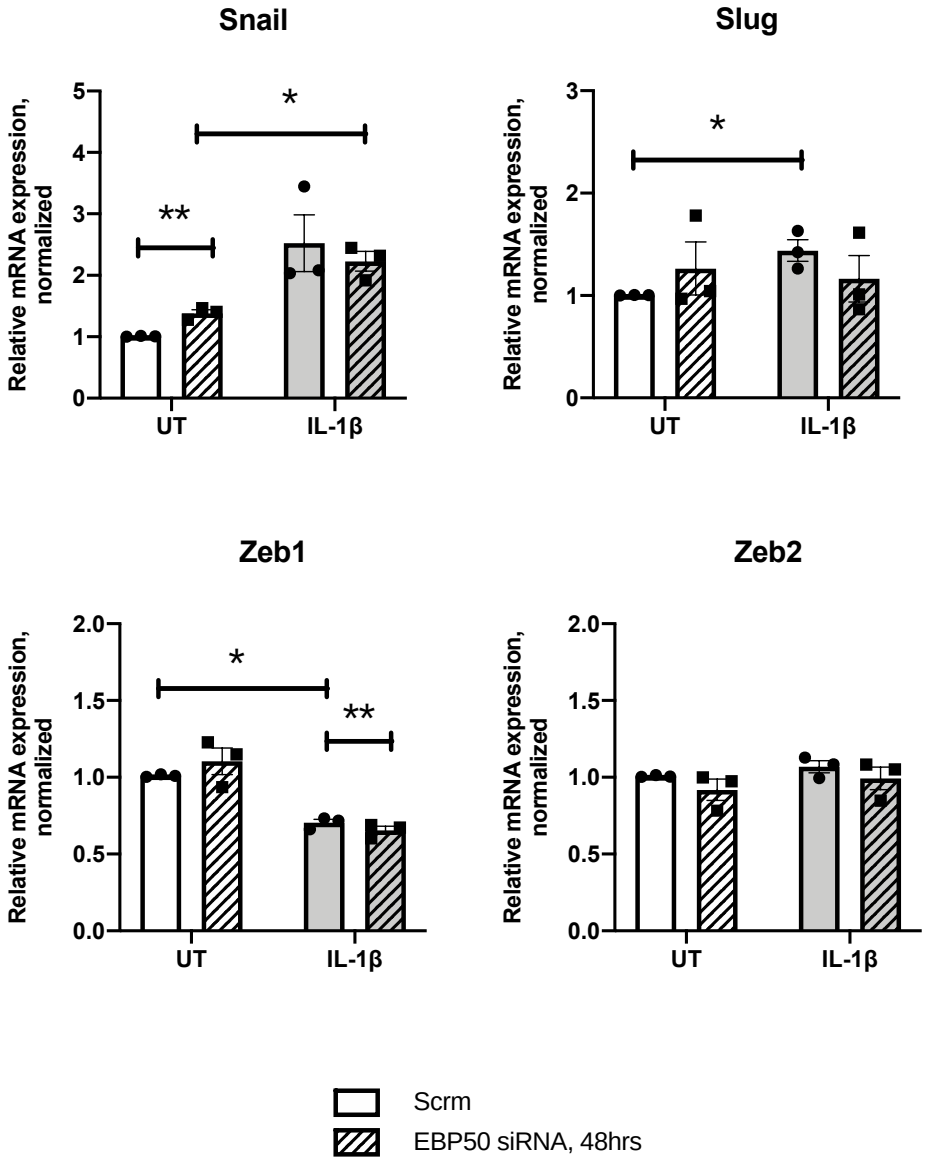


Appendix Figure 3. IL-1β promotes a loss of endothelial markers and a gain of mesenchymal markers by the pulmonary endothelial cells.

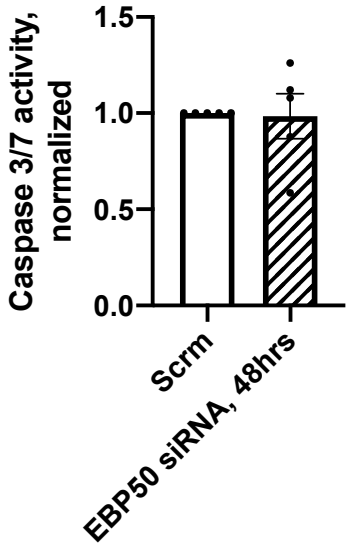
A. mRNA expression of endothelial markers PECAM and VE-cadherin is downregulated, while mesenchymal markers N-cadherin and SM22 are upregulated following 72 hrs of 10 ng/ml IL-1β treatment (* - $p < 0.05$, ** - $p < 0.01$, **** - $p < 0.0001$ from Scrambled siRNA, n =). mRNA normalized to 18s was quantified using ΔΔCt rt-PCR method.

B. Representative Western immunoblots (left) and densitometric quantification (right) of PECAM and VE-cadherin demonstrate a decrease in protein expression following 72 hrs of 10 ng/ml IL-1β treatment (** - $p<0.01$ from Scrambled siRNA, n = 3). Relative protein expression was calculated as a ratio to β-actin level in a respective sample.

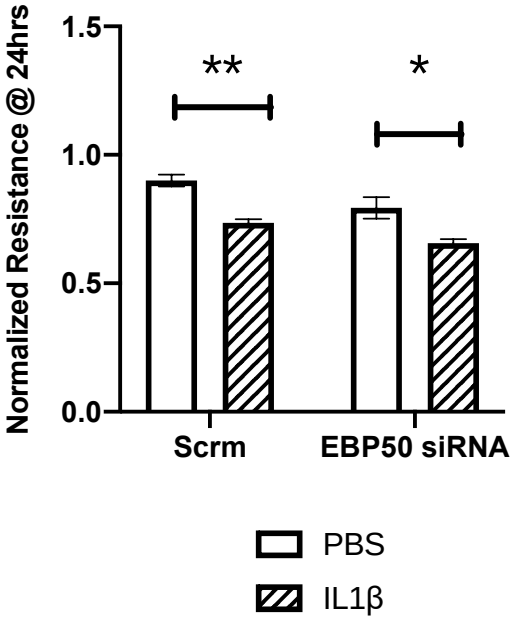
C. Representative Western immunoblots (left) and densitometric quantification (right) of N-cadherin and SM22 demonstrate an increase in protein expression following 72 hrs of 10 ng/ml IL-1β treatment (* - $p<0.05$ from Scrambled siRNA, n = 3). Relative protein expression was calculated as a ratio to β-actin level in a respective sample.



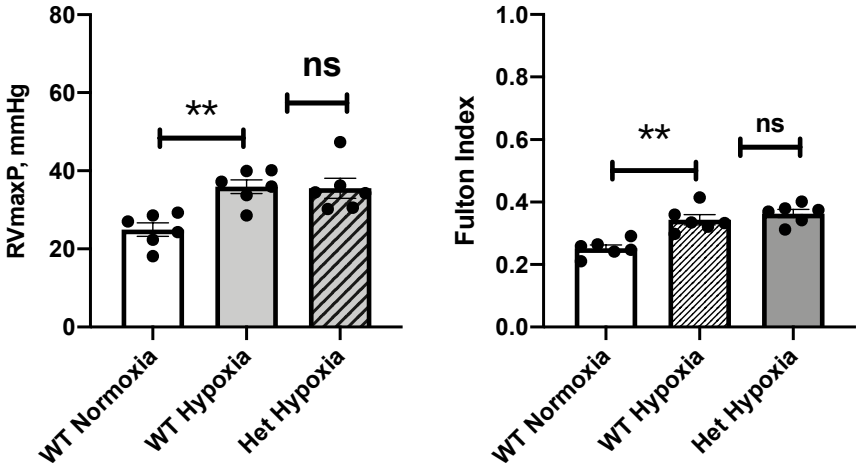
Appendix Figure 4. EBP50 knockdown does not affect IL-1β-induced changes in expression of EndMT transcription factors.
* - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, n = 3. mRNA normalized to 18s was quantified using ΔΔCt rt-PCR method.



Appendix Figure 5. EBP50 knockdown does not induce apoptosis, as indicated by caspase 3/7 cleavage evaluation.
Luminescent assay of caspase-3/7 activity demonstrated no difference between Scrambled and EBP50 siRNA-transfected HPAECs after 48 hrs of transfection.



Appendix Figure 6. EBP50 knockdown does not exacerbate IL-1β-induced increase in endothelial permeability as assesed by the measurement of a monolayer resistance
As measured by the ECIS technology, normalized resistance of the HPAECs monolayer was decreased following 24 hrs of treatment with 10 ng/ml IL-1β to a similar extent in Scrambled and EBP50 siRNA-transfected wells.
* - $p < 0.05$, ** - $p < 0.01$, n = 3.



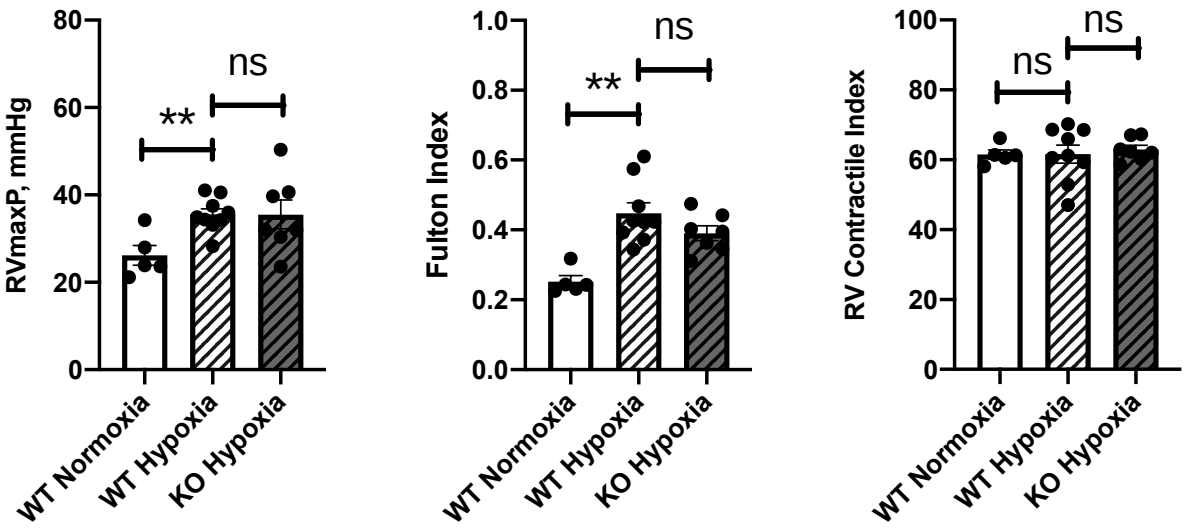
Appendix Figure 7. Male EBP50 Heterozygous mice subjected to chronic hypoxia exposure do not exhibit worsened hemodynamic outcomes compared to WT controls.

Experimental animals were placed in hypoxia (pO_2=10%) for 4 weeks. At the end of the treatment period, pressure-volume loop measurements were acquired through high-fidelity admittance catheter and hemodynamic parameters were calculated.

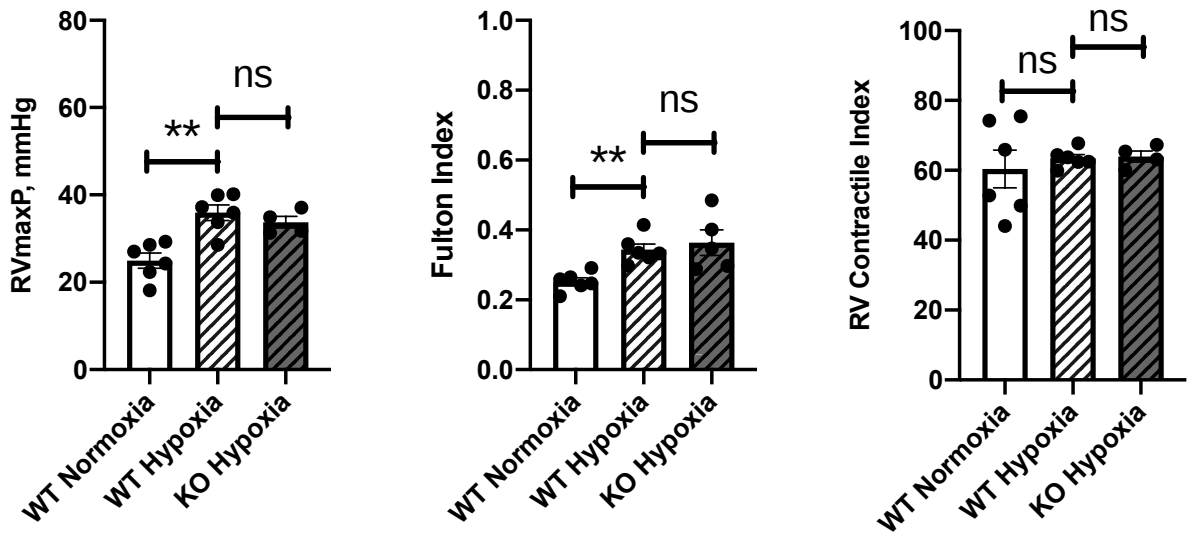
A. Hypoxia-induced elevation of the RVmaxPressure was not more pronounced in EBP50 heterozygous female mice compared to wild-type controls.
B. Het Hypoxia mice did not exhibit an exacerbated RV hypertrophy (elevated Fulton Index) compared to WT Hypoxia group.

** - p<0.01, *** - p<0.001, **** - p<0.0001, n=5-9.

Data is represented as mean ± SEM.



Appendix Figure 8. Female global EBP50 knockout mice subjected to chronic hypoxia exposure do not exhibit worsened hemodynamic outcomes compared to WT controls.
Hypoxia-induced elevation of the RVmaxPressure was not more pronounced in EBP50 knockout female mice compared to wild-type controls (*left*). EBP50 knockout female mice did not exhibit an exacerbated RV hypertrophy (elevated Fulton Index) compared to WT Hypoxia group (*center*). RV Contractile idex remained stable among all three experimental groups (*right*).
** - $p<0.01$, n=5-9. Data is represented as mean ± SEM.



Appendix Figure 9. Male global EBP50 knockout mice subjected to chronic hypoxia exposure do not exhibit worsened hemodynamic outcomes compared to WT controls.
Hypoxia-induced elevation of the RVmaxPressure was not more pronounced in EBP50 knockout female mice compared to wild-type controls (*left*). EBP50 knockout female mice did not exhibit an exacerbated RV hypertrophy (elevated Fulton Index) compared to WT Hypoxia group (*center*). RV Contractile idex remained stable among all three experimental groups (*right*).
** - $p<0.01$, n=5-9. Data is represented as mean ± SEM.

Appendix B Supplementary Tables

Appendix Table 1. Demographic and clinical characteristics of non-PAH control and PAH patient population used for immunofluorescent staining.

Group	ID #	Sex	Age	PH group	Type PH
control	1	Male	72	N/A	N/A
	2	Female	69	N/A	N/A
	3	Male	68	N/A	N/A
	4	Male	65	N/A	N/A
PAH	1	Female	34	Group 1 PH	Idiopathic
	2	Female	64	Group 1 PH	Idiopathic
	3	Female	68	Group 1 PH	Scleroderma
	4	Male	12	Group 1 PH	Familial (BMPR2 mutation)
	5	Male	16	Group 1 PH	Idiopathic
	6	Male	1	Group 1 PH	Trisomy 21
	7	Male	19	Group 1 PH	Idiopathic
	8	Female	42	Group 1 PH	Scleroderma

Appendix Table 2. Demographic and clinical characteristics of non-PAH control and PAH patient populations used for pulmonary endothelial cell isolation.

Group	ID #	Sex	Age	PH group	Type PH	mPAP, mmHg	PCWP, mmHg	PVR, WU
control	1	female	53	N/A	N/A	N/A	N/A	N/A
	2	male	25	N/A	N/A	N/A	N/A	N/A
	3	female	34	N/A	N/A	N/A	N/A	N/A
	4	male	24	N/A	N/A	N/A	N/A	N/A
	5	female	36	N/A	N/A	N/A	N/A	N/A
	6	female	50	N/A	N/A	N/A	N/A	N/A
PAH	7	male	53	group 1 PH	idiopathic	56	5	3.86
	8	male	21	group 1 PH	idiopathic	69	12	19.74
	9	female	62	group 1 PH	familial	48	8	8
	10	female	29	group 1 PH	idiopathic	110	19	6.29
	11	female	16	group 1 PH	idiopathic	N/A	10	N/A
	12	female	40	group 1 PH	idiopathic	N/A	N/A	N/A

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