AKT-MEDIATED PATHWAY REGULATING ENDOTHELIAL-TO-MESENCHYMAL TRANSITION (ENDMT) AND PULMONARY VASCULAR REMODELING

BY

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ABSTRACT

Endothelial-to-mesenchymal transition (EndMT) is greatly implicated in various cardiopulmonary diseases. Although TGFβ has been shown to be involved in inducing EndMT, its downstream mechanisms especially in relationship with Akt1, a key serine threonine kinase vastly involved in regulating endothelial homeostasis and its role in EndMT remain unknown. The objective of this project is to understand the Akt-mediated regulation of EndMT and its contributions to pulmonary vascular remodeling. Using lentiviral mediated Akt1 knock down and pharmacological inhibition of Akt1 in endothelial cells we showed that endothelial loss of Akt1 enhances VEGF induced vascular permeability in the short term while blocks the VEGF- and Ang1-induced barrier stability in the long term. TGFβ1 inhibits Akt in the long term while activating Src resulting in enhanced vascular permeability indicating the protective role of Akt1 in regulating endothelial barrier integrity. Endothelial loss of Akt1 results in increased expression of mesenchymal markers N-cadherin and αSMA, and decreases the expression of endothelial marker eNOS via upregulation of mesenchymal transcription factor Snail. This is mediated by the upregulation of the most predominant EndMT inducing TGFβ isoform, TGFβ2 and its downstream pro-fibrotic Smad, Smad2/3. In Chronic Hypoxia and Hypoxia-SUGEN models, endothelial specific loss of Akt1 in tamoxifen-inducible VE-cad-CreAkt1 mice resulted in exacerbated hypoxia-induced vascular remodeling compared to that of their WT controls. Inhibition of β-catenin using ICG-001 reversed the hypoxia- and hypoxia-SUGEN-induced-

vascular remodeling. SUGEN (SU-5416), a selective VEGFR2 inhibitor induced irreversible vascular remodeling in mice via suppression of Akt1. Together these results demonstrate the involvement of Akt1 in regulating endothelial barrier and homeostasis and that loss of Akt1 results in increased vascular permeability and EndMT *in vitro* and vascular remodeling *in vivo*. Targeting nuclear localization of β-catenin might be a potential therapeutic option to prevent excessive vascular remodeling in several cardio-pulmonary diseases such as COPD, PAH etc.

INDEX WORDS: Akt1; EndMT; Pulmonary arterial hypertension; β-catenin; TGFβ2; FoxC2

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DEDICATION

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This thesis is a tribute to you both.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Endothelium and endothelial to mesenchymal transition (EndMT)

The endothelium plays a key role in regulating vascular integrity. Under normal conditions, the vascular endothelium secretes a variety of vasoactive substances including NO and prostacyclin, which protect the vascular wall against vasoconstriction, inflammatory and proliferative changes, and thrombus formation [1, 2]. Recent studies suggest that impairment of endothelial function, as observed in the presence of cardiovascular risk factors, is not only a marker but also contributes to the pathogenesis of cardiovascular disease. Thus, improving endothelial function is an important therapeutic target for reducing vascular diseases such as arteriosclerosis and pulmonary arterial hypertension [3, 4]. Endothelial cells (ECs) act as gatekeepers, controlling the infiltration of blood proteins and cells into the vessel wall. This unique characteristic is largely exerted by the coordinated opening and closure of cell–cell junctions. Cell–cell junctions that comprise of adherens junctions (AJs) and tight junctions (TJs) can also act as signaling structures that communicate cell position, limit growth and apoptosis and, in general, regulate vascular homeostasis [5, 6].

Endothelial to mesenchymal transition (EndMT) is defined as the phenomenon in which endothelial cells lose their endothelial specific features and acquire mesenchymal properties [7, 8]. EndMT is characterized by a loss of cell-cell adhesion and changes in cell polarity inducing a spindle-shaped morphology. These changes are accompanied by reduced expression of the endothelial such as VE-cadherin and CD31, and increased expression of the mesenchymal markers including fibroblast specific protein-1 (FSP-1), alpha smooth muscle actin (α-SMA), N-cadherin, and fibronectin [9]. Loss of cell-cell adhesion is mediated by transcription factors such as Snail, Slug, ZEB-1, SIP-1, Twist, and LEF-1 that suppress transcription of genes encoding proteins involved in formation of AJs and TJs which are critical in maintaining a healthy intact endothelium [10, 11].

EndMT is an essential mechanism implicated not only in cardiac development but also in the progression of diseases like atherosclerosis, pulmonary hypertension, diabetic nephropathy and cardiac fibrosis [12-15]. EndMT is also implicated in cancer where Xiao et al showed that TGF-β driven EndMT produces a spectrum of endothelial cell phenotypes with different functions that define the plasticity and heterogeneity of tumor vasculature [16]. Aberrantly regulated EndMT results in unscheduled conversion of endothelial cells (EC) into diverse mesenchymal-lineage cell types, especially myofibroblasts (MF), which may dissociate from the vessel wall and can be found throughout the affected tissue. Myofibroblasts are specialized cells within the body that aid in wound healing. Upon activation by biochemical and mechanical signals, myofibroblasts secrete and organize ECM, develop specialized matrix adhesions [17], and exhibit cytoskeletal organization characterized by contractile actin filaments [18]. Together, these features allow for re-establishment of mechanical integrity and stability to the damaged tissue and enable the myofibroblasts to exert large contractile forces on their microenvironment thus assisting in both the closure of the wound and remodeling of the tissue which can lead to pathological remodeling when aberrantly stimulated [19, 20].

1.2. Endothelial injury and barrier breakdown trigger EndMT

Intact cell-cell junctions are critical in maintaining a healthy and functional endothelium. Any change in junction organization results in complex consequences that compromise endothelial reactions with blood elements or modify the normal architecture of the vessel wall. At both tight junctions (TJs) and adherens junctions (AJs), adhesion is mediated by transmembrane proteins that promote homophilic interactions and are linked inside the cells to specific intracellular partners that mediate their anchorage to the actin cytoskeleton. At AJs, adhesion is mediated by cadherins [5, 6]; while at TJs it is mediated by members of the claudin family [21]. ECs express cell-type-specific transmembrane adhesion proteins such as VE-cadherin at AJs and claudin-5 at TJs [22]. In ECs, there is a requirement for AJs (more specifically, VE-cadherin) for vascular development in the embryo. Genetic inactivation of VE-cadherin causes early embryo lethality due to lack of vascular remodeling [23]. In the adult, VE-cadherin-blocking antibodies induce a marked increase in vascular permeability [24]. Thus, VE-cadherin expression and organization at AJs is a crucial determinant for vascular stabilization. Besides its adhesive properties, VE-cadherin acts by transferring intracellular signals by engaging signaling proteins such as phosphatidylinositol-3-OH kinase (PI(3)K) or growth factor receptors, or indirectly by tethering and retaining

transcription factors at the cell membrane such as beta-catenin or p120 catenin and thereby limiting their translocation to the nucleus [25]. Taddei et al demonstrated that transcriptional upregulation of claudin-5 requires expression and clustering of VE-cadherin at junctions. This effect is due to the inactivation of the forkhead box factor FoxO1 and beta-catenin, which, through binding to the claudin-5 promoter, inhibit transcription [26]. VE-cadherin also modulates VEGF mediated c-Src activation by the recruitment of protein tyrosine phosphatases SHP2 to VE-cadherin signaling complex which leads to the release of Csk (C-terminal Src kinase), a negative regulator of Src activation [27].

Vascular endothelial growth factor (VEGF) and Angiopoietin 1 (Ang1) play essential and complementary roles in vascular development during embryogenesis. Whereas VEGF is required for the formation of the initial vascular plexus early in development, Ang1 is necessary for the subsequent vascular remodeling into mature blood vessels [28, 29]. Both VEGF and Ang1 share the ability to promote endothelial survival, proliferation, and migration, by acting on their cognate cell-surface tyrosine-kinase receptors, VEGFR2 (Flk1, KDR) and Tie2 (Tek), respectively [30]. While VEGF causes vascular permeability and tissue edema, Ang1 contributes to the stabilization and the maturation of growing blood vessels [31, 32]. VEGF Esser et al demonstrated that VEGF transiently phosphorylates the components of adherens junctions: VE-cadherin, β-catenin, plakoglobin, p120 and PECAM-1 and hence modulates intercellular contacts during angiogenesis and regulates vascular permeability [33].

The Src family is composed of non-receptor tyrosine kinases, which regulate cell proliferation, migration, apoptosis, and ECM adhesion functions [34]. Src regulates endothelial barrier function and also plays a key role in mediating inflammatory vascular hyperpermeability [35]. These protein kinases are expressed by macrophages, neutrophils, endothelial cells, alveolar epithelial cells, and fibroblasts in the lung [36]. The role of Src family kinases in regulating biological responses is associated with their ability to link other signaling proteins within oligomeric complexes as an adapter protein and to phosphorylate some components of these signaling complexes on critical tyrosine residues [37, 38]. The structural and functional interaction between Src family kinases and cellular proteins integrates a large amount of upstream signaling that coordinately regulates cellular activities. Gavard et al showed that Ang1 interferes with the ability of VEGF to disrupt the endothelial barrier by preventing Src activation, which is an obligatory

component of the pathway by which VEGF provokes vascular permeability [39] and that this process involves the activation of RhoA by Ang1 and the consequent association of mDia, a RhoA downstream target, with Src, thereby preventing the activation of Src by the VEGF receptor VEGFR2. Ultimately, by uncoupling Src to VEGFR2, Ang1 may interfere with the ability of VEGF to initiate the activation of a Src-dependent intracellular signaling route that culminates in the serine phosphorylation-dependent internalization of VE-cadherin and the disassembly of interendothelial adherens junctions, thus preventing VEGF-induced endothelial permeability [27, 39].

Src contributes to pulmonary vascular inflammation and hence acute lung injury (ALI) by phosphorylating ICAM-1 and promotes transcellular transport by phosphorylating caveolin-1 as well as regulate downstream signaling leading to disruption of adherens junctions [35, 40]. In a very recent study, Li et al reported that activation of Src dependent smad3 signaling is required to mediate ventilator induced lung injury [41]. It has been shown by Piegeler et al that inhibition of early onset of Src signaling by Ropivacaine attenuates acute lung injury [42]. In a rat model of cerebral ischemia it was shown that increased phosphorylation of Src aggravates the leakage of blood brain barrier via upregulation of VEGFA [43]. This along with evidences that Src is overactivated following cerebral ischemic injury present the possible involvement of Src in disrupting pulmonary endothelial barrier integrity following endothelial injury.

Recently, it has been hypothesized and supported by few reports that endothelial injury and barrier disruption trigger a myriad of responses by endothelial cells (Figure 1) [44]. The early responses include apoptosis and secretion of inflammatory mediators that recruit macrophages and other inflammatory cells assisting in wound repair while in the later stages the apoptotic resistant endothelial cells either communicate with surrounding cell types in the vasculature including vascular smooth muscle cells and fibroblasts to undergo phenotypic transformation into myofibroblasts that assist in wound healing which when uncontrolled results in extensive vascular remodeling due to excessive ECM deposition or ECs by themselves undergo cellular transdifferentiation by a process called endothelial to mesenchymal transition [45, 46]. This leads to uncontrolled proliferation, migration and invasion of transformed ECs contributing to pathological vascular remodeling as seen in many diseases including pulmonary arterial hypertension [44].

1.3. PI3 Kinase (PI3K)-Akt signaling in ECs

Numerous signaling pathways in ECs, such as those involving G protein–coupled receptors, mitogen activated protein kinases (MAPKs), protein kinase B/Akt, and small G proteins, are involved in normal endothelial functioning, particularly, intracellular signaling pathways mediated by protein kinase Akt are involved in the regulation of cell survival, proliferation, migration, glucose metabolism, and gene expression [47, 48].

Akt, originally identified as a cellular counterpart of the oncogene derived from murine AKT8 retrovirus [49, 50] was later independently isolated as a protein kinase related to protein kinase A and C and was therefore named as protein kinase B (PKB) or RAC (related to protein kinase A and C) [47]. Mammalian genomes contain three Akt genes, Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ which encode proteins containing a pleckstrin homology (PH) domain in the amino terminus, a central kinase domain, and a carboxy terminal regulatory domain [51, 52]. All 3 mammalian Akt genes are widely expressed in various tissues but Akt1 is most abundant in brain, heart, and lung, whereas Akt2 is predominantly expressed in skeletal muscle and embryonic brown fat, and Akt3 is predominantly expressed in brain, kidney, and embryonic heart [47, 53, 54]. In unstimulated cells, Akt protein exists in cytoplasm and the two regulatory phosphorylation sites at threonine at 308 and serine at 473 are in an unphosphorylated state. Upon growth factor stimulation, the PH domain binds to the lipid products of phosphoionositide-3 kinase (PI3K), and Akt is recruited to plasma membrane. Akt is then sequentially phosphorylated at T308 and S473 by upstream kinases referred to as 3-phosphoinositide–dependent protein kinase 1 (PDK1) and PDK2, respectively, which yields a fully activated kinase [55]. PDK1 has been isolated and characterized [56], but the identity of PDK2 is still controversial. Several candidate molecules have been suggested to be a potential S473-kinase including integrin-linked kinase (ILK), MAP kinase–activated protein kinase 2 (MK2), PDK1 (conversion of substrate specificity in association with protein kinase C–related kinase-2 [PRK2]) and Akt itself (autophosphorylation) [57, 58]. Fully activated Akt becomes available to phosphorylate its downstream substrates and a portion of these molecules detach from the plasma membrane and translocate to various subcellular locations including nucleus [59]. Akt is then dephosphorylated and inactivated by protein phosphatases such as protein phosphatase 2A (PP2A) [60].

Akt is a critical regulator of PI3K-mediated cell survival [61]. A large number of studies have demonstrated in various cell types that constitutive activation of Akt signaling is sufficient to block cell death induced by a variety of apoptotic stimuli and that transduction of dominant-negative Akt inhibits growth factor–induced cell survival [62]. It is interesting to note that the vascular permeability factor VEGF enhances the endothelial cell survival via Flk1/VEGFR2-PI3K-Akt pathway [63] following which it was shown that several other endothelial cell stimuli including angiopoietin-1 (Ang-1) [64], insulin, insulin-like growth factor-I (IGF-I), sphingosine-1-phosphate (S1P), hepatocyte growth factor and the small proteoglycan decorin [65-67], also activate PI3K-Akt signaling, illustrating the central role of this pathway in controlling endothelial cell viability.

In ECs and possibly endothelial progenitor cells (EPCs), a variety of growth factors, such as VEGF and insulin; HMG-CoA reductase inhibitors, or statins; and fluid shear stress activate the Akt pathway in a PI3K-dependent manner [48]. The activation of Akt leads to the phosphorylation and activation of eNOS and increased production of NO [48, 68, 69]. Other downstream targets of Akt, such as glycogen synthase kinase-3 (GSK-3) [70], the Forkhead in rhabdomyosarcoma (FKHR) family of transcriptional factors, and BAD [71], regulate cellular metabolism and survival of ECs in response to growth factors. Thus, it is likely that the endothelial Akt pathway is critically involved in many biological aspects of the vascular wall [48]. Indeed, Akt signaling plays an important role in the proliferation and migration of ECs, both of which may contribute to some of its proangiogenic and transdifferentiation effects [48, 72, 73]. However, it is not known whether activation of Akt in the endothelium alone could alter the process of lesion formation in the vascular wall following injury.

1.4 EndMT in pulmonary vascular remodeling of various cardio-pulmonary diseases

Embryonically, EndMT is involved in the development of cardiac septa and valves [10], pulmonary artery development [74, 75] and maturation of pulmonary arteries and veins [76] However, in adults EndMT contributes to several cardiopulmonary diseases including but not limited to different types of hypertension including chronic pulmonary hypertension 17384082 Idiopathic portal hypertension, and chronic thromboembolic pulmonary hypertension [77], cardiac fibrosis [15, 78], pulmonary fibrosis [79, 80] idiopathic and radiation induced pulmonary fibrosis

[79, 81, 82], and pulmonary arterial hypertension (PAH) [83], transplant atherosclerosis and restenosis [84, 85], and chronic obstructive pulmonary disease (COPD) [86].

A healthy pulmonary endothelial barrier is integral to maintaining vascular homeostasis and dysfunction of the endothelial barrier occurs in response to inflammatory mediators such as IL-6 and tumor necrosis factor α (TNFα), as well as pathogens [87, 88]. There has been an increasing attention to the contribution of endothelial cell dysfunction in the development and progression of human pathologies [89]. Loss of endothelial barrier integrity, disordered endothelial proliferation, and enhanced inflammatory cell infiltration are common features believed to contribute to the pathologic vascular remodeling [89, 90]. Pulmonary vascular remodeling, a hallmark of several cardio pulmonary diseases are characterized by intimal thickening, medial hypertrophy, and plexiform lesions. Intimal thickening consists of immature smooth muscle cells (SMCs) associated with determined extracellular matrix components, which play an important role in vascular lesions [91]. However, the nature and origin of these cells remain controversial. Several groups have been trying to characterize the phenotype of these cells located in the pulmonary artery wall. Increasing evidence suggests that exposure of endothelial cells to chronic stresses and inflammatory factors can promotes endothelial cells to undergo endothelial-to-mesenchymal transition (EndoMT) and contribute to vascular smooth muscle cells (SMCs) and cardiac fibroblast populations during both embryogenesis and pathological conditions [15, 92, 93].

Pulmonary artery remodeling (PAR) is a major feature of pulmonary hypertension (PH). It is characterized by cellular and structural changes affecting all three layers of the vessel wall of the pulmonary arteries 17384082 15863631. Common pulmonary vascular remodeling changes include increased intimal and/or medial stiffening and thickening, elevated expression of smooth muscle α-actin, collagen synthesis/deposition, and inflammation [12]. Abnormal deposition of smooth muscle-like (SM-like) cells in normally non-muscular, small diameter vessels and a deregulated control of endothelial cells are considered important pathological features of PAR [94]. Also, increased production of extracellular matrix proteins, with deposition of collagen and elastin contribute to lumen narrowing and PH [95]. It is this remodeling process inside the pulmonary vessels that is responsible for elevation of pulmonary vascular resistance, progressive PH, right ventricular failure and finally death [96]. Trans-differentiation of endothelial cells to smooth muscle cells (EndMT) plays an important role in vascular remodeling [97]. The origin of

the SM-like cells and the mechanisms underlying the development and progression of this remodeling process are not completely understood. It is been thought that muscularization of the intimal layer of the vessel wall is caused by proliferation of resident vascular smooth muscle cells of the medial layer which migrate to the intima [12] other possible sources of SM-like cells in the intimal layer of pulmonary vessels have been postulated, resident endothelial cells within the intima may delaminate from their organized layer of cells in the vessel lining, transition to mesenchymal or SM-like phenotype in a process called endothelial-mesenchymal transition (EnMT) and migrate to their underlying tissue [12, 98].

Arcinegas et al reported the presence of intimal thickenings composed of mesenchymal cells that may arise from the endothelium. Using a chick embryo model, they also reported that embryonic pulmonary endothelial cells undergo a transition to mesenchymal cells and participate in intimal thickening and hence contribute to pulmonary vascular remodeling [74]. Supporting this observation was a report from Good et al in which they showed the presence of Willebrand factor/α-smooth muscle actin–positive endothelial cells in pulmonary vessels of patients with systemic sclerosis-associated PAH and the hypoxia/SU5416 mouse model and an upregulation of mesenchymal markers and reduction in endothelial markers in PAECs upon stimulation with inflammatory cytokines including IL-1β, tumor necrosis factor α, and transforming growth factor β indicating the presence of EndMT in PAR [99]. Ranchoux et al also provided evidence for the contribution of EndMT to the development of pulmonary hypertension in which they show the occurrence of EndMT in pulmonary artery endothelial cells from human PAH patients compared to those from control subjects. They identified that overexpression of transcription factor twist is responsible for the upregulation of mesenchymal marker vimentin [100]. In patients with PAH, examination of pulmonary artery plexiform lesions revealed that luminal endothelial cells were swollen and some cells expressed both endothelial markers and α-smooth muscle actin, a finding not observed in controls [100]. The presence of EndoMT was also confirmed in PAH lungs by showing expression of mesenchymal genes, including fibronectin, N-cadherin, and vimentin and the EndMT-related transcription factor Twist [100].

EndMT of the pulmonary endothelial cells was shown as a possible consequence of radiation induced hypoxia resulting in radiation induced pulmonary fibrosis (RIPF) and injury by Choi SH et al. They went on to show that radiation-induced vascular hypoxia triggered EndMT in vascular

endothelial cells and in fact, this was observed prior to the onset of alveolar EMT and thus could further trigger EMT[81, 101]. Chronic obstructive pulmonary disease (COPD) is a progressively irreversible disease associated with an abnormal inflammatory response of the airways in response to noxious particles and gases[102]. Unfortunately, the research effort directed into this has been disproportionately weak compared to its clinical and scientific importance, and indeed COPD itself is the least researched of all common chronic conditions compared to its social importance. Pathologically, it involves structural changes in lung parenchyma, airways, and vessels [103] manifested as intimal and medial thickening leading to reduction of lumen diameter and muscularization of arterioles[104]. It was reported by Reinman et al., recently that EndMT is also active in COPD in addition to EMT[97], contributing to pulmonary vascular remodeling [97, 105]. Interestingly, cells coexpressing endothelial and mesenchymal cell-specific molecules are present in the lungs of patients with SSc-associated interstitial lung diseases (ILD). CD31+CD102+ ECs isolated from SSc lungs simultaneously expressed mesenchymal cell- and EC-specific transcripts and proteins. demonstrating the occurrence of EndMT in the lungs of patients with SSc-associated ILD[106].

Acute lung injury (ALI) and its most severe manifestation, acute respiratory distress syndrome, are life-threatening complications in critically ill patients [107, 108]. The morbidity and mortality of moderate and severe acute respiratory distress syndrome remain high, ranging between 30 and 50%, despite improvements in critical care patients' management and new technological developments[109]. The leading cause of acute lung injury is sepsis, and converging data suggest that endothelial activation and damage are hallmarks of acute lung injury pathophysiology [110, 111]. There have been several clinical trials targeting aggressive factors correlated with the development of acute lung injury, such as inflammatory cells and proinflammatory cytokines, but limited reports indicating validity and efficacy[112]. Thus, a paradigm shift in the treatment strategy designed to protect vascular endothelial cells and pulmonary epithelial cells against injury, and/or enhancement of the tissue repair is promising. Supporting this hypothesis is the report showcasing that lipopolysaccharide (LPS) induces a fibrotic-like phenotype in human umbilical vein endothelial cells (HUVECs)[113], a process involving EndMT, via NAD(P)H oxidase-dependent reactive oxygen species (ROS) production[113]. This observation was also supported by the evidence that LPS induces EndMT of tissue-resident PVECs during the early phase of acute lung injury, partly mediated by ROS, contributing to increased proliferation of PVECs[114].

EndMT is operative during human vein graft remodeling [115]. It was recently shown that EndMT contributes to the vascular remodeling and neointimal formation that arises following vein graft transplantation into the arterial circulation via early activation of the Smad2/3-Snail2 signaling pathway, with antagonism of TGFβ signaling resulting in decreased EndMT and reduced neointimal formation[116]. Evrard et al., showed that EndMT-derived fibroblast-like cells are common in intimal atherosclerotic plaques using both *Cre-lox* endothelial lineage tracking in mice, and in human plaques by detecting cells co-expressing endothelial and fibroblast/mesenchymal proteins, indicative of EndMT. They demonstrated that the number of transitioning cells was associated with an unstable and ruptured human plaque phenotype, which appears mechanistically driven by altered collagen-matrix metalloproteinase (MMP) production in EndMT-derived fibroblast-like cells. Thus, EndMT contributes to atherosclerotic patho-biology and is associated with complex plaques that may be prone to rupture and cause clinical events[116]. A supporting report demonstrated that the extent of coronary atherosclerosis in patients strongly correlates with the loss of endothelial FGFR1 expression, activation of endothelial TGFβ signaling, and the extent of EndMT, demonstrating a link between development of EndMT, and progression of atherosclerosis[117].

Chronic Obstructive Pulmonary Disease (COPD)

Chronic obstructive pulmonary disease (COPD) is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases [118] COPD is one of the most important causes of death in most countries. The Global Burden of Disease Study has projected that COPD, which ranked sixth as the cause of death in 1990, will become the third leading cause of death worldwide by 2020 [119] [120]. It is also an important cause of morbidity and mortality, resulting in over 3 million deaths globally in 2005 [121]. The optimal management of chronic obstructive pulmonary disease (COPD) requires a multifaceted approach which incorporates non-drug as well as drug-management strategies. It is a complex disease, with both pulmonary and systemic manifestations, and an increased risk of serious comorbidity and mortality. For most patients, it has a major impact on lifestyle and quality of life [122].

Risk Factors

Although cigarette smoke and environmental pollutions are major etiological factors for causing COPD, cigarette smoke has been proven to be the predominant risk factor in many studies [123, 124]. It has been shown that cigarette smoking causes abnormal inflammatory responses, cellular senescence, mitochondrial dysfunction and metabolic dysregulation, suggesting their involvement in the development of COPD [125]. While cigarette smoking is the well-studied COPD risk factor, epidemiologic studies demonstrate that nonsmokers may also develop chronic airflow limitation [126]. Compared with smokers with COPD, never smokers with chronic airflow limitation have fewer symptoms, milder disease, and a lower burden of systemic inflammation [127]. Never smokers with chronic airflow limitation do not have an increased risk of lung cancer or cardiovascular comorbidities; however, they have an increased risk of pneumonia and mortality from respiratory failure [127]

Vascular remodeling in COPD- Role of the endothelium

The pulmonary vasculature is critical to gas exchange in the lung and the entire vascular system is lined by endothelial cells which form a continuous monolayer [128]. Endothelial cells are encased by a basement membrane, a thin protein sheet (50nm thick) that consists of laminins, collagen and proteoglycans[129]. It was demonstrated that alveolar septa in COPD patients were almost avascular. This led to the hypothesis that vascular atrophy resulted in the destruction of alveoli [130]. Supporting this concept, increased levels of apoptotic endothelial cells have been identified in the lungs of patients with COPD[131]. In addition to altered levels of endothelium in patients with COPD, the endothelium appears to behave in a dysfunctional manner. Endothelial dysfunction is defined as disturbed endothelial dependent vasodilatation. It results in a breakdown of the microvascular endothelial barrier and loss of the anti-adhesive and anti-thrombotic functions of the endothelium [132] suggesting that endothelial dysfunction may be important in the pathogenesis of COPD. This hypothesis was supported by a report from Peinado et al.'s group that endothelial dysfunction is already present in the pulmonary arteries of patients with early COPD [133]. Dysfunction is also related to clinical outcomes: patients with increased endothelial dysfunction have reduced 6-minute walk test (6MWT) results and a worse overall prognosis. Endothelial dysfunction is also increased in patients with exacerbations of COPD [134, 135]. Therefore, it has been postulated that increased endothelial dysfunction may induce the development of systemic atherosclerosis and therefore the increased cardiac events seen in these

patients[135]. Magnetic resonance imaging (MRI) studies have demonstrated that pulmonary microvascular blood flow appears to be reduced in COPD. These changes were apparent in patients with mild COPD and were worse in patients with severe COPD. This suggests that microvascular disease may represent an early part of the development of COPD and potentially is important in driving the progression of COPD [136].

Vascular remodeling has also been reported in COPD, main structural changes involve intimal and medial thickening, leading to reduction of lumen diameter and muscularization of arterioles [104] [98]. There are two major pathologic changes in subjects with COPD: emphysema and chronic bronchitis. The former refers to the destruction of alveoli, whereas the latter involves chronic inflammation and subsequent airway remodeling [137].

Link with PAH and other CVDs

Changes in the lung function characteristic of COPD are similar to the changes in lung morphology seen in emphysema and lung cancer. It seems that a common thread of smoking-induced lung injury can be traced to all three diseases[138]. Patients with COPD have structural evidence of lung disease manifested by the presence of emphysema, airway wall thickening, and gas trapping [139][140]. In most patients, COPD is associated with significant concomitant chronic diseases, which increase morbidity and mortality [120].

1.5 Pulmonary arterial hypertension (PAH)

"Pulmonary arterial hypertension (PAH) is a progressive, fatal syndrome characterized by increased pulmonary vascular resistance that leads to right-sided heart failure and, eventually, death" [141] A mean pulmonary artery pressure (PAP) of >25 mmHg, pulmonary vascular resistance >3 Wood units, pulmonary capillary wedge pressure <15 mmHg, and normal or reduced cardiac output in absence of other causes of pulmonary hypertension is regarded as PAH [142] The term pulmonary hypertension (PH) refers to a condition with abnormally high pulmonary vascular pressure. It is important to note that PAH is a sub-category of PH and the two terms are not synonymous.

Pulmonary arterial hypertension results from restricted blood flow through the pulmonary arterial circulation resulting in increased pulmonary vascular resistance ultimately causing right sided

heart failure. During the past few decades of extensive research on mechanisms contributing to the development of PAH, multiple pathogenic pathways have been implicated, including those at the molecular and genetic levels in the endothelial cells, vascular smooth muscle cells, and fibroblasts. It has been believed until recently that the imbalance in the vasoconstrictor/vasodilator milieu served as the root cause for the development of PAH and hence the basis for current medical therapies, although increasingly it is recognized that an imbalance of proliferation and apoptosis contributes to PAH.

While previously considered a rare disease, recent evidence suggests that the prevalence of PAH is about 15 per million [143] There is no cure for this fatal disease which affects mainly young to middle aged women and survival rate is estimated at 55-65% at 3 years post diagnosis despite recent improvements in PAH therapies.[144] It is associated with poor prognosis, with an approximately 15% mortality within 1 year on modern therapy.[145] A few predictors of a poor prognosis include: advanced functional class of the disease, poor exercise capacity as measured by 6-minute walk (6MW) test or cardiopulmonary exercise test, high right atrial (RA) pressure, right ventricular (RV) dysfunction, evidence of RV failure, low cardiac index, elevated brain natriuretic peptide (BNP), and underlying diagnosis of scleroderma spectrum of diseases.

1.6 PAH symptoms and diagnosis

The most commonly reported physiological symptoms of PAH are shortness of breath, dyspnea on exertion, and fatigue. Other symptoms may include syncope, chest pain, abdominal distention, and peripheral edema [146] PAH may also present psychological symptoms including anxiety, depression, panic attacks, and panic disorders [147, 148]

Over the last decade, the age at diagnosis of PAH has been gradually shifting to older age [149] while the mean age of diagnosis is 50 years [150]. Patients at a great risk for the development of PAH who need to be screened periodically include those with a known mutation in BMPR2, scleroderma spectrum of diseases, and portal hypertension. The most fundamental principles in the diagnostic workup of patients with clinical suspicion of PH/PAH remain unchanged over years. Any patient who presents with otherwise unexplained dyspnea on exertion, syncope, and/or signs of right ventricular dysfunction should be suspected of PAH and screened immediately. By far, the most appropriate study to obtain in patients suspected of having PH based on physical examination, medical history, chest x-ray (CXR), and electrocardiogram (ECG) is an

echocardiogram. Transthoracic echocardiography continues to be the most important noninvasive screening tool but right heart catheterization (RHC) remains mandatory to establish the diagnosis. RHC is the gold-standard procedure for the diagnosis and classification of pulmonary hypertension and is recommended for all patients suspected of having PAH before the initiation of any PAH-specific therapy and should include measurement of pulmonary artery pressure, pulmonary wedge pressure, cardiac output, and oxygen saturations in the central veins, cardiac chambers, and pulmonary artery; calculation of pulmonary vascular resistance; and performance of vasodilator testing. [151, 152] Acute vasodilator testing should be performed in all IPAH patients who might be considered potential candidates for long-term calcium-channel blocker therapy. It involves administration of pharmacologic agents to test the presence of pulmonary vasoreactivity and should not be performed on patients with right heart failure or hemodynamic instability.[153] Evaluation for other potential etiologies, such as thromboembolic disease, is appropriate in all patients suspected of having PAH.

In a very recent study, Staniszweska-Slezak et al., demonstrated that FTIR spectroscopy supported by principal component analysis (PCA) has the potential to become a fast and non-destructive method for biochemical characterization of plasma that consequently could have a diagnostic significance in pulmonary arterial hypertension [154].

1.7 **Treatment of PAH**

Goal of treatment for PAH not only includes survival of the patients but also improving the patient's symptoms and quality of life. Assessments to measure response to treatment include improvement in exercise capacity (6 minute walk test, cardiopulmonary exercise test, and treadmill test), hemodynamics, and survival. An important aspect of PAH pathogenesis comprises of endothelial dysfunction leading to an imbalance of vasodilator and vasoconstrictor mediators characterized by sustained reduction in vasodilator mediators (such as PGI2 and NO), and upregulation of vasoconstrictors (such as ET-1).[155] The understanding of molecules involved in the regulation of pulmonary vasomotion has led to the development of currently approved PAH therapies, targeting one of the following key pathways: (1) prostacyclin (PGI2), (2) nitric oxide (NO), and (3) endothelin-1 (ET-1) pathways.

1.7.1 Prostacyclin pathway

Endogenous prostaglandin 12 (PGI2), produced mainly by endothelial cells is a vasodilator, antithrombotic, anti-inflammatory, and antiproliferative agent that binds to prostaglandin I (IP) receptors with downstream effect on cAMP levels.

a) ***Epoprostenol*** - Intravenous (IV) epoprostenol, the first targeted therapy approved for the treatment of PAH is a prostacyclin analogue. Till date, it remains the only drug exhibiting survival benefit in randomized control trials (RCTs). [156, 157]
b) ***Iloprost*** – Iloprost can be administered via both inhaled and IV routes, although the inhaled route has only been tested in RCTs so far. To achieve clinical efficacy, inhaled iloprost must be administered at least 6 times a day as it has a very short half-life of 25 minutes.[158]
c) ***Treprostinil*** – Treprostinil can be administered via the IV, subcutaneous (SC), inhaled, and oral routes as it has greater chemical stability and longer half-life than epoprostenol. Continuous SC administration of treprostinil has an added advantage as it bypasses the practical difficulties and complications associated with indwelling central venous catheters [159].

1.7.2 Endothelin pathway

Endothelin-1 (ET-1) is one of the most potent vasoconstrictors and exerts its effects on two distinct receptor subtypes, ETA and ETB receptors. ETA receptor is highly localized to PASMC, while ETB receptor is predominantly expressed on the endothelium but can also be found on PASMC. Although both ETA and ETB receptor regulate smooth muscle constriction and proliferation, ETB receptor is the major isoform that is involved in the local clearance of ET-1 and induces vasodilation via the release of NO and PGI2 from endothelial cells. Besides promoting proliferation of PAECs and PASMCs, ET-1 is also greatly implicated in fibroblast activation, contraction, and synthesis of the extracellular matrix proteins.[160]

a) ***Bosentan*** – The first oral agent approved for the treatment of PAH, Bosentan, is a nonselective dual ET-1 receptor antagonist. It has also been shown to improve both exercise capacity and hemodynamics in congenital heart disease-PAH. Increase in the levels of liver transaminase is a common side effect and hence, periodic monitoring of liver function tests is mandatory in patients undergoing Bosentan therapy [161, 162].

b) ***Ambrisentan*** – Ambrisentan, a selective ETA receptor antagonist, demonstrated improved outcomes in 6MWD and NYHA FC in patients with PAH.[163] Risk of liver injury and potential drug-drug interaction is low when compared to bosentan. However, patients should be monitored for peripheral edema, a common side effect associated with ambrisentan therapy. Interestingly, despite the theoretical advantage of selective ETA receptor blockade considering the role played by ETB receptor in local ET-1 clearance, clinical efficacy appears broadly similar between bosentan and ambrisentan.[163]

c) ***Macitentan*** - Macitentan is a dual ET-1 receptor antagonist that demonstrates high lipophilicity, increased receptor affinity, and prolonged receptor binding than bosentan due to modification in the structure.[164] Therefore, macitentan has enhanced tissue penetration capabilities with sustained antagonism against ET-1 receptors.[164, 165]

1.7.3. Nitric oxide-cGMP pathway

Reduced nitric oxide (NO) production and bioavailability in endothelial cells is a key feature of endothelial dysfunction that initiates the pathogenesis of PAH. NO released from ECs activates soluble guanylate cyclase resulting in smooth muscle relaxation. In addition, NO is also a potent inhibitor of smooth muscle proliferation and platelet activation. This pathway is highly implicated in PAH and can be manipulated via direct administration of NO, inhibition of phosphodiesterase-5 (PDE-5), an enzyme responsible for cGMP degradation or stimulation of sGC.

a) ***Sildenafil*** – Phosphodiesterases (PDEs) are the enzymes that regulate the intracellular degradation of cGMP. PDE-5 plays a key role in the regulation of smooth muscle tone in the pulmonary vascular bed and the corpus cavernosum. Sildenafil, the first PDE-5 inhibitor approved for the treatment of PAH, demonstrated that the majority (60%) of patients improved or maintained their functional status at 3 years of treatment at a dose of 80 mg 3 times a day.[161]

b) ***Tadalafil*** - Tadalafil is a structurally distinct PDE-5 inhibitor in comparison with sildenafil, which accounts for its different pharmacokinetic properties and longer half-life. Tadalafil is used to improve the ability to exercise in patients with PAH.[150, 166]

c) ***Riociguat*** - Riociguat is a sGC stimulator that not only maintains sGC in its active configuration when low levels of NO are available but also produces cGMP even in the absence of NO.[167] Since there is a deficiency in endothelial NOS and, hence, NO

production in patients with PAH, riociguat offers a better symptomatic relief. Riociguat and NO together have a more pronounced therapeutic effect than either of their single therapies.

1.7.4. Procedures/nonmedical therapies

a) ***Lung transplantation*** - Despite targeted PAH therapy, lung transplantation remains a destination therapy for most PAH patients. Considering limited organ availability and high mortality rates of PAH patients awaiting transplantation, eligible patients for whom first-line treatment strategies have failed should be immediately referred for transplantation assessment. Although double-lung transplantation is the preferred option for PAH, heart-lung transplantation remains necessary for some patients and is adopted for patients with refractory right heart failure.[168]

b) ***Atrial septostomy and Potts shunt*** - Balloon atrial septostomy is a procedure which creates artificial right-to-left shunt to decompress the right ventricle as there is an excessive right ventricular hypertrophy in patients with progressive PAH. It can be accomplished percutaneously with careful graded balloon dilation and can improve peripheral oxygen delivery despite a fall in systemic arterial saturation because of a compensatory rise in cardiac output.[169] Atrial septostomy is not a highly recommended procedure because it is associated with high periprocedural mortality in patients with markedly elevated right atrial pressure and should only be considered as a palliative therapy or bridge to transplantation in centers with experience in this procedure.

   An alternative method of right ventricular decompression is the Potts shunt which involves creation of an anastomosis between the descending aorta and left pulmonary artery. In the setting of suprasystemic pulmonary artery pressure, Potts shunt has a theoretical advantage over septostomy which is the sparing of the cerebral and coronary circulation from deoxygenated blood. Interestingly, a small case series of adults with NYHA FC IV PAH demonstrated the feasibility of the creation of Potts shunt via a minimally invasive percutaneous approach.[170] However, further investigation is required to assess the safety and efficacy of this innovative technique.

c) ***Pulmonary artery denervation*** –A recently emerging novel nonmedical therapy for PAH, pulmonary artery denervation involves borrowing the concept of renal artery denervation.

The presumed mechanism of action of this technique is via the abolishment of sympathetic nerve supply to the pulmonary circulation.[171] In a recent first-in-human single-center study, 13 patients underwent pulmonary artery denervation with the use of a dedicated radiofrequency ablation catheter resulting in significant reduction of mean PAP and improvement in 6MWD. However, these preliminary results require further confirmation, because the negative findings from a trial of renal denervation for resistant hypertension serves as a cautionary reminder that device-based therapy must be subjected to rigorous evaluation before adoption into clinical practice [141, 172].

Recent understanding on left ventricular failure suggests alterations in electromechanical coupling and hemodynamics that have responded dramatically to cardiac resynchronization therapy. Given the similarities between RV and LV remodeling in response to dyssynchrony, it is reasonable to investigate the possibility of resynchronization therapy for the failing RV. Multiple studies including animals, a computer simulation analysis, and one study involving patients with chronic thromboembolic pulmonary hypertension have shown acute improvements in cardiac function and hemodynamics with RV pacing.[173] However, it is too soon to conclude as it is not clear if these results would apply to other types of pulmonary hypertension, Further investigation is required to determine whether resynchronization therapy for RV failure in patients with PAH will become a viable treatment option [174].

2. Pathogenesis of PAH

The major factors contributing to increased pulmonary vascular resistance in PAH include vasoconstriction, proliferative, and obstructive remodeling of the pulmonary vessel wall, inflammation, resistance to apoptosis, formation of plexiform lesions, and thrombosis *in situ.* [143, 155, 175-177] A common feature to all vascular remodeling processes is that in early stages of the disease, a significant increase in oxidative stress and inflammatory processes are observed, causing irreversible DNA damage and cell death. This leads to 1) endothelial injury and 2) enhanced inflammatory response, which could be the underlying causes of exaggerated vasoconstriction and vascular cell proliferation and contribute to the occlusive pulmonary vascular remodeling that characterizes PAH [178, 179].

1.8 Molecular mechanisms contributing to PAH

In order to develop an effective treatment strategy, it is very important to understand the molecular mechanisms that trigger and maintain the PASMC and PAEC proliferation/apoptosis imbalance which greatly contributes to vascular remodeling and hence the development of PAH.

In PAH, there is a high turnover of hyperproliferative and apoptosis-resistant PASMC and PAEC populations which contribute to PA remodeling. Several mechanisms have been identified as important players in this proliferation/apoptosis imbalance of which the most described pathway is the transforming growth factor (TGF) β pathway. Mutations in bone morphogenic protein receptor 2 (BMPR2), a member of the TGF-β superfamily are found in 50% of familial PAH cases and disruption of this pathway is most commonly found in other forms of PAH as well.[180] Mutations in the BMPR2 gene activate the v-Src sarcoma (Schmidt-Ruppin A-2 [Src]) pathway, leading to subsequent activation of downstream signal transduction pathways including STAT3 which leads to a disruption of the BMPR2 pathway. Additionally, cytokines and growth factors such as PDGF and VEGF also activate STAT3 by binding to tyrosine kinase receptors. From the wealth of literature it is evident that the Src/STAT3 axis is a central integrative hub in PAH which controls multiple pathways implicated in PASMC and PAEC proliferation, migration, and resistance to apoptosis. In addition to affecting BMPR2, Src/STAT3 also regulates several signaling molecules including NFATc2, HIF-1, survivin, provirus integration site for Moloney murine leukemia virus-1 (Pim-1), and MMPs. It is important because overexpression of Pim-1/NFATc2 upregulates PASMC proliferation by promoting mitochondrial hyperpolarization and antiapoptotic proteins, such as survivin. The extracellular matrix modifiers MMPs, notably MMP-2 and MMP-9 play a significant role in PAH vascular remodeling by promoting PAEC and PASMC proliferation and migration. Src/STAT3 activation also regulates PASMC migration via phosphorylation of focal adhesion kinase (FAK). Therapeutically, inhibition of the Src/STAT3 pathway has been shown to reverse the PAH phenotype in many pre-clinical studies.

a) ***TGF-beta superfamily*** – The transforming growth factor-beta (TGF-β) and TGF-β like molecules are members of a large superfamily of more than 40 structurally related polypeptide cytokines including TGFβ, bone morphogenetic proteins (BMPs), activins, nodal, lefty, myostatin, anti-müllerian hormone (AMH), and growth differentiation factors (GDFs). These pleiotropic cytokines are expressed in complex temporal and tissue specific patterns and control numerous biological functions such as proliferation, apoptosis, embryonic patterning, stem cell

maintenance, cell differentiation, migration, and regulation of the immune system. Together, these growth factors account for a significant portion of intracellular signals regulating cell fate. Unraveling the complexity that underlies their mode of action has remained challenging because these effects are known to be highly cell type-specific and context-dependent.[181-183] TGF-β/SMAD signaling plays crucial roles in both embryogenesis and carcinogenesis. In embryonic development, Nodal proteins are essential for mesoderm induction in vertebrates [184]; BMP signals are essential for embryonic patterning and early skeletal formation [185]; TGF-β/Activin regulates vascular function and angiogenesis due to their promotion of both EMT (epithelial to mesenchymal transition) and EndoMT (endothelial to mesenchymal transition) [186, 187]. In carcinogenesis, TGF-β/SMAD signaling has dual functions. It can inhibit cell cycle progression or induce apoptosis and, thereby, be cytostatic or cytotoxic in premalignant tumor cells. However, in advanced tumors, in which oncogenic mutations have inactivated these tumor-suppressing functions, TGF-β/SMAD signaling can induce or enhance EMT, invasion, and metastasis [188, 189].

The three TGFβ isoforms, TGFβ1, TGFβ2, and TGFβ3, are the most widely studied members of the family, mostly because they are ubiquitously expressed and can influence the majority of tissue types. On the other hand, the expression of other cytokines is limited to only a few tissues, such as myostatin, or particular developmental stages, such as the AMH [190, 191]. The TGFβ molecules are initially synthesized in an inactive pro-TGFβ form, which consists of TGFβ associated with latency associated proteins (LAPs). The TGFβ large latent complex (LLC) consists of the LAPs and the latency-TGFβ-binding proteins (LTBPs) assembled together with disulfide bridges between specific cysteine residues [192-195]. In turn, the LLC is covalently associated to the extracellular matrix (ECM) via the N-terminal region of LTBPs [196, 197]. The presence of the TGFβ ligand within the LLC complex maintains the cytokine in an inactive form by preventing the interaction with its receptors [198].

b) TGF-β receptors and signaling – TGF-β and related factors signal through a family of transmembrane protein serine/threonine kinases referred to as the TGF-β receptor family. Extensive evidence that has accumulated indicates that TGF-β family members signal through receptor serine/threonine kinases. One exception is the glial cell–derived neurotrophic factor (GDNF), which signals through the receptor tyrosine kinase Ret [199]. Based on their structural and functional properties, the TGF-β receptor family is divided into two subfamilies: type I

receptors and type II receptors. Type I receptors have a higher level of sequence similarity than type II receptors, particularly in the kinase domain. Seven type I receptors and five type II receptors exist in humans. Interactions with contiguous or non-contiguous molecular surfaces determine the specificity of ligand–receptor pairings [200, 201]. TGFβ binds exclusively to the type I receptor TGFBR1 (also known as ALK5 and TβRI) and the type II receptor TGFBR2. Activin, Nodal and BMPs share the type II receptors activin receptor type 2A (ACVR2A) and ACVR2B. Activin and Nodal, but not BMPs, share the type I receptors ACVR1 (also known as ALK2), ACVR1B (also known as ALK4) and ACVR1C (also known as ALK7), whereas BMPR1A (also known as ALK3) and BMPR1B (also known as ALK6) primarily act as type I receptors for BMPs and anti-Muellerian hormone (AMH). BMPR2 is another type II receptor for BMPs, and AMHR2 is the type II receptor for AMH. Activin receptor-like 1 (ACVRL1; also known as ALK1) is a BMP9 (also known as GDF2) and BMP10 type I receptor, but it can be collaterally engaged by high TGFβ concentrations [202, 203]. In most cells, the TGF-β signaling pathway involves the TβRII/ALK5 complex, which induces Smad2/3 phosphorylation. However, in endothelial cells, TGF-β activates two distinct type I receptors, ALK5 and ALK1, which transmit signals via the ALK5/Smad2/3 and ALK1/Smad1/5 pathways, respectively [204]. ALK5/Smad2/3 inhibits and ALK1/Smad1/5 stimulates endothelial-cell proliferation and migration.

c) Structural features of the TGF-β receptors

- **The extracellular domain:** Type I and II receptors are glycoproteins of approximately 55 kDa and 70 kDa, respectively, with core polypeptides of 500 to 570 amino acids including the signal sequence [205-207]. The extracellular region is relatively short (approximately 150 amino acids), N-glycosylated [208], and contains 10 or more cysteines that may determine the general fold of this region. Three of these cysteines form a characteristic cluster near the transmembrane sequence [209]. The spacing of other cysteines varies and is more conserved in type I receptors than in type II receptors.

  The transmembrane region and the cytoplasmic juxtamembrane region of type I and II receptors have no singular structural features. However, Ser213 in this region of TβR-II is phosphorylated by the receptor kinase in a ligand-independent manner and is required for signaling activity [210]. Ser165 in the juxtamembrane region of TβR-I is phosphorylated by TβR-II in a ligand-

dependent manner, and this appears to selectively modulate the intensity of different TGF-β responses [211].

- **The GS domain:** A unique feature of type I receptors is a highly conserved 30–amino acid region immediately preceding the protein kinase domain. This region is called the GS domain because of a characteristic SGSGSG sequence it contains. Ligand-induced phosphorylation of the serines and threonines in the TTSGSGSG sequence of TβR-I by the type II receptor is required for activation of signaling [211, 212], and the same happens with the activin type I receptor ActR-IB [213]. Immediately following the SGSGSG sequence, all type I receptors have a Leu-Pro motif that serves as a binding site for the immunophilin FKBP12 [214]. FKBP12 may act as a negative regulator of the receptor signaling function. The penultimate residue in the GS domain, right at the boundary with the kinase domain, is always a threonine or a glutamine [215].
- **The kinase domain:** The kinase domain in type I and II receptors conforms to the canonical sequence of a serine/threonine protein kinase domain. Consistent with this, type I receptors have been shown to phosphorylate their substrates—SMAD proteins—on serine residues [216, 217], whereas type II receptors phosphorylate themselves and type I receptors on serine and threonine residues but not tyrosine residues [205, 207, 211, 212].

d) TGF- β signaling -– TGF-β exerts its downstream effects via canonical SMAD dependent pathway or non-canonical pathways like ERK/MAPK/PI3K-Akt and so forth. These effects are cell and context dependent. TGFβ family members regulate gene expression by receptor-mediated activation of SMAD transcription factors. Activated SMAD proteins regulate the transcriptional output of active genes and can also open repressive chromatin. Additionally, SMAD proteins serve as hubs for the integration of regulatory inputs and context-dependent modulation of TGFβ signaling. BMP type I receptors phosphorylate SMAD1, SMAD5 and SMAD8, and BMP type I receptors for TGFβ, activin and Nodal mainly phosphorylate SMAD2 and SMAD3. Receptor-mediated phosphorylation of this set of SMAD proteins (collectively known as receptor-regulated SMAD proteins (R-SMAD proteins)) then binds to MH2 domain of SMAD4 which facilitates its translocation to the nucleus. SMAD4 itself is not a receptor substrate but functions as a shared partner for all R-SMAD proteins. Trimers with two R-SMAD molecules and one SMAD4 are thought to be the principal functional units [218]. In the basal state, SMAD proteins constantly shuttle between the cytoplasm and the nucleus via contact with

nucleoporins for transit through the nuclear pore [219]. However, in the receptor-induced oligomeric state, SMAD proteins require nuclear import and export factors. In the nucleus, R-SMAD proteins in activated SMAD4–R-SMAD complexes bind other DNA-binding transcription factors as partners for target gene recognition and transcriptional regulation [220-222].

Although TGFβ signals mainly via the SMAD pathway, TGF-β can also activate other pathways that are collectively referred to as 'non-canonical' TGF-β signaling and complement SMAD action. Effects of TGFβ and BMP on the activity of various MAPKs and also on the phosphoinositide 3-kinase (PI3K) pathway can be either immediate and transient or delayed and secondary, depending on the cell type and the culture conditions and are implicated in multiple pathological disorders [189, 223]. MAPK and PI3K activation has been proposed to complement and converge with SMAD signaling [72,106,[202], although these pathways can also antagonize SMAD signaling in other contexts. Due to a lack of structural information it is not known whether activation of the MAPK and PI3K pathways is directly coupled to TGF-β receptors, whether it is the result of collateral activation of other receptors or whether it is a consequence of network-wide signaling crosstalk. Regardless, the MAPK and PI3K pathways on their own are major signaling routes for receptor tyrosine kinases, metabolic inputs and environmental stresses, and in cancer these pathways are often activated by key oncoproteins. These considerations raise questions about what an extra TGF-β input into PI3K or MAPKs can accomplish in this context. Although recent data shows conflicting results in terms of TGF-β associated Akt activation, it was widely believed that TGF-β family proteins activate PI3K-Akt pathway [224]. It has been shown that, silencing Akt1 expression was seen to enhance TGF-β-induced EMT [225]. This might be the same phenomenon happening in TGF-β induced endothelial to mesenchymal transition contributing to vascular remodeling in PAH.

e) ***Role of TGF-β in PAH*** – It is clear that PAH has a multifactorial pathobiology, and that one factor or gene mutation will not explain all forms and cases of PAH. However, the current understanding of the mechanisms underlying PAH has shed light on the involvement of TGF-β superfamily in the development of pathological vascular remodeling and hence PAH. Germline mutations in the gene coding for BMP type-II receptor (BMPR2) have been identified in 60% of familial PAH and 10% to 30% of idiopathic PAH [226-228]. The absence of BMPR2 mutations in some families and in the majority of sporadic and associated cases suggests that

there may be further genes, possibly related to the BMP/TGF-pathway, to be identified. Indeed, mutations in the TGF-receptors, ALK-1 and endoglin, have been identified in PAH patients with a personal or family history of hereditary hemorrhagic telangiectasia [229, 230].

The net result of TGF-β signaling on vascular growth and structure is complex. Whether the TGF-β superfamily inhibits or promotes cell proliferation is highly context-specific. In pulmonary arteries, endothelial cells seem to be the main target of TGF-β, particularly in iPAH. Gore et al reported that lung tissue and PECs from patients with iPAH expressed increased amounts of ALK1 and endoglin, located predominantly on endothelial cells compared to that of control specimens; and patients with iPAH had higher serum and lung TGF-β levels than the controls [231]. In pulmonary artery smooth muscle cells from patients with idiopathic PAH, TGF-β1 causes enhanced cell proliferation in contrast to the growth inhibitory effect observed in normal cells [232]. This is not due to alterations in TGF-β1 receptor ratios or downregulation of TGF-β1 type-II receptor [233]. Transforming growth factor-beta is also known to increase production of extracellular matrix. In human lung fibroblasts, TGF-β increases elastin expression by stabilization of elastin mRNA, and thus it is possible that increased elastin expression observed in PAH may be due to alterations in the pathway. The TGF superfamily may regulate the activity of other factors implicated in vascular remodeling. The TGF-β1 induces ET-1 in human pulmonary artery cells probably via activation of protein kinase A [234]. Connective tissue growth factor production can also be stimulated by TGF-β in pulmonary fibroblasts [235]. Clearly much remains to be learned of the interaction of the TGF-β/BMP pathway with other factors already demonstrated to play important roles in the control of vascular tone and growth.

Upregulation of TGF-β has also been reported in several animal models of PAH [236, 237] and decreased TGF-β signaling related to dominant negative TGF-β type II receptor (TGF-βRII) overexpression [238] or anti-TGF-β antibody protects against PAH.

f) Role of TGF-β in myofibroblast differentiation and fibrosis – Fibrosis arises from deregulation of wound healing processes and is characterized by a stiff and collagen-rich extracellular matrix (ECM) that is resistant to degradation. Fibrotic diseases promote loss of function in a variety of organs including the heart, liver, lung, and kidney resulting in a significant number of deaths worldwide [239, 240]. Synthesis of collagen is influenced by a variety of mediators, including

growth factors, hormones, cytokines and lymphokines. Transforming growth factor-beta (TGF-β) is a pro-fibrotic growth factor implicated in the development of fibrotic lesions. It causes deposition of extracellular matrix (ECM) by increasing the synthesis of matrix protein like collagen and decreasing the degradation by stimulating various inhibitor mechanisms. While TGF-β is essential for healing, overproduction leads to scar tissue and fibrosis. Although both the TGF-β isoforms 1 and 2 are implicated ECM remodeling and protein synthesis, TGF-β1 is a key regulator of fibrosis [241] and plays a critical role not only in synthesis and degradation of ECM but also in response of cells to ECM mediated through integrin receptors; moreover, specific components of the ECM, in turn, can both deliver TGF-β and regulate its activity. The cytokine TGF-β1 is considered to have a central role in inducing myofibroblastic phenotype, and its expression is increased under numerous fibrotic conditions.

Inappropriate activation and accumulation of myofibroblasts is responsible for pathological fibrosis. Myofibroblasts in fibrotic tissues are derived from at least three sources: activation of resident tissue fibroblasts, tissue migration of bone marrow-derived, circulating fibrocytes, and transition of epithelial cells into mesenchymal cells (EMT) [80]. TGF-β plays an important role in many fibrotic diseases also by inducing EMT. Kamath et al recently reported that TGF-β1 and 2 act synergistically in the fibrotic pathway contributing to the development of oral submucous fibrosis [242]. TGFβ1 promotes myocardial fibrosis [243, 244] and TGF-β up-regulation after myocardial infarction has been described by several groups [245-247].

g) Role of TGF in EndMT – Endothelial to mesenchymal transition (EndMT) is defined as the phenomenon in which endothelial cells lose their endothelial specific features and acquire mesenchymal properties. [7, 8]. It is an essential mechanism implicated not only in cardiac development but also in progression of diseases like atherosclerosis, pulmonary hypertension, diabetic nephropathy and wound healing [12-14]. EndMT is also implicated in cancer where Xiao et al showed that TGF-β driven EndMT produces a spectrum of endothelial cell phenotypes with different functions that define the plasticity and heterogeneity of tumor vasculature [120]. In these diseased states aberrantly regulated EndMT results in unscheduled conversion of endothelial cells (EC) into diverse mesenchymal-lineage cell types, especially myofibroblasts (MF), which may dissociate from the vessel wall and can be found throughout the affected tissue. Myofibroblasts are specialized cells within the body that aid in wound healing. Upon activation by biochemical and mechanical signals, myofibroblasts secrete and organize ECM, develop

specialized matrix adhesions [17], and exhibit cytoskeletal organization characterized by contractile actin filaments [18]. Together, these features allow for re-establishment of mechanical integrity and stability to the damaged tissue and enable the myofibroblasts to exert large contractile forces on their microenvironment thus assisting in both the closure of the wound and remodeling of the tissue which can lead to pathological remodeling when aberrantly stimulated.

EndMT is characterized by loss of cell-cell adhesion and changes in cell polarity inducing a spindle-shaped morphology. These changes are accompanied by reduced expression of the endothelial markers such as VE-cadherin and CD31, and increased expression of the mesenchymal markers like fibroblast specific protein-1 (FSP-1), alpha smooth muscle actin (α-SMA), N-cadherin, and fibronectin [9]. Loss of cell-cell adhesion is mediated by transcription factors such as Snail, Slug, ZEB-1, SIP-1, Twist, and LEF-1 that suppress transcription of genes encoding proteins involved in formation of adherens junctions and tight junctions [10, 11]. Transforming growth factor-beta (TGF-β) signaling ligands are potent inducers of converting epithelial cells to mesenchymal cells [248] and given the similarities between EMT and EndMT, one would expect a similar mechanism of disease development. Although both TGF-β1 and 2 promote EndMT, activity of TGF-β2 isoform appears to be predominant [10, 249, 250]. Ablation of TGF-β2 in mice prevents EndMT-mediated cardiac development. TGF-β1 or TGF-β3 knockout mice show no significant effects on EndMT and heart development [8]. Supporting this observation, Medici et al demonstrated that TGF-β2 stimulates EndMT in human microvascular endothelial cells through the Smad, MEK/ERK, PI3K and p38 MAPK pathways [251].

The important role of TGF-β in wound repair has been studied using cultured endothelial cells: TGF- β inhibits endothelial cell growth over a 24 hour period by enhancing fibronectin secretion, decreases cell migration, and abolishes high affinity EGF binding and EGF-dependent competence gene induction [252-254]. These effects would be expected to complement the lack of PDGF receptors on vascular endothelial cells, with the result that stimulation of smooth muscle and connective tissue regeneration would occur more rapidly than reformation of the vascular endothelium in response to platelet degranulation. More recently however, the use of a three-dimensional matrix in which to culture cells has revealed that TGF-β might not inhibit endothelial cell proliferation, but may in fact stimulate angiogenesis [117]. Chrobak et al

demonstrated in a recent study that interferon-γ contributes promotes endothelial to mesenchymal transition contributing to vascular remodeling in systemic sclerosis via upregulation of ET-1 and TGF-β2 [255]. TGF-β2 is expressed at much higher levels than the other TGF-β isoforms in the vitreous humor and is a likely mediator of EMT in RPE cells in vivo [256, 257]. Feng et al showed that combined silencing of TGF-β2 and snail genes inhibit EMT of retinal pigment epithelial cells under hypoxia [258]. TGF-β2 is a more potent inducer of fibrosis than TGF-β1 [259]. It was reported that TGF-β2 alone can induce differentiation of animal hemisphere progenitors to mesodermal cells during embryonic differentiation in amphibians [260].

1.9 Context and phase dependent effects of loss of Akt1 in barrier protection and injury

PI3K-Akt signaling has been highly implicated in endothelial cell survival and normal functioning. However, effects of Akt1 signaling are highly dependent on the cell type, context and phase of the disease development. It is still a debate whether Akt1 is necessary or detrimental in maintaining barrier integrity. Our lab has been studying the role of Akt1 signaling in multiple cell types in various disease conditions and at various stages of development (early vs late phases). In this regard, we showed some novel yet conflicting results in terms of loss of Akt1 that challenges the existing dogma that Akt1 is just a survival protein and its inhibition is protective in proliferative or fibrotic disorders. The precise roles of different Akt isoforms in neovascularization remain elusive. Chen and Somanath et al, showed that Akt1 is the predominant isoform in vascular cells and in Akt1(-/-) mice there was enhanced impairment of blood vessel maturation and increased vascular permeability. In bladder cancer, ShRNA-mediated Akt1 knockdown resulted in impaired T24 cell survival, proliferation, colony formation, migration and microinvasion. Pharmacological inhibition of Akt1 resulted in impaired T24 and UM-UC-3 cell motility, viability and proliferation indicating that Akt1 promotes tumorigenic and invasive properties of bladder cancer cells [261]. In fibroblasts, Akt1 activation is crucial for TGFβ-induced MF formation and persistent differentiation. Sustained activation of Akt1 was associated with an increase in αSMA expression and assembly; an effect that is blunted in cells expressing inactive Akt1 despite TGFβ stimulation. Akt1 deficiency was associated with decreased myocardin, SRF, and αSMA expressions in vivo. These findings highlight Akt1 as a novel potential therapeutic target for fibrotic diseases [262]. Interestingly, we also showed that Akt1-/- mice were protected from chronic hypoxia-induced peripheral vascular pruning while hyperactivation of Akt1 induced focal fibrosis similar to TGFβ-

induced fibrosis. Pharmacological inhibition of Akt, but not the Akt substrate mTOR, inhibited hypoxia- and TGFβ-induced pulmonary vascular rarefaction and fibrosis. We have previously shown that Akt1 is necessary for wound healing through matrix regulation. Our lab has shown for the first time that Akt1 and Mek1 pathways cooperate in the regulation of ECM remodeling by the fibroblasts via activation of integrin β1[263]. We also showed that TNFα treatment induced a dose-dependent increase in TSP-1 expression in HMECs associated with increased endothelial permeability, apoptosis, and reduced proliferation. Whereas TNFα activated Akt, ERK, and P38 mitogen-activated protein kinase (P38 MAPK) simultaneously in hMEC, inhibitors of Akt and P38 MAPK, but not ERK blunted TNFα-induced TSP-1 expression. This study identified the novel role of TNFα in inducing inflammatory stress response in HMEC through Akt- and P38 MAPK-mediated expression of TSP-1, independent of NFκB signaling [264]. In cardiac setting of ischemia reperfusion following MI, although no difference in infarct size following short-term MI was observed between Akt1(+/+) and Akt1(-/-) mice, I/R caused substantially more cardiomyocyte apoptosis and tissue damage in Akt1(-/-) mice compared with Akt1(+/+). Akt1(-/-) hearts exhibited improved cardiac function following long-term MI compared with Akt1(+/+) and were associated with reduced fibrosis in the left ventricle (LV). However, in the long term, Akt1 contributes to fibrosis in post-MI hearts and might exacerbate cardiac dysfunction showing dichotomous role for Akt1 in cardiac remodeling after MI [265]. Recent data from our lab (unpublished) also shows that inhibition of Akt1 suppresses tumor growth but enhances metastasis via barrier breakdown and increased vascular permeability. Hence, Akt1 plays differential roles in different cell types based on the context and phase. It remains unknown till date whether and how Akt regulates EndMT. My research is focused on elucidating the cross-talk between Src and Akt1 in endothelial-barrier modulation, study the effect of endothelial injury as a result of Akt1 depletion on EndMT, and identify the molecular mechanisms.

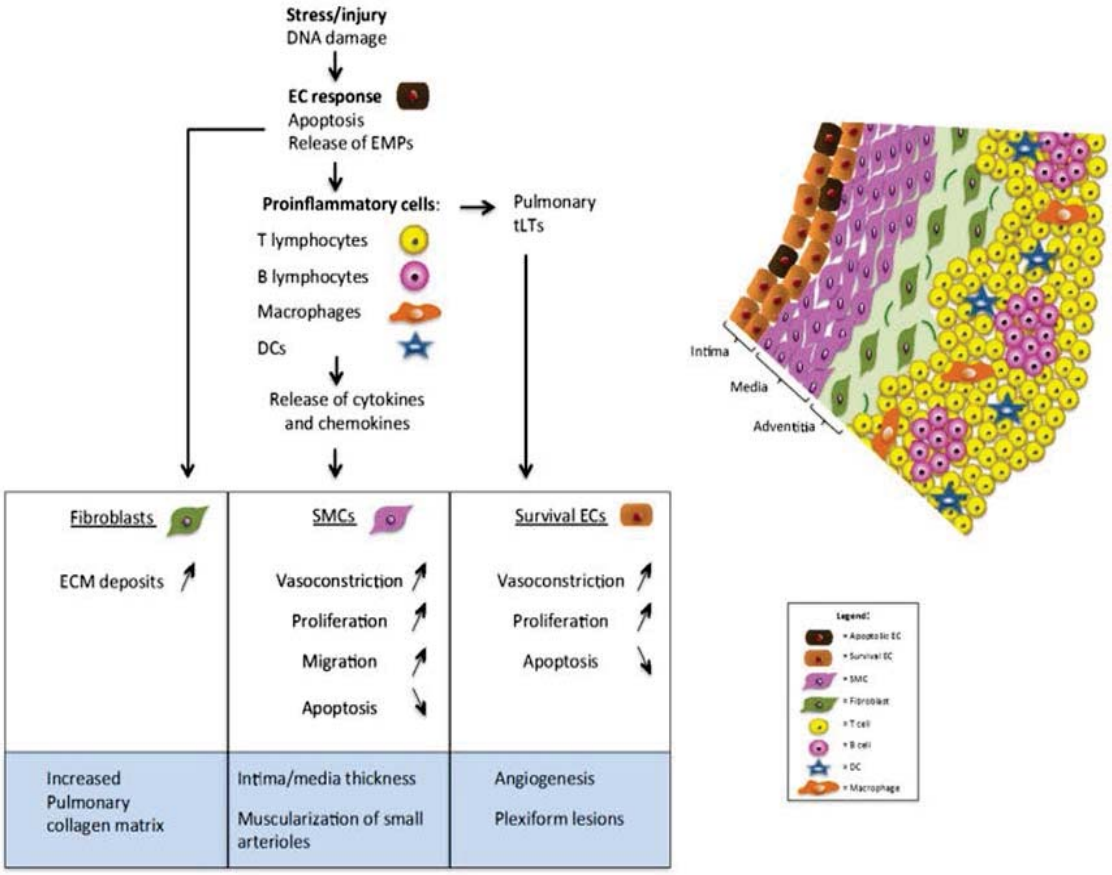
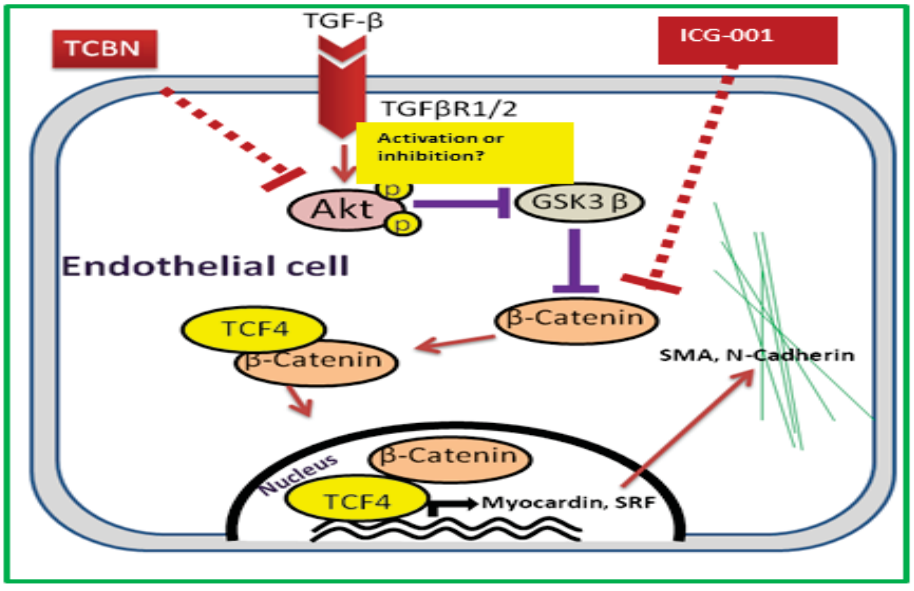


Figure 1: Stress induced endothelial injury progresses into transdifferentiation of endothelial cells. In pulmonary arterial hypertension, stress and injury induces DNA damage that leads to endothelial cell (EC) apoptosis. This will lead to increased inflammatory cell infiltration, which will trigger proliferation and suppressed apoptosis of smooth muscle cells (SMCs), fibroblasts, and ECs. DC, dendritic cells; ECM, extracellular matrix; EMP, endothelial microparticles; tLT, tertiary lymphoid tissue**. Figure adopted from Vaillancourt et al, Canadian J of Cardiology, 2015**

OBJECTIVE AND CENTRAL HYPOTHESIS

Endothelial to mesenchymal transition (EndMT) is a phenomenon in which endothelial cells lose their cell specific markers and acquire mesenchymal markers thus rendering them motile, proliferative and invasive. EndMT is implicated not only in embryonic development of septa and valves of the heart, and maturation of pulmonary arteries and veins, but also in the development and progression of fibrotic and vascular diseases including cardiac, pulmonary and kidney fibrosis, acute lung injury, chronic obstructive pulmonary disease, chronic pulmonary hypertension, and pulmonary arterial hypertension. Although profibrotic (TGFβ) and inflammatory cytokines (TNF-α, IL-10) are reported to be involved in promoting EndMT, the trigger that initiates EndMT and the mechanisms through which it progresses remain elusive. Given the critical role played by PI3K-Akt signaling in regulating survival and functioning of ECs, it is important to address the controversies existing on the protective vs destructive roles of Akt on barrier integrity and its cross-talk with Src (if any) in inducing endothelial injury leading to EndMT.

Our **central hypothesis** is that, "*Akt1 suppression by TGFβ1 induces GSK3β-mediated nuclear translocation of β-catenin, promoting endothelial injury, EndMT and hypoxia-induced pulmonary vascular remodeling*"



SPECIFIC AIMS

Aim 1: Compare the effects of TGFβ1, VEGF and Ang-1 on Akt1 pathway and its cross-talk with Src in regulating EC-barrier function

In order to study the effect of endothelial specific loss of Akt1 on regulating endothelial barrier integrity leading to endothelial injury, a stable ShRNA mediated Akt1 knock down human microvascular endothelial cell (HMEC) line will be prepared. ECIS assay will be performed to measure the endothelial barrier resistance to elucidate the effect of Akt1 loss on VEGF, Ang-1 mediated vascular permeability. Immunofluorescence studies will be performed to investigate the effect of loss of endothelial Akt1 in gap formation and hence barrier protection. Western blot analysis of Akt1 null HMECs and HMECs treated with triciribine, a pan Akt inhibitor, or pp2 a selective Src family kinase inhibitor will be performed to investigate the phosphorylation patterns of Akt1 and Src to be able to understand the cross-talk between them, if it exists.

Aim 2: Determine the effects of TGFβ1, TGFβ2 and TGFβ3 on the activation of canonical (Smad) and non-canonical (p38 MAPK) signalling in ECs and promotion of EndMT

To accomplish this aim HMECs will be stimulated with three different TGF-β isoforms 1, 2 and 3 in a dose and time dependent manner to examine if TGFβ induces EndMT *in-vitro.* If it does, which one of the three isoforms is a more potent inducer of EndMT will be determined. These objectives will be studied using western blot analysis for upregulation of mesenchymal markers N-cadherin, α-SMA and downregulation of endothelial markers VE-cadherin, eNOS. The activation of canonical (Smad2/3) and non-canonical (p-38MAPK) along with mesenchymal transcription factors Snail and Foxc2 will be examined.

Aim 3: Investigate the effect of endothelial specific loss of Akt1 on EndMT *in-vitro* and hypoxia-induced pulmonary vascular remodelling *in-vivo*

In order to study the effect of endothelial specific loss of Akt1, stable ShRNA mediated Akt1 null HMECs will be generated and subjected to western blot analysis and immunofluorescence staining for changes in endothelial and mesenchymal markers. Gene array and protein array analyses will be performed to identify the genes and proteins that are being modulated to elicit the EndMT effects following the loss of Akt1 and identify any changes in the expression of endothelial and mesenchymal genes. To study the effect of EC specific loss of Akt1 *in-vivo* VE-cadherin driven tamoxifen inducible Cre-Akt1 mice and C57BL6 mice will be subjected to a 21 day chronic

hypoxia along with a weekly i.p administration of 20mg/kg SU-5416, a VEGFR2 inhibitor to induce pulmonary arterial hypertension. One of the groups will be treated with 10 mg/kg i.p ICG-001, a β-catenin inhibitor during exposure to hypoxia once a day. Lungs, hearts, pulmonary arteries will be harvested and subjected to histopathological studies to examine the development of vascular remodeling and western blot analysis to elucidate the downstream signaling molecules involved. Heart chambers will be isolated and weighed separately to measure Fulton index, to study right ventricular hypertrophy. Co staining of endothelial marker VE-cadherin/CD31 and mesenchymal marker α-SMA in pulmonary arteries will be performed to identify the percentage of endothelial population that is transitioning into mesenchymal cells. Comparative analysis in the development of vascular remodeling due to loss of Akt1 will be examined and if it is reversed with the administration of ICG-001.

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CHAPTER 2

MODULATION OF LONG-TERM ENDOTHELIAL-BARRIER INTEGRITY IS CONDITIONAL TO THE CROSS-TALK BETWEEN AKT AND SRC SIGNALING

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[1]Sabbineni H, [1]Gao F, Artham S and Somanath PR. J Cell Physiol. 2017 Oct;232(10):2599-2609. Reprinted here with the permission of the publisher.

ABSTRACT

Although numerous studies have implicated Akt and Src kinases in vascular endothelial growth factor (VEGF) and Angiopoietin-1 (Ang-1)-induced endothelial-barrier regulation, a link between these two pathways has never been demonstrated. We determined the long-term effects of Akt inhibition on Src activity and *vice versa*, and in turn, on the human microvascular endothelial cell (HMEC) barrier integrity at the basal level, and in response to growth factors. Our data showed that Akt1 gene knockdown increases gap formation in HMEC monolayer at the basal level. Pharmacological inhibition of Akt, but not Src resulted in exacerbated VEGF-induced vascular leakage and impaired Ang-1-induced HMEC-barrier protection *in-vitro* at 24 hours. Whereas inhibition of Akt had no effect on VEGF-induced HMEC gap formation in the short term, inhibition of Src blunted this process. In contrast, inhibition of Akt disrupted the VEGF and Ang-1 stabilized barrier integrity in the long-term while inhibition of Src did not. Interestingly, both long-term Akt inhibition and Akt1 gene knockdown in HMECs resulted in increased Tyr416 phosphorylation of Src. Treatment of HMECs with transforming growth factor-β1 (TGFβ1) that inhibited Akt Ser473 phosphorylation in the long-term, activated Src through increased Tyr416 phosphorylation and decreased HMEC-barrier resistance. The effect of TGFβ1 on endothelial-barrier breakdown was blunted in Akt1 deficient HMEC monolayers, where endothelial-barrier resistance was already impaired compared to the control. To our knowledge, this is the first report demonstrating a direct cross-talk between Akt and Src in endothelial-barrier regulation.

Keywords: Akt; Src; vascular permeability; endothelial-barrier; VE-cadherin

1. INTRODUCTION

Vascular permeability is a complex yet a highly coordinated process that not only regulates vesicular trafficking but also integrates complex junction rearrangements, and refined cytoskeletal dynamics (Goddard and Iruela-Arispe 2013). The endothelium plays a key role in regulating vascular integrity. Recent studies suggest that impairment of endothelial function, as observed in the presence of cardiovascular risk factors, is not only a marker but also contributes to the pathogenesis of cardiovascular diseases (Landmesser, Hornig et al. 2004). Thus, improving endothelial function is an important therapeutic target for reducing vascular diseases (Bonetti, Lerman et al. 2003, Melo, Gnecchi et al. 2004). It is evident that Akt1 is highly involved in the vascular endothelial growth factor (VEGF)-mediated vascular permeability as the phosphorylation of Akt1 increases considerably in VEGF-stimulated endothelial cells (Chen, Somanath et al. 2005). However, since Akt1 is activated by not only vascular permeability-inducing agents such as VEGF and tumor necrosis factor-α (Fairaq, Goc et al. 2015, Gao, Artham et al. 2016) but also by agents that promote barrier integrity such as angiopoietin-1 (Ang-1), roundabout guidance receptor-4 and sphingosine-1-phosphate, the precise role of Akt1 in regulating vascular permeability was not clear until recently (Daly, Wong et al. 2004, De Palma, Meacci et al. 2006, Somanath, Kandel et al. 2007). Studies from our laboratory have demonstrated that Akt1 is important for vascular maturation and that suppression of Akt1 activity leads to increased vascular permeability (Chen, Somanath et al. 2005). There are also reports demonstrating the role of Akt1-FoxO signaling in the transcriptional up-regulation of claudin-5 as a result of vascular endothelial-cadherin (VE-cadherin) clustering at the endothelial junctions (Taddei, Giampietro et al. 2008) and in response to long-term treatments with VEGF and Ang-1 (Gao, Artham et al. 2016). Apart from Akt, Src family of non-receptor tyrosine kinases is well known for its contributions to the VEGF-induced vascular leakage (Ha, Bennett et al. 2008). Pathological levels of VEGF result in endothelial barrier breakdown via Src-mediated degradation of VE-cadherin (Gavard, Patel et al. 2008, Azzi, Hebda et al. 2013).

Although a lot has been reported about the involvement of Akt and Src in the modulation of growth factor-mediated endothelial-barrier integrity and vascular permeability, it is not clear until today whether there is any cross-talk between Akt and Src in mediating endothelial-barrier function. Given the importance of Akt and Src in endothelial function, survival, and barrier regulation, we wanted to study the effect of Akt inhibition on Src activity and *vice versa*, in turn,

regulating the endothelial-barrier. Our results indicated that whereas Src, but not Akt is necessary for the early VEGF-induced HMEC-barrier breakdown, in the later stages, Akt, but not Src is responsible for the long-term HMEC-barrier stability offered by VEGF and Ang-1. Interestingly, whereas long-term pharmacological inhibition of Akt and ShRNA-mediated Akt1 gene knockdown in HMECs resulted in increased activating Src Tyr416 phosphorylation and increased HMEC-barrier breakdown, long-term treatment with transforming growth factor-β1 (TGFβ1) resulted in decreased Akt1 Ser473 phosphorylation, increased Src Tyr416 phosphorylation and increased HMEC-barrier breakdown. Effect of TGFβ1 on HMEC-barrier was blunted in Akt1 deficient HMEC monolayers apparently due to the already reduced Akt activity and increased Src activity in Akt1 deficient HMECs. Together, we show for the first time that Akt and Src maintain a reciprocal phosphorylation/activity pattern in HMECs and thus provide novel insights into the cross-talk between Akt and Src, and their interplay in the regulation of endothelial-barrier function.

2. MATERIALS AND METHODS

2.1 Cell culture and preparation of ShAkt1 stable cell lines

Telomerase-immortalized HMECs (CRL-4025; ATCC, Manassas, VA) were maintained in EBM-2 with a Growth factor-2 Bullet Kit (Lonza; Walkersville, MD). Cultures were maintained in a humidified 5% CO2 incubator at 37 ℃ and routinely passaged when 80– 90% confluent.

Stable ShControl, ShAkt1 (sequence: ACGCTTAACCTTTCCGCTG) HMECs were generated using SMART vector 2.0 lentivirus particles (10^9 pfu) (Thermo Scientific, Waltham, MA). Lentiviral particles mixed in 1ml Hyclone SFM4Transfx-293 (Fisher, Hanover Park, IL) were added along with 1 µl Polybrene (10 mg/ml, American bioanalytical, Natick, MA). Three days later, transfection efficiency was tested through Turbo-GFP expression and subjected to 4 µg/ml Puromycin (Life Technologies, Grand Island, NY) selection until all the cells expressed GFP.

2.2 Measurement of endothelial-barrier resistance

Endothelial-barrier integrity was measured as the electrical resistance of the HMEC monolayer using ‘electric cell-substrate impedance sensing’ (ECIS) equipment (Applied Biophysics, NY) as described previously (Goc, Al-Azayzih et al. 2013, Gao, Al-Azayzih et al. 2015). To synchronize the HMECs before treatment, cells were cultured in serum-free EBM-2 for 5 hours, followed by

treatment with either 20 ng/ml VEGF or 50 ng/ml Ang-1. Endothelial-barrier resistance was measured at multiple frequency modes for 24 hours. Growth factors for stimulation such as VEGF, Ang-1 and TGFβ1 were obtained from R&D Systems (Minneapolis, MN).

2.3 Immunofluorescence staining

Immunofluorescence staining of HMEC monolayers was performed using chamber slides (Fisher, Hanover Park, IL). Cells were cultured to monolayer, treated with either vector or 20 ng/ml VEGF or 50 ng/ml Ang-1 and co-treated with either 10 μM Triciribine (TCBN) or 1 μM pp-2 for 24 hours and washed twice with ice-cold PBS, fixed using 2% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 15 min, and blocked with 2% BSA in sterile PBS for 1 hour. Cell monolayers were then incubated with antibodies against VE-cadherin (1:100, Rabbit antibodies, Cell Signaling, Danvers, MA) at 4℃ overnight. Immunofluorescence was revealed using goat anti-rabbit AlexaFlour-488 secondary antibodies (1:2000, Life Technologies, Grand Island, NY). Cells were mounted on to a glass slide using DAPI containing mounting medium (Vector Laboratories, PA). Images were captured using a confocal microscope equipped with argon and helium/neon lasers (LSM510, Zeiss, Germany). Controls were performed by omitting primary antibodies. All controls gave negative results with no detectable non-specific labeling.

2.4 Western blot analysis

Cell lysates were prepared using complete lysis buffer (EMD Millipore, San Diego, CA) with protease and phosphatase inhibitor cocktails (Roche Diagnostics, Indianapolis, IN). Protein quantification was performed using DC protein assay (Bio-Rad, Hercules, CA. Western blot analysis was performed as described previously (Goc, Al-Azayzih et al. 2013, Gao, Al-Azayzih et al. 2015). Antibodies used include Src Tyr416, total-Src, Akt Ser473, total-Akt (Cell Signaling, Danvers, MA) and anti- β-actin (Sigma, St. Louis, MO). Densitometry was done using NIH Image J software.

2.5 Statistical Analysis

All the data are presented as Mean $\pm$ SD and were calculated from multiple independent experiments performed in quadruplicates. For normalized data analysis, data was confirmed that normality assumption was satisfied and analyzed using paired sample *t*-test (dependent *t*-test) and/or further confirmed with non-parametric test Wilcoxon signed rank test. For all other analysis,

Student's two-tailed *t*-test or ANOVA test were used to determine significant differences between treatment and control values using the GraphPad Prism 4.03 and SPSS 17.0 software.

3. RESULTS

3.1 Long-term Akt inhibition disrupts, but Src inhibition protects the endothelial-barrier

Whereas the role of Src in VE-cadherin internalization is well known, our immunocytochemistry analysis indicated that ShRNA-mediated Akt1 gene knockdown in HMEC monolayers results in increased gap formation, compared to ShConrol HMEC monolayers (Figure 1A-D). Whereas no significant difference was observed in the number of gaps in Src inhibitor (pp2) treated HMEC monolayers, treatment with Akt inhibitor TCBN resulted in increased number of gaps in the HMEC monolayers, compared to control (DMSO treated) monolayers (Figure 1E-F). A similar result on the Src and Akt inhibitor was also observed in another analysis on endothelial-barrier resistance as measured using the ECIS approach. Whereas Src inhibition, but not Akt inhibition promoted HMEC-barrier resistance at 90 minutes post treatment, Akt inhibition, but not Src inhibition reduced HMEC-barrier resistance in the long-term (24 hours) (Figure 2A-B). To explore the differential effects of Akt and Src on barrier permeability, HMEC monolayers were treated with either 20 ng/ml VEGF (Figure 2C-D) or 50 ng/ml Ang-1 (Figure 2E-F) and co-treated with either 10 μM TCBN, a pharmacological inhibitor of Akt or 1 μM pp2, a selective Src inhibitor. Barrier resistance was measured in real-time using ECIS assay for 24 hours. Inhibition of Akt, but not Src resulted in exacerbated VEGF-induced vascular leakage and reduced Ang-1 induced barrier protection *in-vitro* at 24 hours suggesting the vascular protective role of Akt in the long term.

3.2 Inhibition of Akt, but not Src results in HMEC monolayer gap formation in the long-term

HMEC monolayers were treated with either the vehicle (DMSO), 20 ng/ml VEGF or 50 ng/ml Ang-1, and co-treated with either 10 μM TCBN or 1 μM pp2 for 30 minutes and 24 hours. Immunofluorescence staining was performed using VE-Cadherin antibodies to study the effect of inhibitors on the number of "gaps" formed in the monolayers. As expected, VEGF increased the number of gaps in 30 minutes compared to that of control. While inhibition of Akt did not affect it further, inhibition of Src reduced the VEGF-induced gap formation (Figure 3A-B). In contrast,

at 24 hours, VEGF stabilized the HMEC-barrier integrity, which was disrupted by Akt inhibition (Figure 3C-D). Inhibition of Src had no effect on VEGF-induced stabilization of barrier integrity in the long term. Ang-1-induced long-term HMEC-barrier protection was disrupted by Akt inhibition while Src inhibition had no significant effect (Figure 3E-F).

3.3 Akt and Src maintain a reciprocal relationship in the phosphorylation/activity pattern

To elucidate the relationship between the expression and phosphorylation patterns of Akt and Src upon mutual inhibition and growth factor stimulation in the long-term, HMECs were treated in the presence or absence of TCBN for 1 to 24 hours and changes in phosphorylation of Tyr416 Src were studied using Western blot analysis. Inhibition of Akt promoted Tyr416 phosphorylation of Src starting from 12 hours after treatment with TCBN (Figure 4A-B). No changes in the Src Tyr416 phosphorylation were observed during the early stages of TCBN treatment. Alternately, HMECs were subjected to ShRNA-mediated Akt1 knockdown followed by treatment with either 20 ng/ml VEGF or 50 ng/ml Ang-1, and changes in Src Tyr416 phosphorylation were examined. Loss of Akt1 by itself induced an increase in Src Tyr416 phosphorylation, which was further increased in the presence of Ang-1 or 20 ng/ml VEGF (Figure 4C-D), but not 50 ng/ml VEGF. In order to directly compare the changes in Akt phosphorylation upon TCBN treatment and its correlation with changes in Src phosphorylation, we determined a time course effect of TCBN on Akt Ser473 phosphorylation. Our data indicates that significant increase in Src Tyr416 phosphorylation at 12 and 24 hours after TCBN treatment correlates with a significant decrease in Akt Ser473 phosphorylation (Figure 4E-F).

3.4 TGFβ1 inhibits Ser473 phosphorylation of Akt and enhances Tyr416 phosphorylation of Src to induce HMEC-barrier breakdown in a time-dependent manner

To investigate the cross-talk between the activity of Akt and Src upon stimulus from a growth factor known to induce endothelial injury in the long-term, HMECs were treated with 5 ng/ml TGFβ1 for 0-24 hours. Western blot analysis of the cell lysates collected at various time points following TGFβ1 treatment indicated a time-dependent reduction in Akt Ser473 phosphorylation (Figure 5A and 5B). In contrast, although TGFβ1 treatment inhibited Tyr416 Src phosphorylation early on (6 hours), prolonged stimulation resulted in increased Tyr416 Src phosphorylation (Figure 5C and 5D), showing a reciprocal relationship with Akt in the long-term. The barrier permeability of HMEC monolayers was increased with TGFβ1 treatment in a time-dependent manner, an effect

that was blunted in already permeable Akt1 knockdown cells (Figure 5E-F), suggesting that TGFβ1 inhibits Akt and activates Src in the long-term to induce endothelial injury (Figure 6).

4. DISCUSSION

Vascular permeability is the hallmark of several diseases including cancer, acute kidney and lung injuries, cerebral edema, diabetes-related vascular complications, chronic inflammatory diseases, and cardiovascular diseases. (Leu, Berk et al. 2000, Azzi, Hebda et al. 2013, Moughon, He et al. 2015). The healthy and intact endothelium is essential for vascular homeostasis. Although numerous signaling pathways are involved in regulating the endothelial-barrier function, PI3K-Akt and Src pathways are of prime importance in regulating endothelial cell survival, proliferation, migration, endothelial-barrier function and gene expression (Coffer, Jin et al. 1998, Eliceiri, Paul et al. 1999, Minshall, Tiruppathi et al. 2002, Shiojima and Walsh 2002, Laird, Li et al. 2003, Yeatman 2004). A variety of stimuli including growth factors, cytokines, vascular permeability-inducing agents such as VEGF, and barrier protective agents such as Ang-1 activate Akt and Src and hence are greatly implicated in the regulation of vascular wall integrity (Eliceiri, Paul et al. 1999, Shiojima and Walsh 2002, Gavard and Gutkind 2006, Wallez, Cand et al. 2007, Aghajanian, Wittchen et al. 2008, Guignabert and Montani 2013). While there have been contradicting reports about the role of Akt in endothelial-barrier regulation, our recent study has demonstrated the integral role of Akt1 in the long-term protection of endothelial-barrier in response to VEGF and Ang-1 (Gao, Artham et al. 2016). The src family of non-receptor tyrosine kinases has been implicated in vascular permeability and injury (Paul, Zhang et al. 2001, He, Liu et al. 2015). Following ischemia and injury, phosphorylation of Src is highly upregulated causing activation of downstream signaling to induce barrier breakdown and vascular permeability (Esser, Lampugnani et al. 1998, Ha, Bennett et al. 2008). Src is activated in pathological conditions following elevated levels of VEGF and causes internalization of VE-cadherin, a key component of adherens junctions which is responsible for maintaining intact endothelium (Azzi, Hebda et al. 2013). Although a great amount of light has been shed on the independent roles of Akt and Src and their specific roles in the regulation of endothelial-barrier function and vascular permeability, a cross-talk between these two pathways in response to various stimuli, if there is one, has not been studied yet.

The objective of the current study was to investigate the changes in Ser473 Akt and Tyr416 Src-activating phosphorylations in response to growth factors that make or break endothelial-barrier junctions, determine the effect of direct activity modulation of Akt on Src and *vice versa*, and finally establish a cross-talk between Akt and Src in the modulation of endothelial-barrier function. Our results show that although VEGF-induced endothelial-barrier permeability is short-term and is reversed on due course of time, TCBN-mediated Akt inhibition blunts the reversal and diminishes the long-term barrier protective effects of VEGF and Ang-1. In contrast, pp2-mediated Src inhibition reversed VEGF-induced short-term vascular permeability but had no significant effect on the VEGF and Ang-1-mediated long-term barrier protection. Genetic loss of Akt1 achieved by ShRNA-mediated knockdown resulted in increased gap formation at the basal levels compromising the barrier integrity, thus confirming our previous observations that Akt1 loss results in increased vascular permeability *in vivo* (Chen, Somanath et al. 2005, Gao, Artham et al. 2016), and a report by Mukai *et al* (Mukai, Rikitake et al. 2006) demonstrating that endothelial-specific activation of Akt1 suppresses lesion formation and maintains integrity of vascular wall. As expected, VEGF increased the number of gaps in the short-term, an effect that was blunted by the inhibition of Src, but not Akt. However, in the long-term (24 hours), both VEGF and Ang-1 stabilized the endothelial-barrier, which was disrupted by inhibition of Akt but not Src, thus indicating that both Src and Akt play different roles in the short- and long-term endothelial-barrier regulation in response to VEGF and Ang-1.

Interestingly, inhibition of Akt although did not exhibit any significant change in the levels of Tyr416 Src phosphorylation in the short-term (0- 6 hours), it resulted in increased levels of activating Tyr416 Src phosphorylation in the long-term (12-24 hours). The increase in Src Tyr416 phosphorylation was accompanied by a decrease in Akt Ser473 phosphorylation at 12 and 24 hours following TCBN treatment, indicating that long-term inactivation of Akt enhances Src activity, thus contributing to the long-term endothelial-barrier disruption following Akt inhibition. Similarly, a significant increase in Tyr416 Src phosphorylation was also observed in Akt1 deficient HMECs. Although Akt-mediated effects are specific to the tight-junction protein turnover, and not adherens junction modulation (Gao, Artham et al. 2016), activation of Src upon long-term inhibition of Akt would explain why we still see gap formations in VE-cadherin stained Akt1 deficient HMEC-monolayers.

To further confirm the Akt and Src cross-talk in the long-term endothelial-barrier regulation, we involved TGFβ, a cytokine known to induce endothelial and vascular injury in the long-term. Stimulation of HMECs with TGFβ1 although did not affect Ser473 Akt phosphorylation in the short-term, it resulted in reduced Ser473 Akt phosphorylation and hence its inhibition in the long-term. In contrast, although TGFβ1 inhibited Tyr416 Src phosphorylation in the short-term, it promoted Tyr416 Src phosphorylation in the long term, once again indicating a reciprocal regulation of Akt and Src activities in the long-term in HMECs. Furthermore, whereas TGFβ1 treatment resulted in HMEC-barrier disruption in the long-term, this effect was blunted in ShAkt1 HMEC monolayers, thus indicating that Akt inhibition is necessary for the TGFβ1-induced endothelial-barrier injury.

Although our data indicate a reciprocal regulation of Akt and Src pathways in the long-term endothelial barrier function, the underlying molecular mechanisms regulating this cross-talk need further extensive analysis. Nevertheless, since the growth factors such as VEGF, Ang-1, and TGFβ that modulate endothelial-barrier function and angiogenesis in various vascular beds in physiological, hypoxic as well as pathological conditions, the existence of Akt-Src cross-talk in these conditions *in vivo* is very likely. However, since Akt is a serine-threonine kinase and Src a tyrosine kinase, it is very clear that the effects are not direct. The fact that Akt-Src cross-talk occurs only in the long-term, the possibility of secondary events including paracrine effects, in this process, cannot be ruled out.

In conclusion, we report for the first time that Akt and Src maintain a reciprocal regulation of their activities in response to various growth factors in the regulation of long-term endothelial-barrier function (Figure 6). Whereas VEGF and Ang-1 activate Akt to return Src activity to the basal levels in the long-term thus protecting the endothelial-barrier, TGFβ stimulation inhibits Akt and activates Src in the long-term leading to endothelial-barrier injury. In support of this newly characterized Akt and Src cooperation by three different growth factors in the modulation of long-term endothelial-barrier function, treatment with Akt inhibitor TCBN that promotes endothelial-barrier breakdown in the long-term is also associated with increased Src activation. Based on these observations and support from our previous findings in this field (Chen, Somanath et al. 2005, Gao, Artham et al. 2016) we demonstrate the programmed orchestration of adherens-junction and

tight-junction protein turnover by Src and Akt respectively, in the modulation of endothelial-barrier function.

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CONFLICT OF INTEREST: The authors declare that no conflict of interest exists.

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FIGURES AND FIGURE LEGENDS

Figure 1

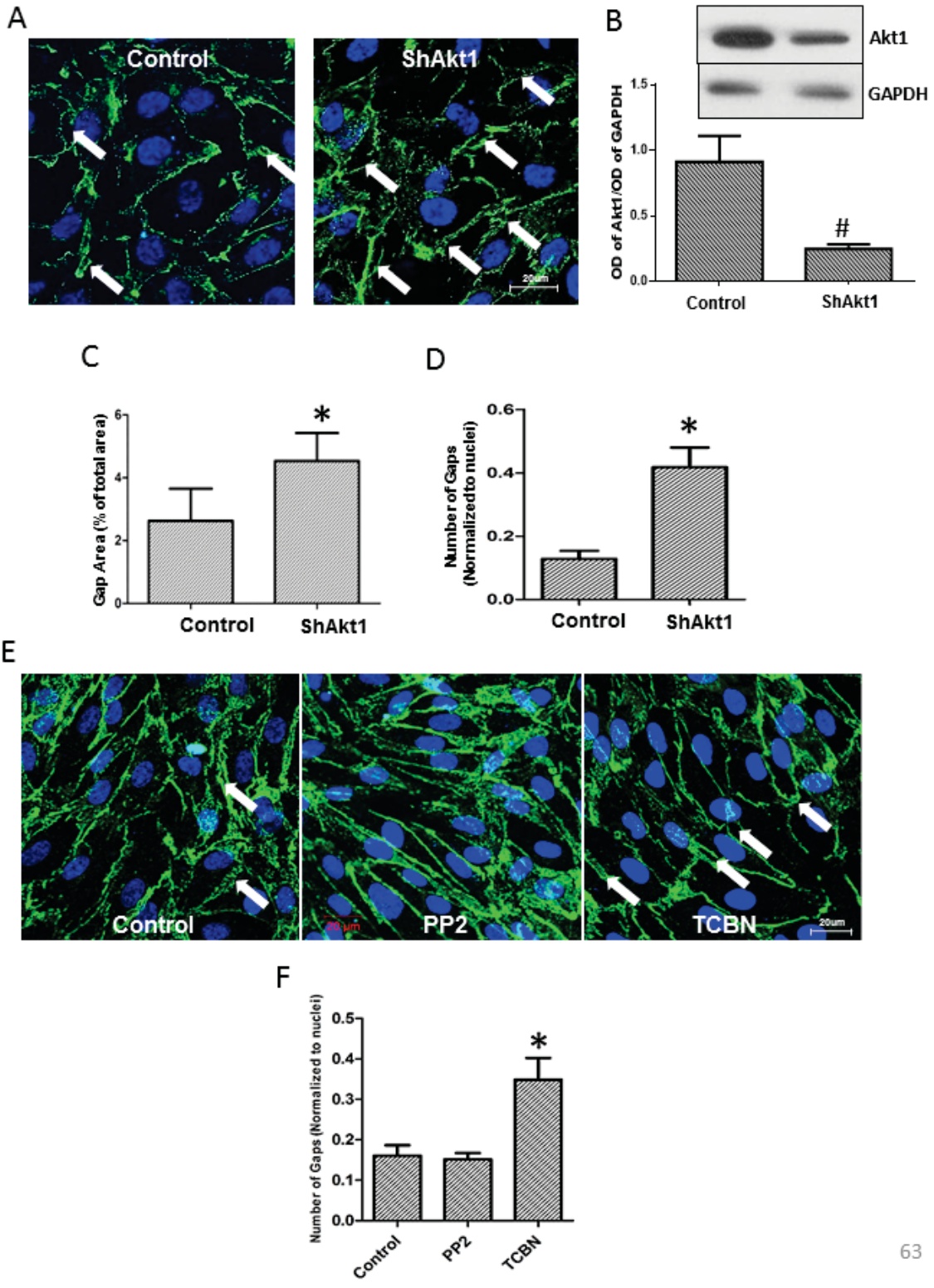


Figure 1: Long-term Akt inhibition disrupts, but Src inhibition protects the endothelial-barrier. (A) Representative confocal images showing immunofluorescence staining of VE-cadherin on HMEC monolayers transfected with either scrambled ShRNA or ShRNA targeting Akt1. **(B)** Representative Western blot images and band densitometry quantification of stable ShControl and ShAkt1 HMEC lysates showing reduced Akt1 expression in ShAkt1 HMEC compared to ShControl HMEC (n=3). **(C)** Quantification of the gap area in control and Akt1 knockdown HMEC monolayers normalized to the total area (n=4). **(D)** Quantification of the number of gaps in control and Akt1 knockdown HMEC monolayers normalized to the number of nuclei per field (n=4). **(E)** Representative confocal images showing VE-cadherin staining on the vehicle (DMSO), Src inhibitor (PP2) and Akt inhibitor (TCBN) treated HMEC monolayers 24 hours after treatment. **(F)** Quantification of the number of gaps in the vehicle (DMSO), Src inhibitor (PP2) and Akt inhibitor (TCBN) treated HMEC monolayers 24 hours after treatment (n=4). Data are represented as mean $\pm$ SD. *$P<0.01$, #$P<0.05$, *scale bar 20 μm*.

Figure 2

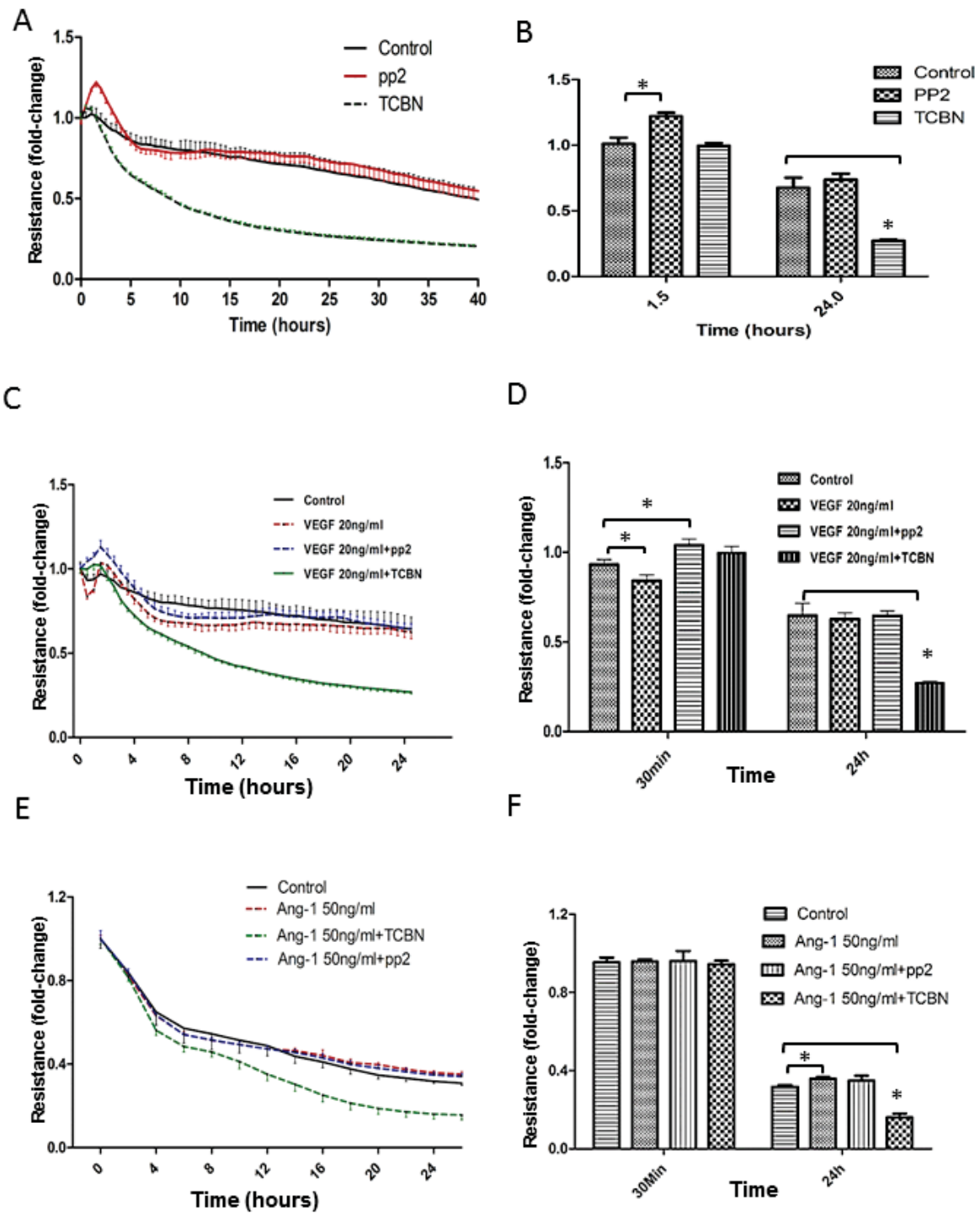
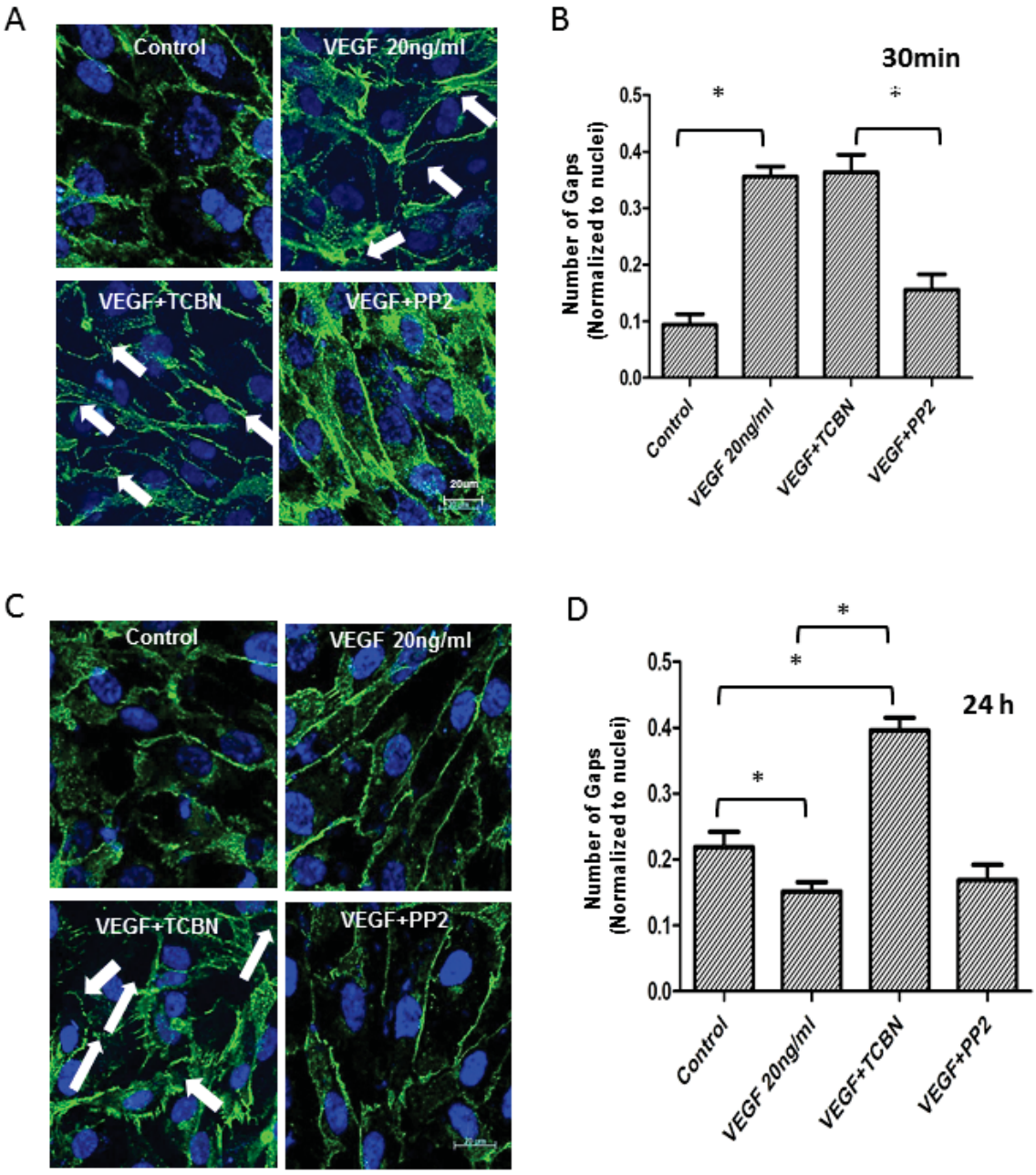
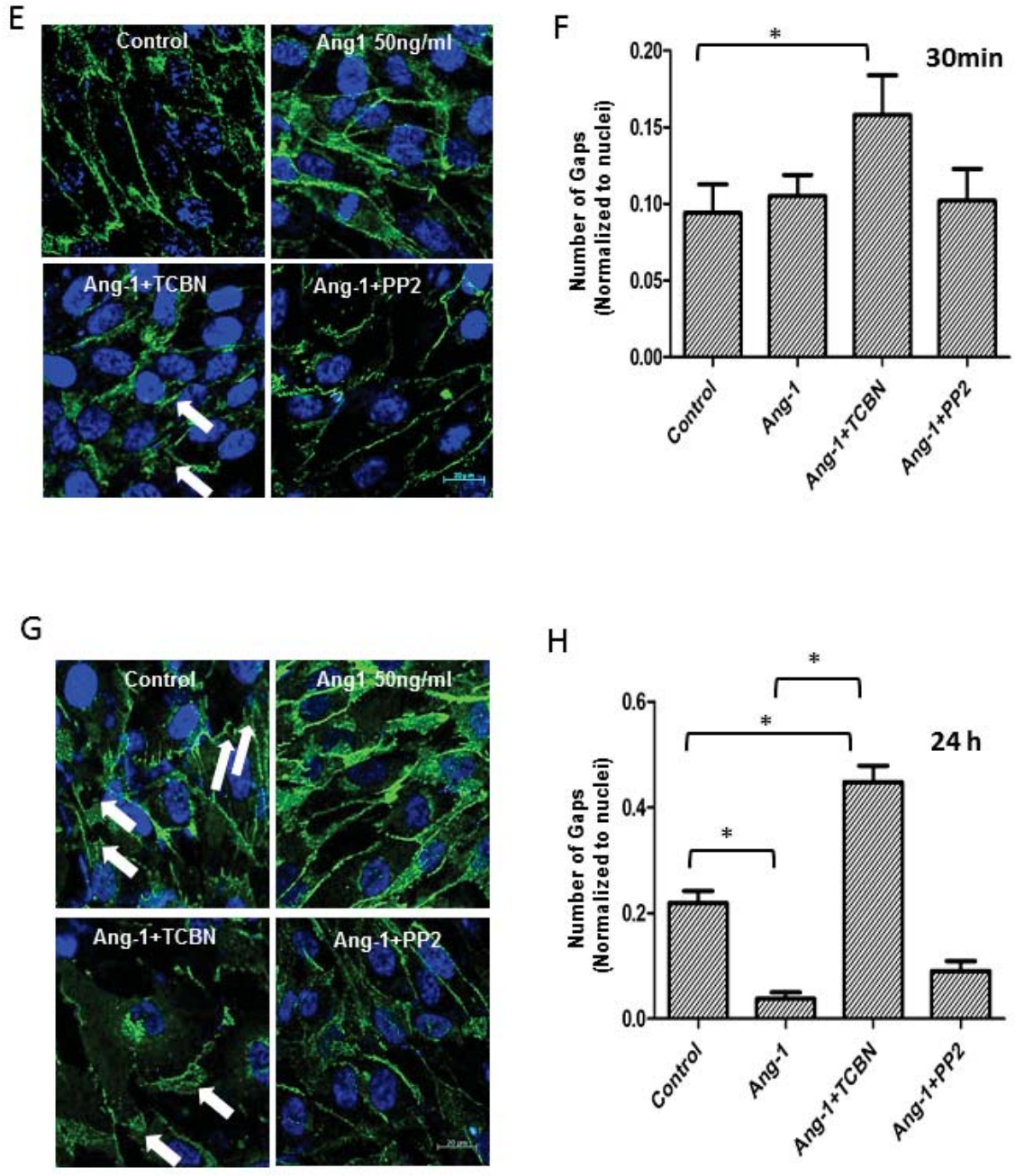

A
Control
pp2
TCBN
Resistance (fold-change)
Time (hours)
B
Control
PP2
TCBN
Time (hours)
C
Control
VEGF 20ng/ml
VEGF 20ng/ml+pp2
VEGF 20ng/ml+TCBN
Resistance (fold-change)
Time (hours)
D
Control
VEGF 20ng/ml
VEGF 20ng/ml+pp2
VEGF 20ng/ml+TCBN
Resistance (fold-change)
30min
Time
24h
E
Control
Ang-1 50ng/ml
Ang-1 50ng/ml+TCBN
Ang-1 50ng/ml+pp2
Resistance (fold-change)
Time (hours)
F
Control
Ang-1 50ng/ml
Ang-1 50ng/ml+pp2
Ang-1 50ng/ml+TCBN
Resistance (fold-change)
30Min
Time
24h

Figure 2: Akt inhibition promotes, but Src inhibition reduces endothelial-barrier leakage. **(A)** Real-time changes in the barrier resistance of control, 1 μM pp2 and 10 μM TCBN treated HMEC monolayers as measured using ECIS equipment. **(B)** Quantification of the changes in barrier resistance between control, 1 μM pp2 and 10 μM TCBN treated HMEC monolayers at 90 minutes and 24 hours after plating an equal number of cells in array wells (n=3). **(C)** Real-time changes in the barrier resistance of control and 20 ng/ml VEGF-treated HMEC monolayers co-treated with either TCBN or pp2 as measured using ECIS equipment. **(D)** Quantification of the changes in barrier resistance between control and 20 ng/ml VEGF-treated HMEC monolayers at 30 minutes and 24 hours after plating an equal number of cells in array wells (n=3). **(E)** Real-time changes in the barrier resistance of control and 50 ng/ml Ang-1 treated HMEC monolayers co-treated with either TCBN or pp2 as measured using ECIS equipment. **(F)** Quantification of changes in the barrier resistance between control and 50 ng/ml treated HMEC monolayers at 30 minutes and 24 hours after plating an equal number of cells in array wells (n=3). Data are represented as mean $\pm$ SD. *$P<0.01$.

Figure 3


A
Control
VEGF 20ng/ml
VEGF+TCBN
VEGF+PP2
B
30min
Number of Gaps
(Normalized to nuclei)
0.5
0.4
0.3
0.2
0.1
0.0
Control
VEGF 20ng/ml
VEGF+TCBN
VEGF+PP2
C
Control
VEGF 20ng/ml
VEGF+TCBN
VEGF+PP2
D
24 h
Number of Gaps
(Normalized to nuclei)
0.5
0.4
0.3
0.2
0.1
0.0
Control
VEGF 20ng/ml
VEGF+TCBN
VEGF+PP2


E
Control
Ang1 50ng/ml
Ang-1+TCBN
Ang-1+PP2
F
30min
Number of Gaps
(Normalized to nuclei)
0.20
0.15
0.10
0.05
0.00
*
Control
Ang-1
Ang-1+TCBN
Ang-1+PP2
G
Control
Ang1 50ng/ml
Ang-1+TCBN
Ang-1+PP2
H
24 h
Number of Gaps
(Normalized to nuclei)
0.6
0.4
0.2
0.0
*
*
*
Control
Ang-1
Ang-1+TCBN
Ang-1+PP2

4

Figure 3: Inhibition of Akt, but not Src results in HMEC monolayer gap formation in the long-term. (A-B) Representative confocal images and quantification of the gap number in VE-cadherin stained HMEC monolayers treated with 20 ng/ml VEGF in the presence and absence of Akt inhibitor TCBN or Src inhibitor pp2 for 30 minutes. **(C-D)** Representative confocal images and quantification of the gap number in VE-cadherin stained HMEC monolayers treated with 20 ng/ml VEGF in the presence and absence of Akt inhibitor TCBN or Src inhibitor pp2 for 24 hours. **(E-F)** Representative confocal images and quantification of the gap number in VE-cadherin stained HMEC monolayers treated with 50 ng/ml Ang-1 in the presence and absence of Akt inhibitor TCBN or Src inhibitor pp2 for 30 minutes. **(G-H)** Representative confocal images and quantification of the gap number in VE-cadherin stained HMEC monolayers treated with 50 ng/ml Ang-1 in the presence and absence of Akt inhibitor TCBN or Src inhibitor pp2 for 24 hours. Data are represented as mean $\pm$ SD. (n=4), *$P<0.01$, *scale bar 20 μm*.

Figure 4

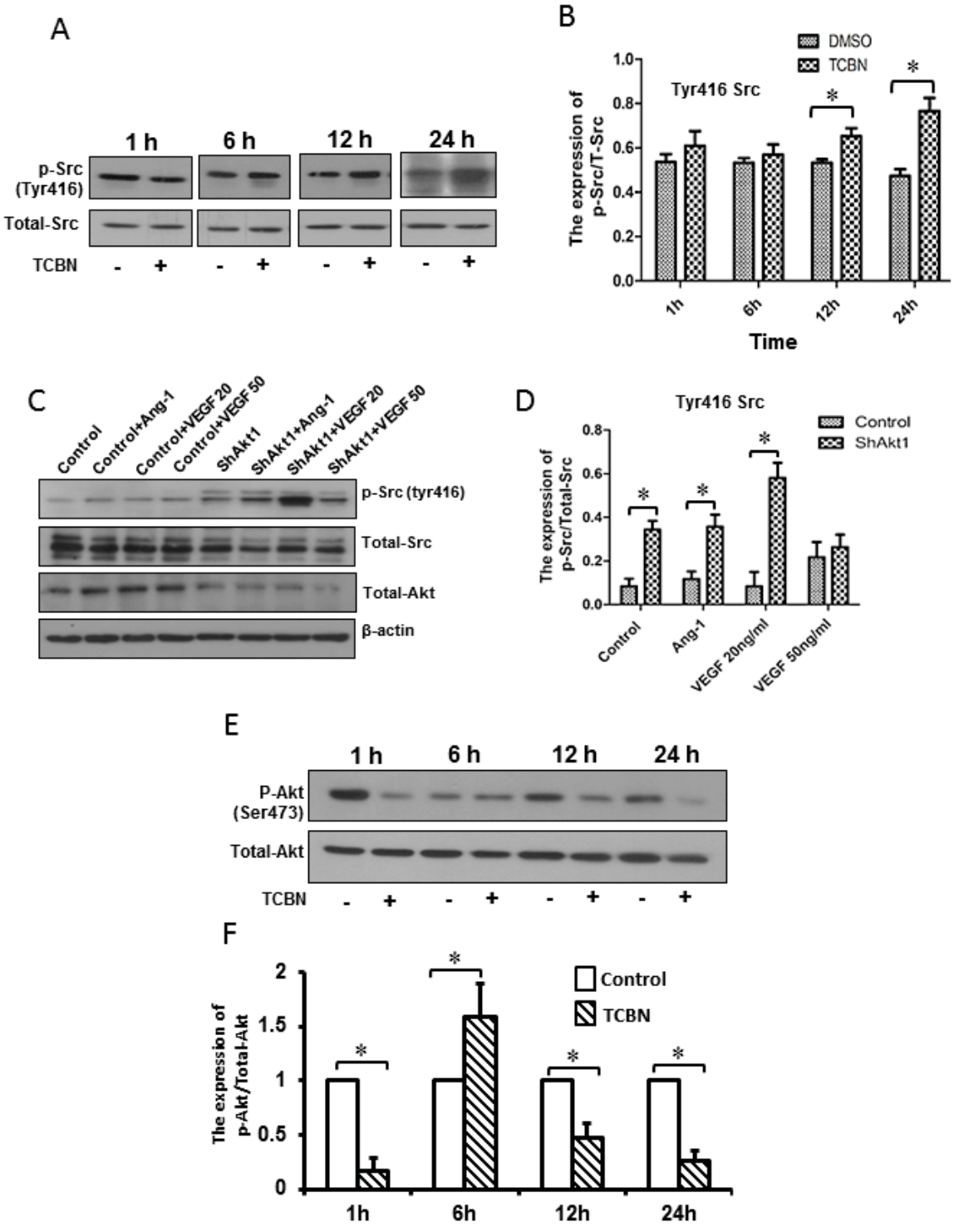

A
1 h
6 h
12 h
24 h
p-Src (Tyr416)
Total-Src
TCBN - + - + - + - +
B
DMSO
TCBN
Tyr416 Src
The expression of p-Src/T-Src
1.0
0.8
0.6
0.4
0.2
0.0
1h
6h
12h
24h
Time
C
Control
Control+Ang-1
Control+VEGF 20
Control+VEGF 50
ShAkt1
ShAkt1+Ang-1
ShAkt1+VEGF 20
ShAkt1+VEGF 50
p-Src (tyr416)
Total-Src
Total-Akt
β-actin
D
Tyr416 Src
Control
ShAkt1
The expression of p-Src/Total-Src
0.8
0.6
0.4
0.2
0.0
Control
Ang-1
VEGF 20ng/ml
VEGF 50ng/ml
E
1 h
6 h
12 h
24 h
P-Akt (Ser473)
Total-Akt
TCBN - + - + - + - +
F
Control
TCBN
The expression of p-Akt/Total-Akt
2
1.5
1
0.5
0
1h
6h
12h
24h

Figure 4: Akt and Src maintain a reciprocal relationship in the phosphorylation/activity pattern. (A-B) Representative Western blot images of HMEC lysates treated with or without TCBN showing Src Tyr416 phosphorylation and total Src, and a corresponding bar graph showing changes in the Src Tyr416 phosphorylation normalized to total Src from 1-24 hours, respectively. **(C-D)** Representative Western blot images of HMEC lysates transfected with scrambled ShRNA or ShRNA targeting Akt1 followed by treatment with either 20 and 50 ng/ml VEGF or 50 ng/ml Ang-1 showing changes in the Src Tyr416 phosphorylation and total Src, and corresponding bar graph showing changes in the Src Tyr416 phosphorylation normalized to total Src at 24 hours, respectively. **(E-F)** Representative Western blot images of HMEC lysates treated with or without TCBN showing Akt Ser473 phosphorylation and total Akt, and a corresponding bar graph showing changes in the Akt Ser473 phosphorylation normalized to total Akt from 1-24 hours, respectively. Data are represented as mean $\pm$ SD. (n=4), $*p<0.01$.

Figure 5

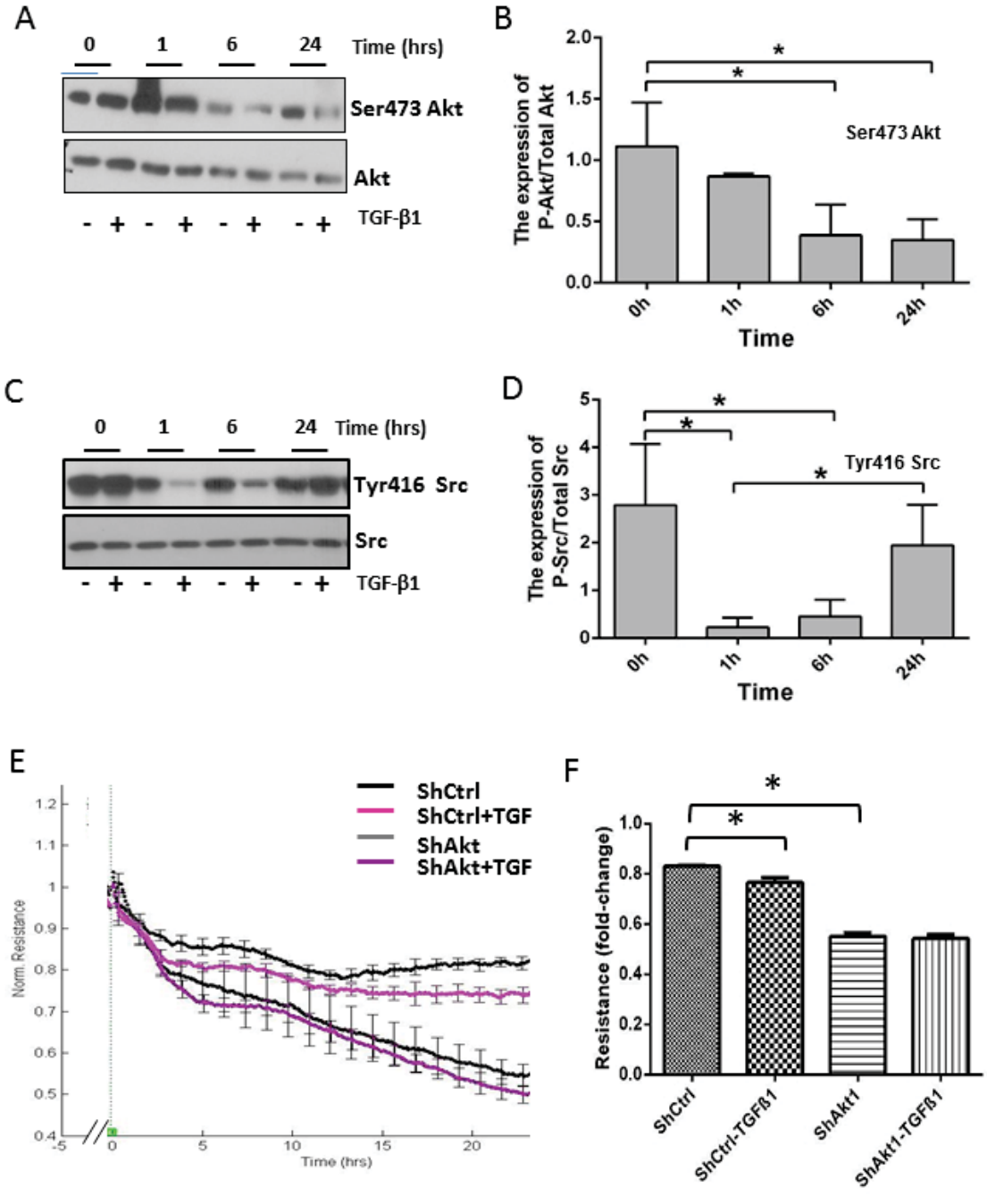

A
0
1
6
24
Time (hrs)
Ser473 Akt
Akt
- + - + - + - +
TGF-β1
B
The expression of P-Akt/Total Akt
Ser473 Akt
0h
1h
6h
24h
Time
C
0
1
6
24
Time (hrs)
Tyr416 Src
Src
- + - + - + - +
TGF-β1
D
The expression of P-Src/Total Src
Tyr416 Src
0h
1h
6h
24h
Time
E
ShCtrl
ShCtrl+TGF
ShAkt
ShAkt+TGF
Norm. Resistance
Time (hrs)
F
Resistance (fold-change)
ShCtrl
ShCtrl-TGFß1
ShAkt1
ShAkt1-TGFß1

Figure 5: TGFβ1 inhibits Ser473 phosphorylation of Akt and enhances Tyr416 phosphorylation of Src to induce HMEC-barrier breakdown in a time-dependent manner. **(A-B)** Representative Western blot images and corresponding bar graph of Akt Ser473 phosphorylation and total-Akt in HMEC lysates treated in the presence and absence of 5 ng/ml TGFβ1 for 1-24 hours, respectively. **(C-D)** Representative Western blot images and corresponding bar graph of Src Tyr416 phosphorylation and total-Src in HMEC lysates treated in the presence and absence of 5 ng/ml TGFβ1 for 1-24 hours, respectively. **(E-F)** Real-time changes and a bar graph showing the quantification of changes in the ShControl and ShAkt1 HMEC-barrier resistance upon treatment with either vehicle (PBS) or 5 ng/ml TGFβ1 as measured using ECIS equipment. Data are represented as mean $\pm$ SD. (n=3), $*p<0.01$.

Figure 6

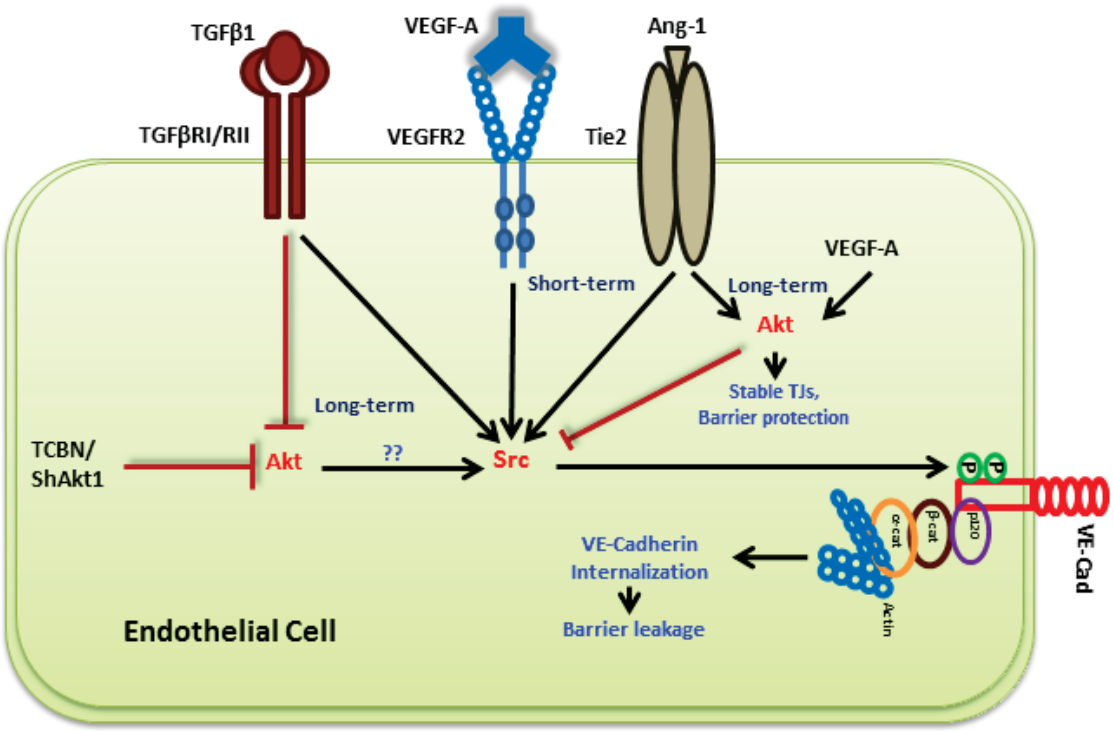


Figure 6: Schematic representation of cross-talk between Akt and Src pathways in the long-term endothelial barrier regulation in response to VEGF, Ang-1, and TGFβ. Our study concludes that whereas long-term stimulation with VEGF and Ang-1 in HMEC increases Akt and normalizes Src activities leading to endothelial-barrier protection, treatment with TGFβ1 promotes endothelial-barrier breakdown in the long-term associated with inhibition of Akt and activation of Src activities. Direct inhibition of Akt activity through pharmacological (TCBN) and genetic (ShRNA for Akt1) approaches also lead to Src activation in the long-term indicating that Akt and Src are reciprocally regulated in the growth factor-induced long-term endothelial-barrier regulation.

CHAPTER 3

ISOFORM-SPECIFIC EFFECTS OF TRANSFORMING GROWTH FACTOR-B ON ENDOTHELIAL TO MESENCHYMAL TRANSITION

Sabbineni H, Verma A and Somanath PR.

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ABSTRACT

Endothelial to mesenchymal transition (EndMT) was first reported in the embryogenesis. Recent studies show that EndMT also occurs in the disease progression of atherosclerosis, cardiac and pulmonary fibrosis, pulmonary hypertension, diabetic nephropathy, and cancer. Although transforming growth factor-β (TGFβ) is crucial for EndMT, it is not clear which isoform elicits a predominant effect. The current study aims to directly compare the dose-dependent effects of TGFβ1, TGFβ2, and TGFβ3 on EndMT and characterize the underlying mechanisms. In our results, all the three TGFβ isoforms induced EndMT in human microvascular endothelial cells (HMECs) after 72 hours, as evidenced by the increased expression of mesenchymal markers N-cadherin and alpha-smooth muscle actin (αSMA) as well as the decreased expression of endothelial nitric oxide synthase (eNOS). Interestingly, the effect of TGFβ2 was the most pronounced. Whereas the highest tested dose (5 ng/ml) of TGFβ1 and TGFβ3 were required to induce EndMT, TGFβ2 was able to induce EndMT at much lower concentrations (1 ng/ml). At 1 ng/ml, only TGFβ2 treatment resulted in significantly increased phosphorylation (activation) of Smad2/3 and p38-MAPK and increased expression of mesenchymal transcription factors Snail and FoxC2. Intriguingly, we observed that treatment with 5 ng/ml TGFβ1 and TGFβ3, but not TGFβ2 resulted in increased expression of TGFβ2 thus indicating that EndMT with TGFβ1 and TGFβ3 treatments was due to the secondary effects through TGFβ2 secretion. Together, our results indicate that TGFβ2 is the most potent inducer of EndMT and that TGFβ1- and TGFβ3-induced EndMT necessitates a paracrine loop involving TGFβ2.

1. INTRODUCTION

Endothelial to mesenchymal transition (EndMT) is a phenomenon in which endothelial cells (ECs) lose their characteristic features and acquire mesenchymal properties (Azhar et al., 2009; Boyer et al., 1999). EndMT is not only an essential mechanism implicated in the embryonic cardiac development (Azhar et al., 2009) but also in the progression of diseases such as atherosclerosis, pulmonary hypertension, diabetic nephropathy, cardiac and pulmonary fibrosis, and many types of cancers (Arciniegas et al., 2007; Kizu et al., 2009; Lee and Kay, 2006; Long et al., 2009). Aberrant EndMT results in the uncontrolled conversion of ECs into mesenchymal cells (Medici et al., 2010), which further switch their phenotype to myofibroblasts (Zeisberg et al., 2008). Myofibroblast is a diverse mesenchymal cell type greatly implicated in wound healing (Gabbiani et al., 1971; Stone et al., 2016) and organ fibrosis (Gerarduzzi and Di Battista, 2016; Liu, 2006; Zeisberg et al., 2000). Upon activation by biochemical and mechanical signals, myofibroblasts secrete and organize ECM, develop specialized matrix adhesions (Hinz et al., 2003), and exhibit cytoskeletal organization characterized by contractile actin filaments (Gabbiani et al., 1971). This allows the re-establishment of mechanical integrity and stability to the damaged tissue thus assisting in both the wound closure and resolution, which can lead to pathological remodeling when aberrantly stimulated and goes unconstrained (Hinz and Gabbiani, 2010; Hinz et al., 2012).

EndMT is characterized by the loss of cell-cell adhesions and changes in cell polarity-inducing a spindle-shaped morphology (Manetti et al., 2011). These changes are accompanied by reduced expression of one or more of the endothelial markers such as VE-cadherin, eNOS, and CD31, and increased expression of mesenchymal markers like fibroblast specific protein-1 (FSP-1), alpha smooth muscle actin (αSMA), N-cadherin, and fibronectin (Potenta et al., 2008). Loss of cell-cell adhesion is mediated by transcription factors such as Snail, Slug, ZEB-1, Twist, and FoxC2 that suppress transcription of genes encoding proteins involved in the formation of adherens junctions and tight junctions (Liebner et al., 2004; Medici et al., 2008) that are integral to an intact endothelium. Transforming growth factor-β1 (TGFβ1) is a potent inducer of epithelial to mesenchymal transition (EMT) (Akhurst and Derynck, 2001), a phenomenon very similar in biology to that of EndMT. Whereas TGFβ2 is a more potent inducer of fibrosis than TGFβ1 in amphibians (Rosa et al., 1988), both TGFβ1 (Wermuth et al., 2016) and TGFβ2 (Kokudo et al., 2008; Liebner et al., 2004; Romano and Runyan, 2000) are implicated greatly in mediating myofibroblast activation, EMT, and EndMT invertebrates

leading to organ fibrosis. Although both TGFβ1 and TGFβ2 promote EndMT, only TGFβ2 gene ablation in mice prevented EndMT-mediated cardiac development, and TGFβ1 or TGFβ3 knockout mice had normal heart development (Azhar et al., 2009). Interestingly, although TGFβ3 is implicated in EMT in cancer (Jalali et al., 2012), there are no reports on the effects of TGFβ3 on EndMT. Thus, it is not clear from the literature on which isoform of TGFβ is a predominant inducer of EndMT.

In the current study, we directly compared the isoform-specific effects of TGFβ1, TGFβ2 and TGFβ3 in inducing EndMT in human microvascular ECs (HMECs) *in vitro* and their effect on the expression of EC markers, mesenchymal markers, transcription factors regulating mesenchymal gene expression and the activity status of TGFβ-mediated canonical and non-canonical pathways. Our results demonstrated that TGFβ2 is the predominant mediator of EndMT in HMECs and that TGFβ1- and TGFβ3-induced EndMT needs EC-mediated paracrine loop through increased TGFβ2 generation.

2. MATERIALS AND METHODS

2.1 Cell culture

Human dermal (Telomerase-immortalized) microvascular ECs (HMEC) (CRL-4025; ATCC, Manassas, VA) were maintained in EC Basal Medium-2 with a Growth Medium-2 Bullet Kit (Lonza; Walkersville, MD). All cultures were maintained in a humidified 5% CO2 incubator at 37 ℃ and routinely passaged when 80– 90% confluent. TGFβ1, TGFβ2, and TGFβ3 were obtained from R&D Systems (Minneapolis, MN) and were reconstituted according to the manufacturer's protocol. HMECs monolayers were treated with 1, 2.5 and 5 ng/ml doses of TGFβ1, TGFβ2 and TGFβ3 in 5% serum-containing medium for 72 hours. The growth factors were replenished every 24 hours.

2.2 Western blot analysis

Cell lysates were prepared using complete lysis buffer (EMD Millipore, San Diego, CA) with protease and phosphatase inhibitor cocktails (Roche Diagnostics, Indianapolis, IN). Protein quantification was performed using DC protein assay from Bio-Rad (Hercules, and CA). Western blot analysis was performed as described previously (Abdalla et al., 2013; Al-Azayzih

et al., 2015). Antibodies used include N-cadherin, VE-cadherin, phosphorylated and total p38-MAPK, phosphorylated and total Smad2/3, FoxC2, Snail, and GAPDH from Cell Signaling Technology (Danvers, MA), αSMA, TGFβ2 and β-actin (Sigma, St. Louis, MO), and CD31, and eNOS from BD Pharmingen (San Diego, CA). Band densitometry was done using NIH Image J software.

2.3 Statistical Analysis

All the data are presented as Mean $\pm$ SD and were calculated from multiple independent experiments performed in quadruplicates. For normalized data analysis, data was confirmed that normality assumption was satisfied and analyzed using paired sample *t-test* (dependent *t-test*) and/or further confirmed with non-parametric test Wilcoxon signed rank test. For all other analysis, Student's two-tailed *t*-test or ANOVA test were used to determine significant differences between treatment and control values using the GraphPad Prism 4.03 and SPSS 17.0 software.

1. RESULTS

3.1 TGFβ1 induces EndMT *in vitro*

To investigate whether TGFβ is involved in inducing EndMT, HMEC monolayers were treated with either vehicle or 5 ng/ml dose of the widely studied TGFβ isoform, TGFβ1 for 72 hours, and the cell lysates were subjected to Western blot analysis. Our results showed that stimulation of HMECs with TGFβ1 results in a significant increase in the expression of mesenchymal markers N-Cadherin, αSMA (Figure 1A-B). Although reduced expression of endothelial markers VE-cadherin and eNOS was observed, these data were not significant (Figure 1A-B). Our results indicate that TGFβ1 induces EndMT *in vitro.*

3.2 TGFβ2 is a more potent inducer of EndMT than TGFβ1 or TGFβ3 *in vitro*

In order to determine whether all three TGFβ isoforms can induce EndMT and further identify which of the three TGFβ isoforms is a predominant inducer of EndMT, HMEC monolayers were treated with 1, 2.5, and 5 ng/ml doses of TGFβ1, TGFβ2, and TGFβ3 for 72 hours and the cell lysates were subjected to Western blot analysis. Our results showed that stimulation of HMEC by TGFβ2 results in significantly higher expression of mesenchymal markers N-

cadherin and αSMA as compared to control, TGFβ1 or TGFβ3 (Figure 2A-B). Interestingly, 1 ng/ml TGFβ2 treatment promoted mesenchymal marker expression to a significantly higher level than the highest dose (5 ng/ml) of TGFβ1 or TGFβ3 (Figure 2A-B), thus indicating that TGFβ2 is the predominant induced of EndMT.

3.3 TGFβ2 is the most potent suppressor of endothelial marker expression *in vitro*

Next, we determined the effect of 1, 2.5, and 5 ng/ml doses of TGFβ1, TGFβ2, and TGFβ3 on endothelial marker expression. Analysis of cell lysates after 72 hours of treatment revealed that TGFβ2 treatment resulted in reduced the expression of endothelial markers eNOS and VE-cadherin, and the effect was much higher than similar doses of TGFβ1 and TGFβ3 (Figure 3A-B). We observed that treatment with 1 ng/ml dose of TGFβ2 down-regulated eNOS and VE-cadherin expression with higher efficiency as compared to the highest dose (5 ng/ml) of TGFβ1 or TGFβ3 (Figure 3A-B).

3.4 TGFβ2 is the most potent activator of canonical and non-canonical TGFβ-mediated pathways in HMEC

TGFβ superfamily ligands exert their downstream signaling effects via either canonical or non-canonical signaling pathways (Gauldie et al., 2007; Hanahan and Folkman, 1996; Kavsak et al., 2000; Santibanez et al., 2011; Suzuki et al., 2002). In order to investigate whether one or both these pathways are activated by TGFβ isoforms in EndMT, HMEC monolayers were treated with 1, 2.5, and 5 ng/ml doses of TGFβ1, 2, and 3 isoforms for 72 hours and the cell lysates were subjected to Western blot analysis. Our results showed that TGFβ2 promoted activation (phosphorylation) of receptor-regulated Smad2/3, the canonical effector of TGFβ signaling and p38-MAPK with higher efficiency as compared to the vehicle, TGFβ1 or TGFβ3 treated cells (Figure 4A-B), suggesting the involvement of both canonical and non-canonical TGFβ signaling in the promoting EndMT.

3.5 TGFβ2, and not TGFβ1 or TGFβ3, is the predominant regulator of mesenchymal transcription factors Snail and FoxC2 expression in HMEC

Next, we wanted to further examine the efficiency of different TGFβ isoforms in inducing EndMT promoting transcription factors Snail and FoxC2. Once again, HMEC monolayers were treated with 1, 2.5, and 5 ng/ml doses of TGFβ1, 2, and 3 isoforms for 72 hours and the cell lysates were subjected to Western blot analysis. Our results indicated that 1 ng/ml dose of

TGFβ2 is more potent in upregulating Snail and FoxC2 in HMEC than 5 ng/ml dose of TGFβ1 or TGFβ3 (Figure 5A-B).

3.6 TGFβ1- and TGFβ3-induced EndMT involve an endothelial TGFβ2-mediated paracrine loop

In order to investigate how higher doses of TGFβ1 or TGFβ3 were able to induce EndMT in HMEC, we investigated if TGFβ1 or TGFβ3 could increase the production of TGFβ2 by HMEC. Our Western blot analysis indicated that treatment with both TGFβ1 and TGFβ3, but not TGFβ2 itself, promoted synthesis of TGFβ2 by the HMEC (Figure 6A-B). These results further confirmed that TGFβ2 is the most potent inducer of EndMT and that the TGFβ1 and TGFβ3 isoforms initiate a TGFβ2 paracrine loop to indirectly promote EndMT in HMECs.

2. DISCUSSION

Myofibroblasts or activated mesenchymal cells play a crucial role in tissue repair and contribute to the pathogenesis of various fibrotic and vascular diseases including but not limited to interstitial pulmonary fibrosis, systemic sclerosis, and liver or cardiac fibrosis (Hinz and Gabbiani, 2010; Hinz et al., 2012; Neilson, 2006). However, the source of these myofibroblasts remains fairly controversial and is gaining more attention recently due to the emergence of a new type of cellular transdifferentiation, a phenomenon known as EndMT (Abraham et al., 2007; Hinz et al., 2007). EndMT is a biological process in which ECs lose one or more of their specific markers such as VE-cadherin, eNOS, and CD31 and acquire mesenchymal markers such as N-cadherin, αSMA, FSP1 and collagen VI (Arciniegas et al., 2005; Arciniegas et al., 1992). EndMT attributes migratory, proliferative and invasive properties to the otherwise non-motile and adherent ECs, transforming them into myofibroblasts (Li and Bertram, 2010; Piera-Velazquez et al., 2011). In the recent past, quite a few studies reported the occurrence of EndMT in various fibrotic disorders like cardiac (Zeisberg et al., 2007), pulmonary (Hashimoto et al., 2010), and renal fibrosis (Li and Bertram, 2010). Although EndMT is implicated in many diseases, the stimuli that trigger the initiation of this cellular differentiation and the mechanisms through which the transformation occurs remain elusive. Several signaling pathways are reported in EndMT, while the most important being TGFβ binding (Nakajima et al., 2000;

Zeisberg et al., 2008; Zeisberg et al., 2007). Given the popularity of TGFβ as a potent cell differentiation cytokine and the extensive involvement of its signaling in the pathogenesis of fibrotic diseases (Rosenbloom et al., 2010; Wynn, 2008), several groups have investigated its role in the generation of myofibroblasts via EndMT (Hinz et al., 2007; Hinz et al., 2012). Although it is widely accepted that TGFβ1 is a potent inducer of fibrosis via generation of myofibroblasts in various fibrotic models, several emerging reports advocate the involvement of TGFβ2 in promoting EndMT (Chrobak et al., 2013; Medici et al., 2011; Nie et al., 2014; Shi et al., 2016). Whereas TGFβ and its downstream effectors as EndMT inducers are being extensively studied by several groups in various fibrotic and vascular diseases, appropriate knowledge on the contributions of different TGFβ isoforms, TGFβ1, 2, and 3 remains unknown.

In order to identify the most potent inducer of EndMT, we directly compared the dose-dependent effects of TGFβ1, TGFβ2, and TGFβ3 on EndMT *in vitro*. We examined changes in the expression of endothelial and mesenchymal markers, transcription factors that promote mesenchymal transition, and the activation of TGFβ-induced canonical and non-canonical pathways by treating HMECs with various doses of these TGFβ isoforms for 72 hours. Stimulation of HMECs with the most widely studied isoform, TGFβ1 revealed the involvement of TGFβ in inducing EndMT which was evident from the upregulation of mesenchymal markers N-cadherin and αSMA. It was also supported by a reduction in expression of endothelial marker VE-cadherin and eNOS. Together these results indicate that TGFβ induces EndMT *in vitro.* It was interesting to note that the lowest dose of TGFβ2 (1 ng/ml) upregulated mesenchymal markers N-Cadherin and αSMA and down-regulated endothelial markers eNOS and VE-Cadherin with significantly higher efficacy than that of the highest doses of TGFβ1 and TGFβ3 (5 ng/ml) implying a predominant role of TGFβ2 on EndMT.

Among the different receptor-regulated Smads involved in TGFβ signaling, Smad3 was reported as the pro-fibrotic member of the Smad family as its activation (phosphorylation) promotes the progression of fibrosis (Darland et al., 2003; Hirschi et al., 1998). On a similar note, we observed that TGFβ2 increases the phosphorylation (activation) of Smad2/3 and p38 MAPK greater than that of TGFβ1 and TGFβ3 suggesting the predominance of TGFβ2 in inducing mesenchymal transition of ECs and the involvement of both canonical and non-canonical TGFβ signaling pathways in promoting EndMT. We also observed that TGFβ2 increases the expression of EndMT promoting transcription factors Snail and FoxC2 with

significantly higher efficiency than the other two TGFβ isoforms further confirming our observation and in agreement with the recent finding reporting a TGFβ2-mediated activation of the ALK5-Smad2/3-Snail pathway (Zeng et al., 2013) leading to EndMT.

Another important question that we wanted to address was how TGFβ1 and TGFβ3 were able to induce EndMT, albeit at higher doses. Are the functions of these three isoforms redundant? To address this, we determined the expression changes in TGFβ2 post 72-hour treatment with TGFβ1, TGFβ2, and TGFβ3. The most intriguing and prominent finding of our study that came from this experiment was that both TGFβ1 and TGFβ3, but not TGFβ2, stimulated the expression of TGFβ2 by the HMECs. This indicated the existence of a positive feedback loop between different TGFβ isoforms via paracrine effects involving TGFβ2 synthesis in inducing EndMT. Together with our results demonstrate that although all the three isoforms of TGFβ induce EndMT, TGFβ2 is the most potent inducer. This is in agreement with observations from several reports that suggested the predominance of TGFβ2 in inducing EndMT of mouse embryonic stem cell-derived ECs (Kokudo et al., 2008) and that TGFβ2 is a more potent fibrotic inducer than TGFβ1 in amphibians (Hsuan, 1989; Rosa et al., 1988). To our knowledge, this is the first study to examine and directly compare the dose-dependent effects of three different TGFβ isoforms- TGFβ1, TGFβ2, and TGFβ3 on EndMT demonstrating a paracrine TGFβ2-mediated EndMT loop in HMECs stimulated by TGFβ1 and TGFβ3. Here we indicate the predominance of TGFβ2 in inducing EndMT thus paving a way to direct future investigations on this pathway in EndMT to yield a better understanding of the mechanisms involved.

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FIGURES AND FIGURE LEGENDS

Figure 1

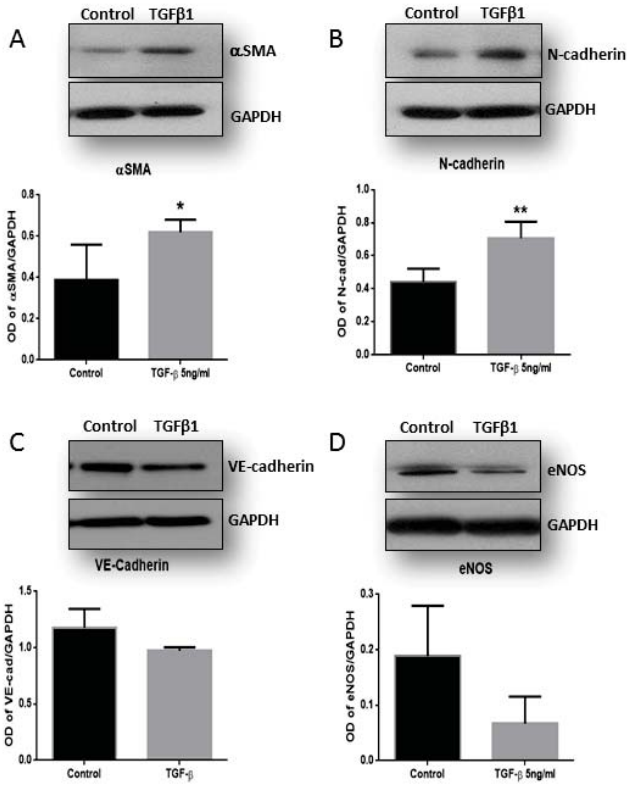

FIGURE 1: TGFβ1 induces EndMT in HMECs. (A) Representative Western blot images and the corresponding bar graph of band densitometry showing increased expression of αSMA in HMECs treated with 5ng/ml TGFβ1for 72 hours **(B)** Representative Western blot images and the corresponding bar graph of band densitometry showing increased expression of N-Cadherin in HMECs treated with 5ng/ml TGFβ1for 72 hours **(C and D)** Representative Western blot images and the corresponding bar graphs of band densitometry showing expression of VE-Cadherin and eNOS in HMECs treated with 5ng/ml TGFβ1for 72 hours, respectively. Data are represented as mean ± SD. (n=3-5), *$p<0.05$; **$p<0.01$.

Figure 2

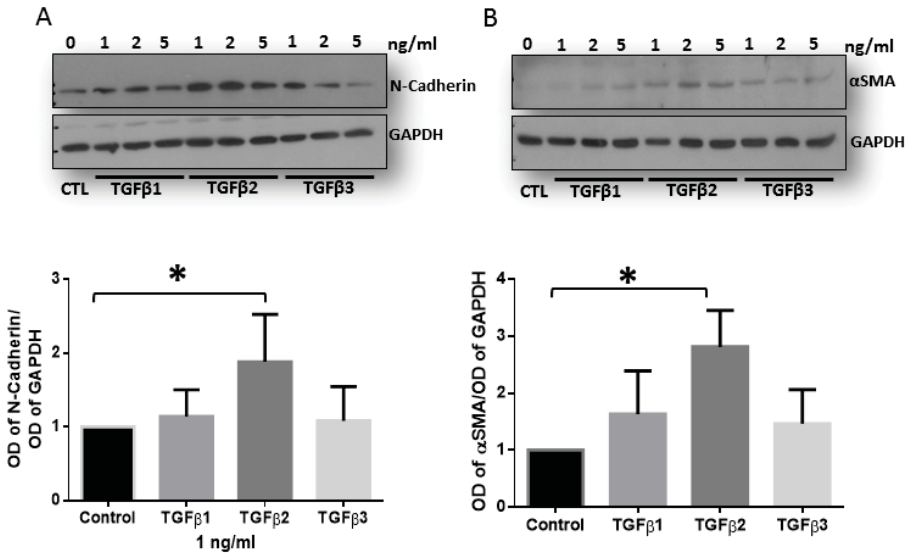

FIGURE 2: TGFβ2 is a more potent inducer of mesenchymal markers in HMECs compared to TGFβ1 and TGFβ3. (A) Representative Western blot images and the corresponding bar graph of band densitometry showing increased expression of mesenchymal marker N-cadherin in HMECs in response to 1 ng, 2.5 ng and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. **(B)** Representative Western blot images and the corresponding bar graph of band densitometry showing increased expression of the mesenchymal marker αSMA in HMECs in response to 1 ng, 2.5 ng and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. Data are represented as mean $\pm$ SD. (n=3-5), *$p<0.05$.

Figure 3

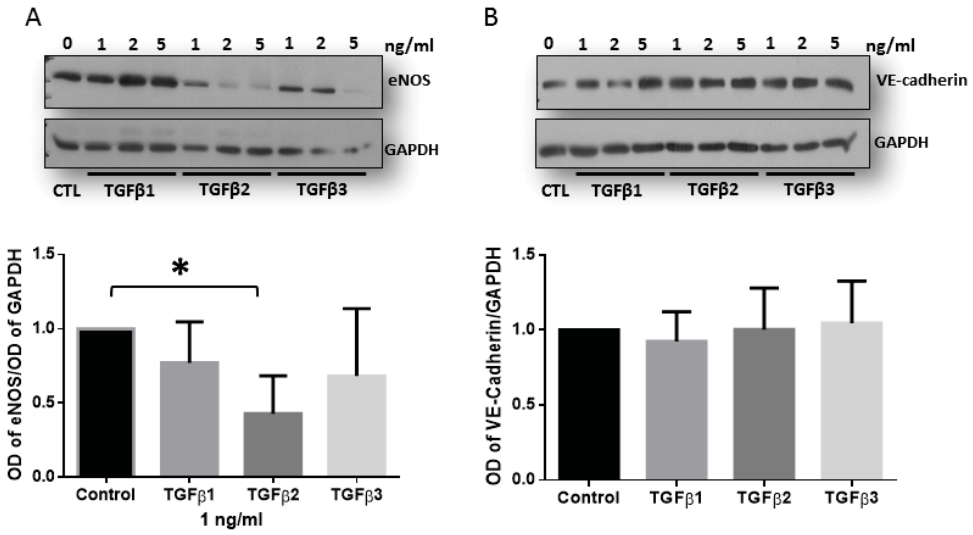

FIGURE 3: TGFβ2 is a more potent suppressor of the endothelial marker expression in HMECs compared to TGFβ1 and TGFβ3. (A) Representative Western blot images and the corresponding bar graph of band densitometry showing reduced expression of endothelial marker eNOS in HMECs in response to 1 ng, 2.5 ng and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. **(B)** Representative Western blot images and the corresponding bar graph of band densitometry showing no significant change in the expression of endothelial receptor VE-cadherin in HMECs in response to 1 ng, 2.5 ng and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. Data are represented as mean $\pm$ SD. (n=3-5), *$p<0.05$.

Figure 4

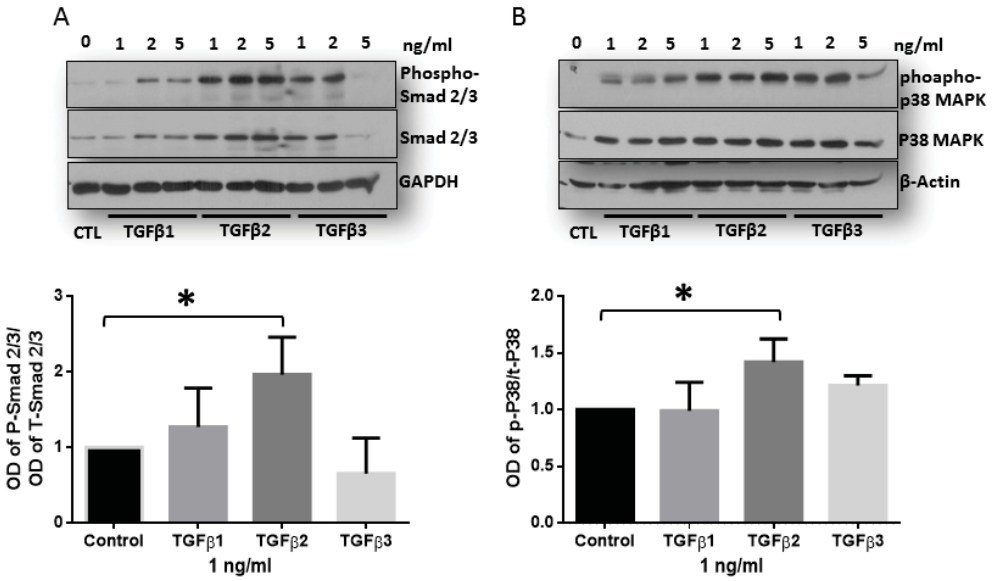

FIGURE 4: TGFβ2 exhibits higher potency in activating both canonical and non-canonical pathways in HMECs compared to TGFβ1 and TGFβ3. (A) Representative Western blot images and the corresponding bar graph of band densitometry showing increased phosphorylation and total expression of canonical transcription factor Smad2/3 in HMECs in response to 1 ng, 2.5 ng and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. **(B)** Representative Western blot images and the corresponding bar graph of band densitometry showing increased phosphorylation of non-canonical, stress-induced p38 MAPK in HMECs in response to 1 ng, 2.5 ng and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. Data are represented as mean $\pm$ SD. (n=3-5), *$p<0.05$.

Figure 5

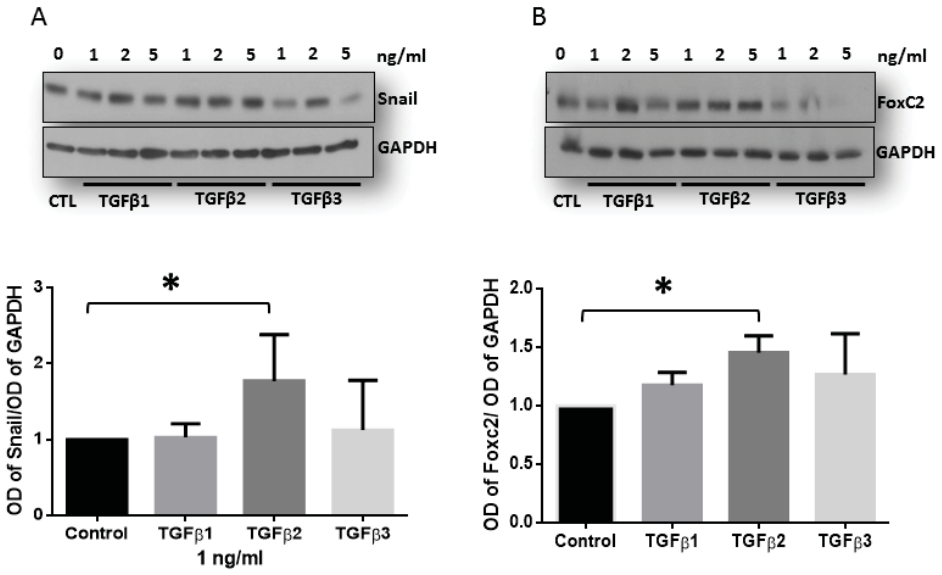

FIGURE 5: TGFβ2 is a more potent stimulator of mesenchymal transcription factor expression in HMECs compared to TGFβ1 and TGFβ3. (A) Representative Western blot images and the corresponding bar graph of band densitometry showing increased expression of mesenchymal transcription factor Snail in HMECs in response to 1 ng, 2.5 ng and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. **(B)** Representative Western blot images and the corresponding bar graph of band densitometry showing increased expression of mesenchymal transcription factor FoxC2 in HMECs in response to 1 ng, 2.5 ng and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. Data are represented as mean $\pm$ SD. (n=3-5), *$p<0.05$.

Figure 6

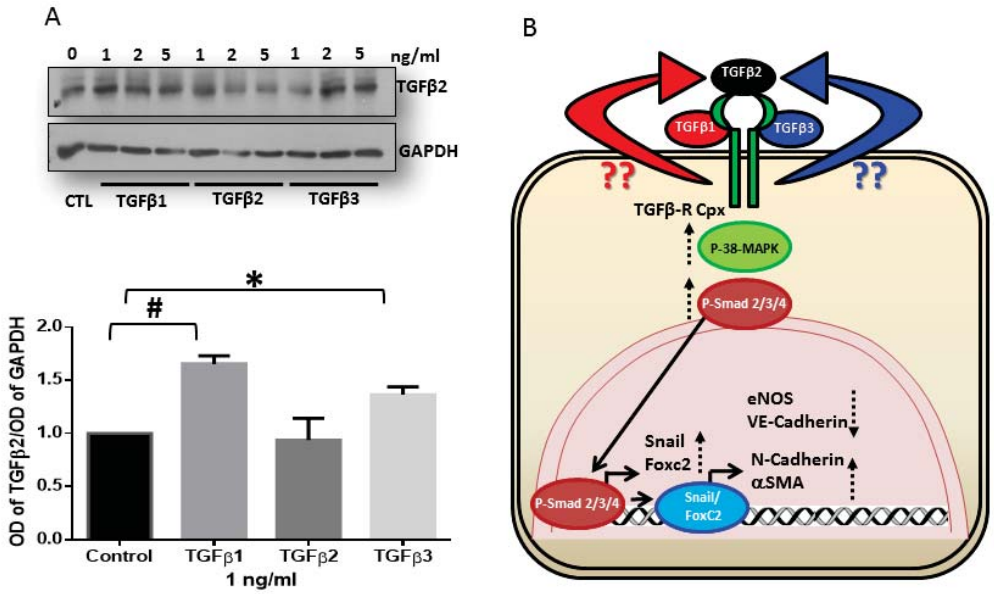

FIGURE 6: TGFβ1- and TGFβ3-induced EndMT needs activation of a paracrine loop in HMECs involving TGFβ2. (A) Representative Western blot images and the corresponding bar graph of band densitometry showing increased expression of the most potent EndMT stimulating TGFβ isoform, TGFβ2, in response to 1 ng, 2.5 ng and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. **(B)** Diagrammatic sketch of the working hypothesis showing both TGFβ1 and TGFβ3, but not TGFβ2 itself, inducing the generation of TGFβ2 in HMECs, which in turn, inducing pathways leading to Snail and FoxC2-induced transcriptional activation of mesenchymal markers and repression of endothelial markers. Data are represented as mean ± SD. (n=3-5), *$p<0.05$.

CHAPTER 4

ENDOTHELIAL SPECIFIC LOSS OF AKT1 INDUCES ENDMT *IN-VITRO* AND PULMONARY VASCULAR REMODELING *IN-VIVO*

1. INTRODUCTION

Endothelial to mesenchymal transition (EndMT) is a phenomenon in which endothelial cells lose their endothelial markers, acquire mesenchymal properties [1, 2], and is characterized by a loss of cell-cell adhesion and changes in cell polarity inducing a spindle-shaped morphology. EndMT is associated with loss of endothelial markers such as VE-cadherin and CD31, and increased expression of mesenchymal markers including fibroblast specific protein-1 (FSP-1), alpha smooth muscle actin (α-SMA), N-cadherin, and fibronectin [3] mediated by one or more of the transcription factors Snail, Slug, ZEB-1, SIP-1, Twist, and LEF-1 that suppress the transcription of genes encoding proteins involved in formation of adherens and tight junctions which are critical in maintaining endothelial barrier integrity[4, 5]. EndMT is involved in the embryonic development [4, 6, 7] and several cardiopulmonary diseases including but not limited to different types of hypertension [8-10], cardiac fibrosis [11, 12], idiopathic pulmonary fibrosis [13, 14], radiation induced pulmonary fibrosis [15] transplant atherosclerosis and restenosis [16, 17] and chronic obstructive pulmonary disease (COPD) [18].

A healthy pulmonary endothelial barrier is integral in maintaining vascular homeostasis. Endothelial barrier dysfunction occurs in response to inflammatory mediators such as IL-6 and tumor necrosis factor-α (TNFα), as well as pathogens [19-21]. Loss of endothelial barrier integrity, disordered endothelial proliferation, and enhanced inflammatory cell infiltration are common features believed to contribute to the pathologic vascular remodeling [22, 23]. Pulmonary vascular remodeling, a hallmark of cardiopulmonary diseases is characterized by intimal thickening, medial hypertrophy, and plexiform lesions. [24]. However, the nature and origin of the cells contributing to the neointimal thickening and plexiform lesion formation remain controversial. Several groups

have been trying to characterize the phenotype of these cells located in the pulmonary artery wall [25]. Increasing evidence suggests exposure of endothelial cells to chronic stress and inflammatory factors promotes endothelial to mesenchymal transition (EndMT) contributing to vascular smooth muscle cells (SMCs) and cardiac fibroblast populations during both embryogenesis and pathological conditions [11, 26, 27].

In endothelial cells, intracellular signaling pathways mediated by protein kinase Akt are greatly implicated in the regulation of cell survival, proliferation, migration, glucose metabolism, and gene expression [28, 29] and hence play an important role in the proliferation and migration of ECs, contributing to angiogenesis and transdifferentiation[29-31]. We have recently shown that endothelial loss of Akt1 enhances VEGF-induced barrier breakdown *in-vitro* and promotes VEGF induced vascular leakage in mice ears [32, 33]. We have also shown that endothelial loss of Akt1promotes lipopolysaccharide (LPS) induced acute lung injury [34] suggesting the involvement of Akt1 in vascular injury, a precursor for the pathological vascular remodeling [35, 36]. Although the involvement of differentiation cytokines TGFβ, Notch, TNFα, etc., in EndMT has been widely reported [37-42], the mechanisms through which they regulate EndMT, the role of Akt1, a critical barrier integrity regulating kinase in EndMT and vascular remodeling remain largely unknown.

Since Akt1 is essential for endothelial barrier integrity, we hypothesized that sustained endothelial loss of Akt1 will promote EndMT *in vitro* and pulmonary vascular remodeling *in vivo*. Our current study demonstrates that Akt1 suppression results in EndMT via increased gene expression of TGFβ2 and FoxC2 *in vitro* and exacerbates pulmonary vascular remodeling *in vivo*, which is revered upon inhibition of β-catenin using ICG001.Together, our study has identified a novel Akt1-mediated pathway regulating EndMT and pathological pulmonary vascular remodeling along with the therapeutic potential of β-catenin inhibitor ICG001 for pulmonary vascular diseases.

2. MATERIALS AND METHODS

2.1. Generation of 'VECad-Cre-Akt1' transgenic mouse model

All experiments were performed with approval by the Charlie Norwood VAMC Institutional Animal Care and Use Committee (Approval reference #13-09-062). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals. Carbon dioxide asphyxiation followed by cervical dislocation was performed for euthanasia. Isoflurane inhalation was used as anesthesia. For our study, we generated an endothelial-specific, tamoxifen-inducible Akt1 knockout mouse model (VECad-Cre-Akt1) as previously described [32]. Age and sex matched 8-12-week-old control WT and VECad-Cre-Akt1 mice on a C57BL/6 background were used in the study. 1mg/10g dose of Tamoxifen (Sigma, St. Louis, MO) was administered using a 27G needle via intraperitoneal (i.p.) injection every 24 hours for 5 consecutive days. Following this, transgene was maintained with a custom-made Tamoxifen diet (Harlan, Madison, WI) for the duration of the experiments.

2.2. Cell culture and preparation of ShAkt1 stable cell lines

Human dermal (Telomerase-immortalized) microvascular endothelial cells (HMEC) (CRL-4025; ATCC, Manassas, VA) were maintained in Endothelial Cell Basal Medium-2 with a Growth Medium-2 Bullet Kit (Lonza; Walkersville, MD). All cultures were maintained in a humidified 5% CO2 incubator at 37 F, and routinely passaged when 80– 90% confluent.

Stable ShControl, ShAkt1 (ACGCTTAACCTTTCCGCTG) HMEC cells were generated using SMART vector 2.0 lentivirus particles (109 pfu) (Thermo Scientific, Waltham, MA). Lentivirus particles were mixed in 1ml Hyclone SFM4Transfx-293 (Fisher, Hanover Park, IL) and added along with 1 µl Polybrene (10mg/ml, American bioanalytical, Natick, MA). Three days later, transfection efficiency was tested through Turbo-GFP expression and subjected for 4 µg/ml puromycin (Life Technologies, Grand Island, NY) selection until all cells expressed GFP.

2.3. Human mesenchymal gene array analysis

Control and ShAkt1 HMEC cell lysates were used for the gene array analysis using human epithelial to mesenchymal transition gene arrays. Briefly, cells were lysed and RNA was isolated using RNAase Mini plus Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Next, cDNA was generated by RT2 First Strand Kit (SABiosciences, Frederick, MD), mixed with qPCR SyberGreen master mix and loaded into human EMT RT2 Profiler PCR Array plates. Reading was completed in Eppendorf Mastercycler realplex-2 equipment (Hauppauge, NY).

2.4. Phalloidin staining

For phalloidin staining HMECs transfected with scrambled or ShRNA targeting Akt1 were grown on cover slips coated with 0.02 % Gelatin (Sigma Aldrich, St. Louis, MO) to monolayer and treated with vehicle or 5ng/ml TGFβ1 for 72 hours. Cells were washed with 1X PBS, fixed with 4% PFA and blocked with 10% goat serum (Sigma Aldrich, St. Louis, MO) followed by incubation with phalloidin (Life Technologies, Grand Island, NY) for 20 minutes at room temperature. Cover slips were mounted on to a glass slide using DAPI containing mounting medium (Vector Laboratories). Samples were observed under a confocal microscope equipped with argon and helium/neon lasers (LSM510, Zeiss, Germany).

2.5. Western blot analysis

Cell lysates were prepared using complete lysis buffer (EMD Millipore, San Diego, CA) with protease and phosphatase inhibitor cocktails (Roche Diagnostics, Indianapolis, IN). Protein quantification was performed using DC protein assay (Bio-Rad, Hercules, CA). Western blot analysis was performed as described previously [43, 44]. Antibodies used include Akt1, β-catenin (ser675), N-cadherin, VE-cadherin, Snail1, Foxc2, phosphor- and total-Smad 2/3, phospho- and total-p-38 MAPK, GAPDH from Cell Signaling (Danvers, MA)., anti- β-actin, alpha-SMA, and TGFβ2 from Sigma Aldrich 9St. Louis, MO), eNOS from BD Pharmingen (SanDiego, CA). HRP-conjugated goat-anti-mouse and goat-anti rabbit secondary antibodies were obtained from Bio-Rad (Hercules, CA). Densitometry was done using NIH Image J software.

2.6. Chronic Hypoxia and Hypoxia-SUGEN models

VE-cad-Cre-Akt1 mice were injected with 20mg/kg Tamoxifen i.p for 5 consecutive days to induce endothelial specific Akt1 knock down. WT mice also received Tamoxifen injections for control. Following this WT and Cre-Akt1 mice were subjected to 21 day chronic hypoxia in a chamber with regulated N2 flow to maintain oxygen levels at 10%. During this time, all the mice in Hy-SU animal model received 10mg/kg SU-5416, a VEGFR2 inhibitor once a week. Treatment groups also received 10mg/kg ICG-001, a β-catenin inhibitor for 21 days, i.p. Lungs and hearts were harvested and subjected to histopathological studies including Masson's trichrome staining, H&E staining and EVG staining.

2.7. Statistical Analysis

All the data are presented as Mean + SD and were calculated from multiple independent experiments performed in quadruplicates. For normalized data analysis, data was confirmed that normality assumption was satisfied and analyzed using paired sample t test (dependent t test) and/or further confirmed with non-parametric test Wilcoxon signed rank test. For all other analysis, Student's two-tailed t test or ANOVA test were used to determine significant differences between treatment and control values using the GraphPad Prism 4.03 software and SPSS 17.0 software.

3. RESULTS

3.1 Endothelial Loss of Akt1 promotes upregulation of mesenchymal markers and downregulation of endothelial markers in vitro

Western blot analysis of the control (ShControl) and Akt1 knockdown (ShAkt1) HMEC lysates post treatment with 5ng/ml TGFβ or vehicle for 72 hours showed that endothelial loss of Akt1 promotes the expression of mesenchymal markers N-Cadherin and α-SMA (Figure 1A-B), and loss of endothelial marker eNOS (Figure 1C) indicating that loss of Akt1 induces EndMT *in vitro*.

3.2 Endothelial Loss of Akt1 promotes the mRNA and protein expression of mesenchymal genes and transcription factors

Gene array and Western blot analysis of the control (ShControl) and Akt1 knockdown (ShAkt1) HMECs showed that endothelial loss of Akt1 increases the expression of mesenchymal genes fibronectin 1 (FN1), keratin 7 (KRT7), mesenchymal transcription factors FoxC2, and a 9-fold increase in the gene expression of the most potent EndMT inducing TGFβ isoform, TGFβ2 (Figure 2A). Western blot analysis of the cell lysates showed an increase in the expression of TGFβ2 (Figure 2B), the mesenchymal transcription factor Snail1 (Figure 2C). We also observed that endothelial loss of Akt1induces the activation (phosphorylation) of the pro-fibrotic Smad, Smad 2/3 (Figure 2D) and p-38 MAPK (Figure 2E) These results suggest that endothelial loss of Akt1 induces the expression of TGFβ 2 which further activates both canonical and non-canonical signaling pathways promoting EndMT.

3.3 Endothelial specific loss of Akt1 induces a change in morphology and promotes stress fiber formation

To examine the effect on morphology, HMECs transfected with scrambled or ShRNA against Akt1 were cultured in 6 well plates and subjected to either normoxia or hypoxia (0.1%). Endothelial loss of Akt1 induced a change in morphology of HMECs where the cobble shaped cells (Figure 3A) acquired a spindle shaped and elongated morphology (Figure 3B) very similar to that of regular HMECs control cells exposed to hypoxia for 72 hours (Figure 3C). To examine the effect of Akt1 on stress fiber formation and phenotypic changes, HMECs transfected with scrambled or ShRNA against Akt1were grown on gelatin coated cover slips and treated with either vehicle or 5ng/ml TGFβ1 for 72 hours and subjected to phalloidin staining to examine. Loss of Akt1 in endothelial cells induced stress fiber formation similar to that of TGFβ (Figure 3D-G) or more which was also associated with a distinct change in phenotype from cobble shape (healthy state) to spindle shape (transformed state).

3.4 Long-term Akt1 inactivity in HMEC results in increased phosphorylation β-catenin. Western blot analysis for phosphorylation and total expression of β-catenin of HMECs transfected with scrambled or ShAkt1RNA and treated with PBS, 20 ng/ml VEGF, 50 ng/ml VEGF and 50 ng/ml Ang1 revealed that long-term loss of Akt1 results in increased phosphorylation of β-catenin while the expression of total protein was not affected (Figure 4A-C).

3.5 Endothelial Loss of Akt1 exacerbates Hypoxia induced vascular remodeling in-vivo.

Masson's trichrome staining of lung sections of VE-cad-Cre-Akt1 mice injected with either vehicle or Tamoxifen to induce endothelial knock down of Akt1 and subjected to either normoxia or 10% hypoxia showed that endothelial specific loss of Akt1 exacerbates hypoxia induced pulmonary vascular remodeling by increasing blood vessel thickness of the pulmonary microvasculature (Figure 5A-D).

3.6 Hypoxia-SU-induced vascular remodeling in mice was reversed by β-catenin suppression

Masson's trichrome staining of lung sections of C57BL6-WT and VE-cad-Cre-Akt1 mice subjected to Hypoxia-SU and treated with either vehicle ICG-001, a β-catenin inhibitor showed that inhibition of β-catenin reduced the Hy-SU induced vessel thickening in both WT and VE-cad-CreAkt1 mice (Figure 6A-E). From our observation that shows Akt1 suppression induces phosphorylation of β-catenin whose nuclear translocation induces the expression of mesenchymal genes and that inhibition of β-catenin with ICG-001 prevents Hy-SU- and Akt1-/- induced vascular

remodeling, it is evident that Akt1 mediated GSK-3β/β-catenin signaling is involved in inducing EndMT thus contributing to pathological pulmonary vascular remodeling.

3.7 SU5416 causes irreversible pulmonary vascular remodeling via Akt suppression

Masson's trichrome staining of lung sections of C57BL6-WT and VE-cad-Cre-Akt1 mice subjected to normoxia, hypoxia alone or SU-Hypoxia revealed that SU-5416, a VEGFR2 inhibitor exacerbates irreversible pulmonary vascular remodeling in mice (Figure 7A-D). This is worsened in the presence of hypoxia. We investigated why there was no significant difference between C57BL6 WT mice and VE-Cad-CreAkt1mice in terms of vascular remodeling when subjected to Hy-SU especially that normoxic CreAkt1 mice exhibited exacerbated vascular thickening compared to that of Hypoxic WT mice. We found that SU-5416 causes vascular remodeling by inhibition of Akt (Figure 6E-F).

DISCUSSION

During EndMT, endothelial cells lose their endothelial specific markers and morphology, and acquire a mesenchymal phenotype characterized by the expression of mesenchymal markers. EndMT, which is extensively studied in its predominant role in the embryogenic process [4, 6, 7] has also been implicated in several cardiopulmonary diseases that include, but not limited to chronic obstructive pulmonary disease (COPD) [18] chronic pulmonary hypertension, idiopathic portal hypertension, and chronic thromboembolic pulmonary hypertension [8-10], cardiac fibrosis [11, 12] pulmonary fibrosis [45] idiopathic pulmonary fibrosis [13, 14] radiation induced pulmonary fibrosis [15], and pulmonary arterial hypertension (PAH) [10]. The most commonly studied molecular mechanisms involved in EndMT include TGFβ, Notch and interleukin and interferon-γ signaling [37-41]. Although TGFβ remains to be the widely studied EndMT inducing cytokine, the trigger for EndMT and molecular mechanisms through which it promotes EndMT have never been studied. Due to its predominant role in endothelial barrier protection through claudin gene regulation, an important candidate to investigate on its role in EndMT regulation would be Akt1.

Akt1 signaling is highly implicated in maintaining normal functioning of ECs and in regulating barrier integrity [29, 46]. We recently showed that TGFβ1 inhibits the activity of Akt1 in endothelial cells [33] and that TGFβ1 treatment results in EndMT in vitro [33]. In the current

study, we demonstrate that endothelial loss of Akt1 results in upregulation of mesenchymal markers N-cadherin, and αSMA, and downregulation of endothelial marker eNOS strongly implying the importance of Akt1 in vascular homeostasis and how its suppression can lead to vascular pathologies involving EndMT. We also demonstrated that endothelial-specific loss of Akt1 increases the mRNA expression of mesenchymal genes fibronectin 1 (FN1), keratin 7 (KRT7), and mesenchymal transcription factor FoxC2 and a dramatic 9-fold increase in the expression of the most potent EndMT inducing TGFβ isoform, TGFβ2. We also noticed a decrease in the expression of bone morphogenic protein 2 (BMP2), one of the TGFβ superfamily ligands that binds to receptor BMPR2 [47]. BMPR2 is highly mutated in all familial PAH, and most idiopathic PAH patients [48, 49] and in other vascular diseases [50]. EndMT is also associated with change in the morphology of ECs facilitating their migratory properties [51]. Our current data agrees with this where we saw the endothelial cells lacking Akt1 transform their cobble shaped morphology to a spindle shaped elongated one, very similar to that of the morphology induced by hypoxia. We also noticed a significant increase in the stress fiber formation with endothelial loss of Akt1.

Pulmonary artery remodeling (PAR) is a major feature of pulmonary hypertension (PH). It is characterized by cellular and structural changes affecting all three layers of the vessel wall of the pulmonary arteries [8, 52]. Common pulmonary vascular remodeling changes include increased intimal and/or medial stiffening and thickening, elevated expression of smooth muscle α-actin, collagen synthesis/deposition, and inflammation [8]. Abnormal deposition of smooth muscle-like (SM-like) cells in normally non-muscular, small diameter vessels and a deregulated control of endothelial cells are considered important pathological features of PAR [53]. It is this remodeling process inside the pulmonary vessels that is responsible for elevation of pulmonary vascular resistance, progressive PH, right ventricular failure and finally death [54]. Trans-differentiation of endothelial cells to smooth muscle cells (EndMT) plays an important role in vascular remodeling[55]. Few groups reported the occurrence of EndMT in PAH and its contribution to vascular remodeling [6, 56, 57]. However, the contributions of Akt1 induced EndMT to vascular remodeling remains unclear. Chen and Somanath et al, showed that Akt1 is the predominant isoform in vascular cells and that there was enhanced impairment of blood vessel maturation and increased vascular permeability in Akt1-/- mice[58]. We recently reported that endothelial loss of Akt1 enhances VEGF induced barrier breakdown *in vitro* and promotes VEGF induced vascular

leakage in mice ears [32]. We have also shown that endothelial loss of Akt1promotes lipopolysaccharide (LPS) induced acute lung injury [34] suggesting the involvement of Akt1 in vascular injury, a precursor for the pathological vascular remodeling [35, 36]. Our current findings support these reports where we demonstrated that endothelial-specific loss of Akt1 in VE-Cad-CreAkt1 mice exacerbated both the hypoxia- and Hy-SUGEN-induced pulmonary vascular remodeling which was evident from increased vascular thickening and collagen deposition around the blood vessels. Since the loss of Akt1 in long term increases the phosphorylation of β-catenin in endothelial cells we wanted to investigate whether inhibition of β-catenin would reverse the vascular remodeling induced by loss of Akt1 and/or hypoxia. When WT and Cre-Akt1 mice were treated with ICG-001, a β-catenin inhibitor, there was a decrease in vascular wall thickening indicating that inhibition of β-catenin prevents/reverses the pathological vascular remodeling induced by hypoxia and/or endothelial loss of Akt1. These observations suggest that Akt1 mediated GSK-3β/β-catenin signaling is involved in inducing EndMT thus contributing to pathological pulmonary vascular remodeling. Although Hypoxia-SU5416 (SUGEN) seems to be a good animal model to study pulmonary vascular remodeling in rodents, it did not work with our study design where we wanted to examine the specific role of endothelial Akt1in vascular remodeling. We noticed no difference in the vascular thickening in WT mice and VE-cad-CreAkt1 mice subjected to Hy-SUGEN. This was due to the irreversible vascular remodeling caused by SUGEN via suppression of Akt1.

In summary, this is the first study to examine the effect of endothelial specific loss of Akt1 on EndMT and its contribution to vascular remodeling. We provide novel insights into the role of Akt1 mediated βcatenin pathway in the mechanism of pathological vascular remodeling and present β-catenin as a potential target for developing pharmacological therapy for various cardio-pulmonary diseases involving vascular remodeling.

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FIGURES AND FIGURE LEGENDS

Figure 1

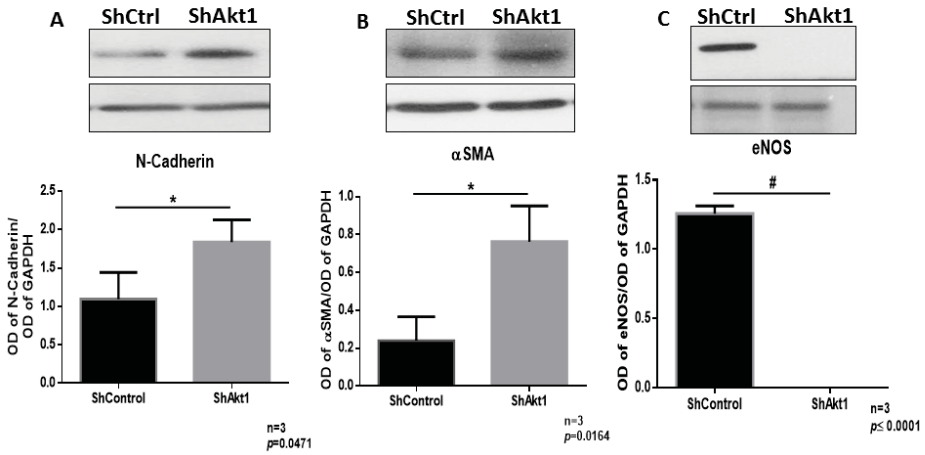


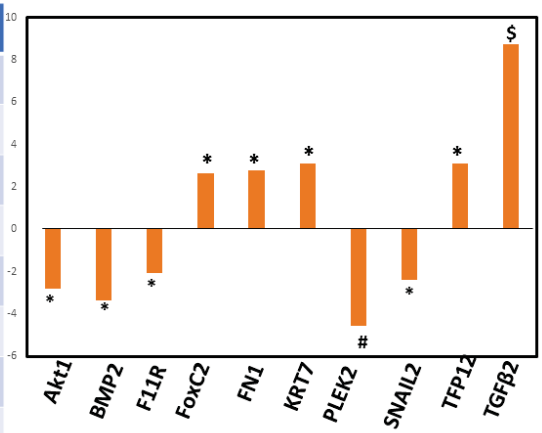
Figure 1. Endothelial loss of Akt1 induces EndMT *in-vitro*.

Representative Western blot images and the corresponding bar graph of band densitometry showing increased expression of N-cadherin **(A)**, and αSMA **(B)** and a decrease in eNOS **(C)** in HMECs transfected with ShRNA targeting Akt1. Data are represented as mean ± SD. (n=3-5), **p<0.05*; ***p<0.01*.

Figure 2

A

Gene	Fold Change
Akt1	-2.81843
BMP2	-3.3475
F11R	-2.08077
FOXC2	2.667933
FN1	2.7755
KRT7	3.1248
PLEK2	-4.57493
SNAIL2	-2.3744
TFP12	3.100367
TGF-β2	8.7498



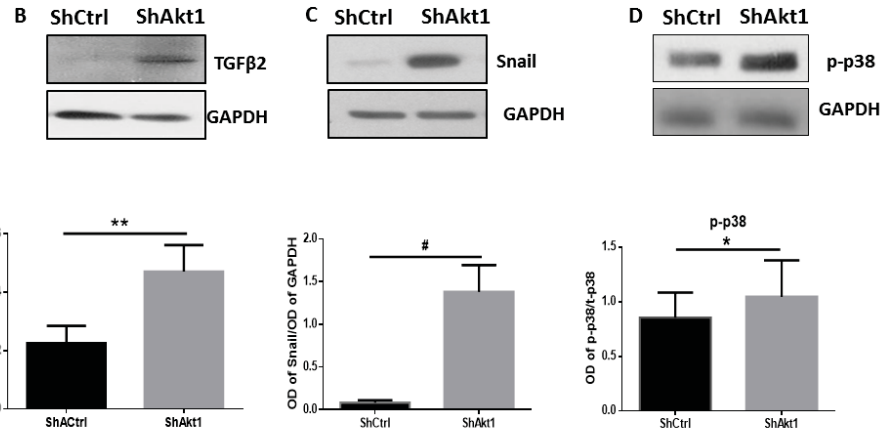


Figure 2. Endothelial loss of Akt1 upregulates mesenchymal genes and transcription factors *in-vito.*

(A) Gene array analysis of the ShCtrl and ShAkt1 HMEC cell lysates demonstrating changes in the expression of mesenchymal genes FN1and KRT7, mesenchymal transcription factors Foxc2, and a 9-fold increase in the expression of TGFβ2. Representative Western blot images and the corresponding bar graph of band densitometry showing increased expression of blot analysis of TGFβ2 **(B)**, Snail and **(C)** and activation (phosphorylation) of p-38 MAPK **(D)** with endothelial loss of Akt1. Data are represented as mean $\pm$ SD. (n=3-5), *$p<0.05$; **$p<0.01$.

Figure 3

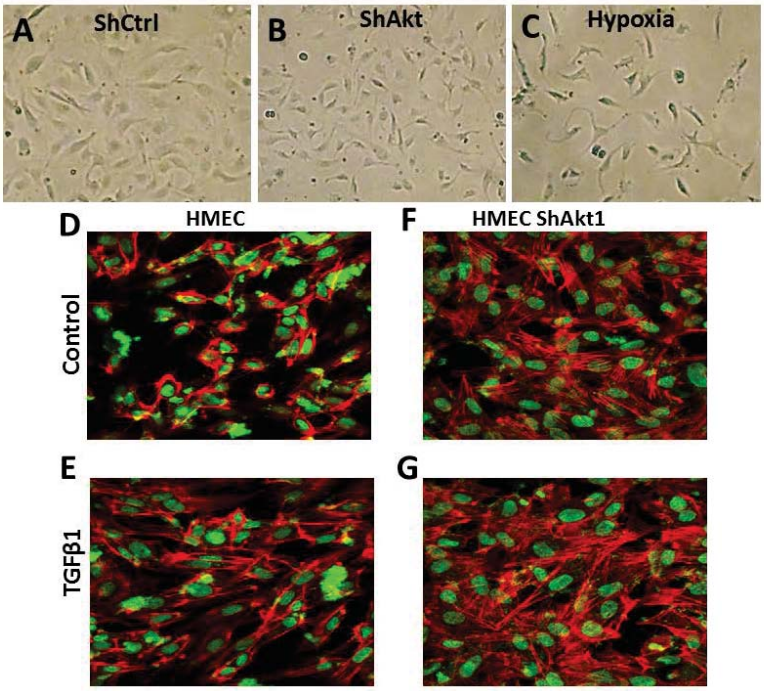


Figure 3. Endothelial specific loss of Akt1 induces a change in morphology and promotes stress fiber formation.

Representative images of normoxic ShCtrl **(A)** and ShAkt1HMECs **(B)** and regular HMECs exposed to 72-hour hypoxia **(C)** demonstrating changes in the morphology of HMECs from cobble shape to an elongated spindle shaped morphology. Representative confocal images of untreated HMECs transfected with scrambled ShRNA **(D)** or treated with 5ng/ml TGFβ1 for 72 hours **(E)** untreated ShAkt1 HMECs **(F)** or treated with 5ng/ml TGF-β for 72 hours **(G)** and subjected to phalloidin staining. Loss of Akt1 in endothelial cells induced stress fiber formation similar to that of TGFβ1.

Figure 4

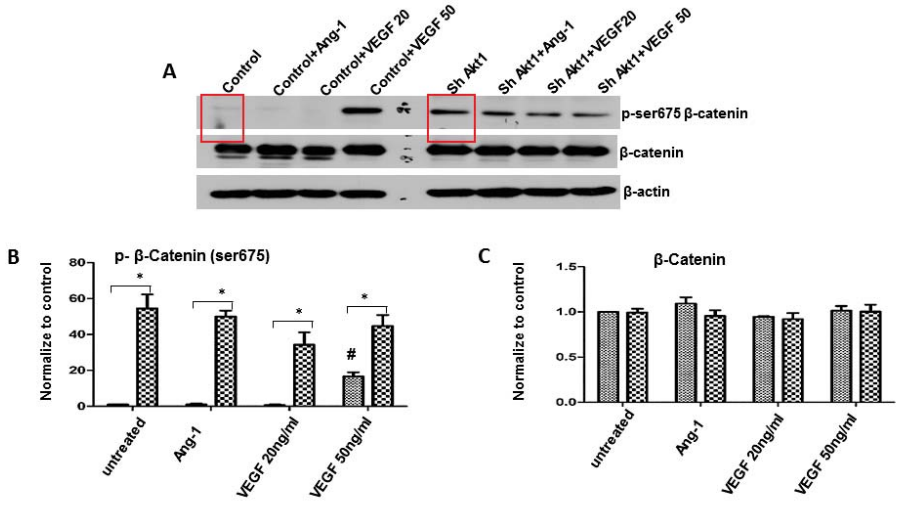


Figure 4. Long-term Akt1 inactivity in HMEC results in increased phosphorylation β-catenin.

(A) Representative western blot images and **(B-C)** bar graphs of the band densitometry of HMECs transfected with scrambled or ShAkt1RNA and treated with PBS, 20 ng/ml VEGF, 50 ng/ml VEGF, and 50 ng/ml Ang1 showing phosphorylation and total expression of β-catenin. Data are represented as mean ± SD. (n=3-5), *$p<0.05$; **$p<0.01$.

Figure 5

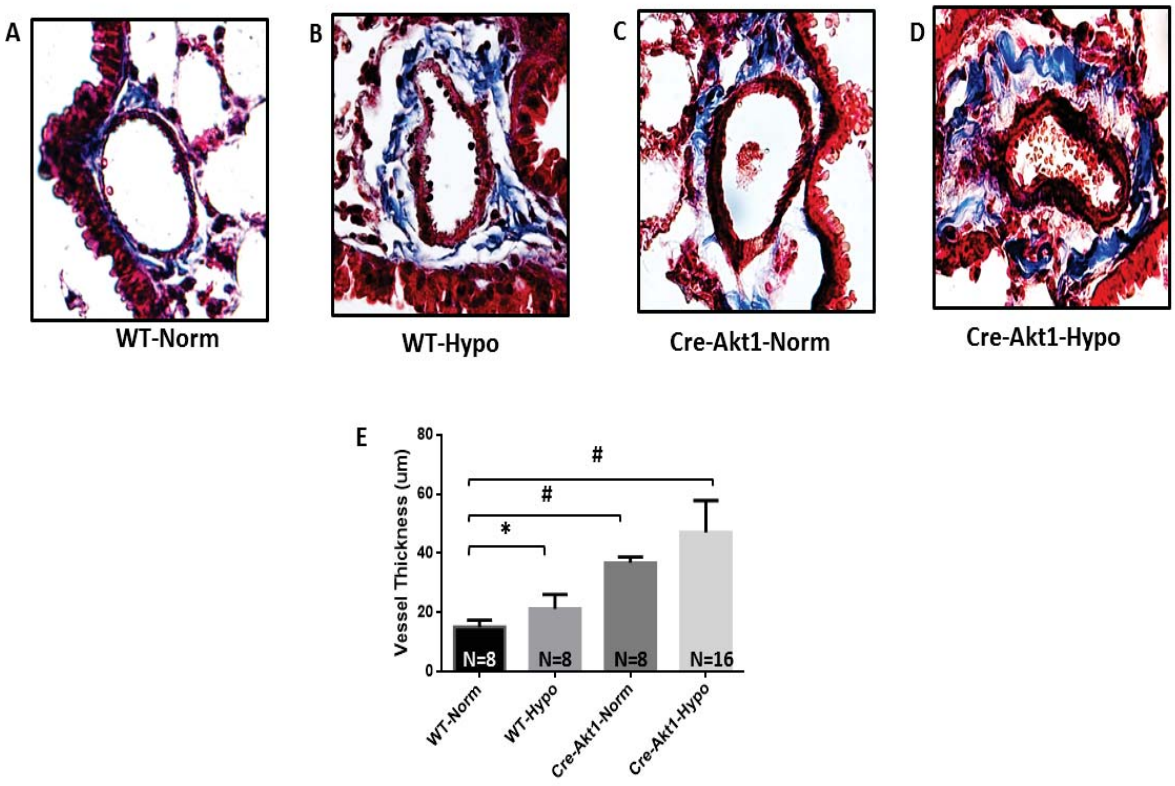


Figure 5. Endothelial Loss of Akt1 exacerbates Hypoxia induced vascular remodeling *in-vivo*.

Representative images of Masson's trichrome staining of lung sections of **(A)** WT- Normoxic, **(B)** WT-Hypoxic, **(C)** Ve-Cad-CreAkt1- Normoxic and **(D)** Ve-Cad-CreAkt1-Hypoxic mice and **(E)** corresponding bar graph of the vessel thickness showing endothelial specific loss of Akt1 exacerbates hypoxia induced pulmonary vascular remodeling by increasing blood vessel thickness. Data are represented as mean $\pm$ SD. (n=3-5), $*p<0.05$; $**p<0.01$.

Figure 6

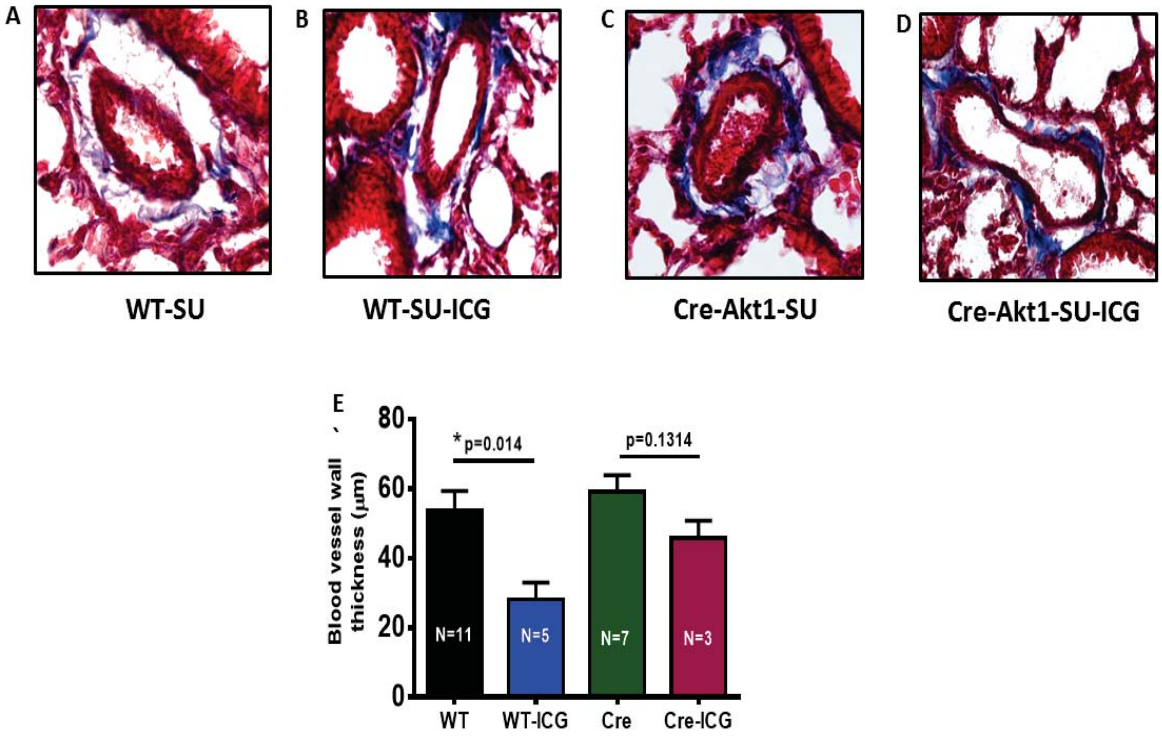


Figure 6. Hypoxia-SU-induced vascular remodeling in mice was reversed by β-catenin suppression

Representative images of Masson's trichrome staining of lung sections of **(A-D)** C57BL6-WT and VE-cad-Cre-Akt1 mice subjected to Hypoxia-SU and treated with either vehicle or 10mg/kg i.p ICG-001, a β-catenin inhibitor and thickness **(E)** corresponding bar graph of vessel showing that inhibition of β-catenin reduced the Hy-SU induced vessel thickening in both WT and VE-cad-CreAkt1 mice. Vessel thickness was measured using NIH Image J software. Data are represented as mean ± SD. (n=3-5), *$p<0.05$; **$p<0.01$. WT- wild type

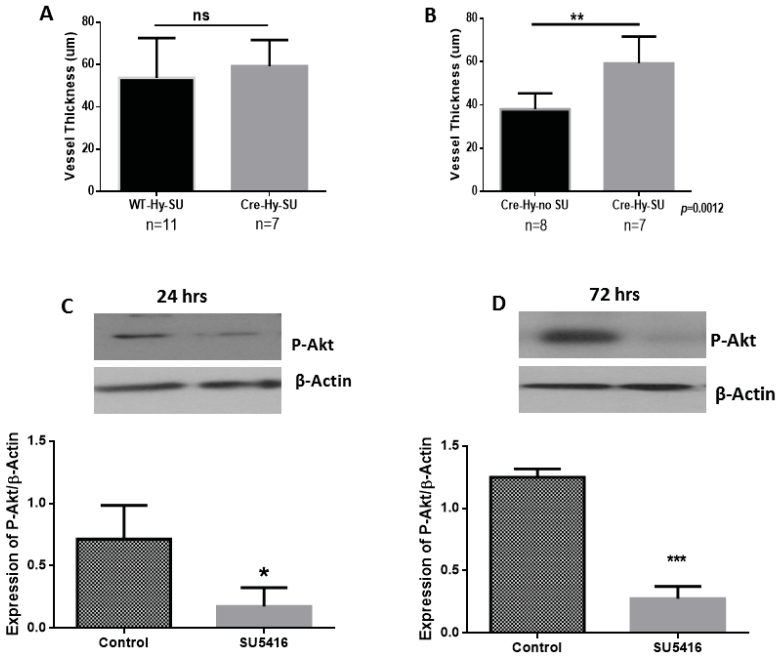


Figure 7. SU5416 causes irreversible pulmonary vascular remodeling via Akt suppression

(A-B) Bar graphs of vessel thickness of C57BL6-WT and VE-cad-Cre-Akt1 mice subjected to normoxia, hypoxia alone or SU-Hypoxia demonstrating that SU-5416, a VEGFR2 inhibitor exacerbates irreversible pulmonary vascular remodeling in mice. This was worsened in the presence of hypoxia. **(C-D)** Representative western blot images and bar graphs of the band densitometry of HMECs treated with 10 μM SU-5416 for 24 and 72 hours respectively, show that SU5416 inhibits Akt. Data are represented as mean ± SD. (n=3-5), *$p<0.05$; **$p<0.01$. WT-wild type

CHAPTER 5

INTEGRATED DISCUSSION, CONCLUSION AND TRANSLATIONAL IMPACT

Discussion

Pulmonary vascular remodeling is a hallmark of many cardio-pulmonary diseases including pulmonary arterial hypertension (PAH), pulmonary fibrosis (PF) and chronic obstructive pulmonary disease (COPD). There is no cure for these diseases except for the treatment of the symptoms. It is also an important cause of morbidity and mortality, resulting in over 3 million deaths globally in 2005. Understanding the mechanisms contributing to the vascular remodeling in these diseases might give us an opportunity to develop potential therapeutics. Endothelial to mesenchymal transition (EndMT) is the trans-differentiation of endothelial cells to mesenchymal cells attributing proliferative, migratory, and invasive properties hence contributing to the pathological conditions including PAH, COPD, PF, acute lung injury (ALI), atherosclerosis and restenosis. Akt1 is the predominant kinase in vascular cells whose contributions to vascular injury ultimately leading to EndMT have been understudied.

The main goal of this dissertation is to identify the role of Akt1 in the development of EndMT *in vitro* and vascular remodeling *in vivo*. To dissect this, we 1- Compared the effects of TGFβ1, VEGF and Ang-1 on Akt1 pathway and its cross-talk with Src in regulating EC-barrier function; 2- Determined the effects of TGFβ1, TGFβ2 and TGFβ3 on the activation of canonical (Smad) and non-canonical (p38 MAPK) signaling in ECs and promotion of EndMT; and 3- Investigated the effect of endothelial specific loss of Akt1 on EndMT *in vitro* and hypoxia-induced pulmonary vascular remodeling *in vivo.* First, we wanted to study the changes in Akt and Src in response to growth factors that regulate endothelial barrier and establish a cross-talk between Akt and Src in the modulation of endothelial-barrier function. We showed that although VEGF-induced endothelial-barrier permeability is short-term which was reversed with time, TCBN-mediated Akt inhibition blocked the reversal and diminished the long-term barrier protective effects of VEGF and Ang-1. In contrast, pp2-mediated Src inhibition reversed VEGF-induced short-term vascular permeability but had no significant effect on the VEGF and Ang-1-mediated long-term barrier

protection. Genetic loss of Akt1 achieved by ShRNA-mediated knockdown resulted in increased gap formation. VEGF increased the number of gaps in the short-term, an effect that was blunted by the inhibition of Src, but not Akt. However, both VEGF and Ang-1 stabilized the endothelial-barrier in the long term which was disrupted by inhibition of Akt but not Src. These results indicate that both Src and Akt play different roles in the short- and long-term endothelial-barrier regulation in response to VEGF and Ang-1.

We also showed that long-term inactivation of Akt enhanced Src activity leading to long-term endothelial-barrier disruption following Akt inhibition. Similarly, we saw a significant increase in Tyr416 Src phosphorylation was also observed in Akt1 deficient HMECs. Further, stimulation of HMECs with TGFβ1 although did not affect Akt phosphorylation in the short-term, resulted in its inhibition in the long-term. In contrast, although TGFβ1 inhibited Src phosphorylation in the short-term, it promoted activating Src phosphorylation in the long term, indicating a reciprocal regulation of Akt and Src activities in the long-term in HMECs. Furthermore, whereas TGFβ1 treatment resulted in HMEC-barrier disruption in the long-term, this effect was blunted in ShAkt1 HMEC monolayers, thus indicating that Akt inhibition is necessary for the TGFβ1-induced endothelial-barrier injury. Hence, Akt1 is necessary for maintaining endothelial barrier integrity to keep Src activity in check.

As TGFβ has been greatly implicated in inducing EndMT we wanted to find out which one of the three TGFβ isoforms is the most potent in executing this effect. In order to examine this, we compared the dose-dependent effects of TGFβ1, TGFβ2, and TGFβ3 on EndMT *in vitro*. Upon examining the changes in the expression of endothelial and mesenchymal markers, transcription factors that promote mesenchymal transition, and the activation of TGFβ-induced canonical and non-canonical pathways by treating HMECs with various doses of these TGFβ isoforms for 72 hours we found that TGFβ induces EndMT which was evident from the upregulation of mesenchymal markers N-cadherin and αSMAand a reduction of endothelial markers VE-cadherin and eNOS. The lowest dose of TGFβ2 (1 ng/ml) induced EndMT significantly greater than the highest doses of TGFβ1 and TGFβ3 (5 ng/ml) implying a predominant effect of TGFβ2 on EndMT. TGFβ2 increased the phosphorylation (activation) of pro-fibrotic Smad, Smad2/3 and p38 MAPK greater than that of TGFβ1 and TGFβ3 suggesting the predominance of TGFβ2 in inducing mesenchymal transition of ECs. We also observed that TGFβ2 increased the expression of

mesenchymal transcription factors Snail and FoxC2 greater than the other two TGFβ isoforms. The most intriguing and prominent finding of our study was that both TGFβ1 and TGFβ3, but not TGFβ2, stimulated the expression of TGFβ2 by the HMECs. This indicated the existence of a positive feedback loop between different TGFβ isoforms via paracrine effect involving TGFβ2 in inducing EndMT. Together with our results demonstrate that although all the three isoforms of TGFβ induce EndMT, TGFβ2 is the more potent than the other isoforms.

Next, we wanted to determine the role of Akt1 in inducing EndMT *in vitro* and vascular remodeling *in vivo.* We demonstrated that endothelial loss of Akt1 results in upregulation of mesenchymal markers N-cadherin, and αSMA, and downregulation of endothelial marker eNOS implying that loss of Akt1 induces EndMT. We also demonstrated that endothelial-specific loss of Akt1 increases the mRNA expression of mesenchymal genes, and mesenchymal transcription factors, and the most potent EndMT inducing TGFβ isoform, TGFβ2. We also noticed a decrease in the expression of bone morphogenic protein 2 (BMP2), one of the TGFβ superfamily ligands that binds to receptor BMPR2, a highly-mutated BMP receptor PAH patients. We also showed that endothelial cells lacking Akt1 transform from cobble shaped morphology to a spindle shaped elongated one, very similar to that of the morphology induced by hypoxia. This was accompanied by a significant increase in the stress fiber formation with endothelial loss of Akt1.

We demonstrated that endothelial-specific loss of Akt1 in VE-Cad-CreAkt1 mice exacerbated both the hypoxia- and Hy-SUGEN-induced pulmonary vascular remodeling which was evident from increased vascular thickening and collagen deposition around the blood vessels. We found that loss of Akt1 in long term increased the phosphorylation of β-catenin in endothelial cells. When WT and Cre-Akt1 mice were treated with ICG-001, a β-catenin inhibitor, there was a decrease in vascular wall thickening, indicating that inhibition of β-catenin prevents/reverses the pathological vascular remodeling induced by hypoxia and/or endothelial loss of Akt1. These observations suggest that Akt1 mediated GSK-3β/β-catenin signaling is involved in inducing EndMT thus contributing to pathological pulmonary vascular remodeling. We noticed no difference in the vascular thickening in WT mice and VE-cad-CreAkt1 mice subjected to Hy-SUGEN due to the irreversible vascular remodeling caused by SUGEN via suppression of Akt1.

Conclusion and Translational Impact

In summary, we provide novel insights into the role of Akt1 mediated βcatenin pathway in the mechanism of pathological vascular remodeling and demonstrate β-catenin as a potential target for developing pharmacological therapy for various cardio-pulmonary diseases involving vascular remodeling. Our findings are clinically relevant as FoxO (AS1842856) and β-catenin (ICG-001) inhibitors will be useful in preventing aberrant vascular injury, EndMT and pathological vascular remodeling in the acute phase of lung injury. Specific TGFβ2 (AP12009) and/or P38 MAPK inhibitors can be utilized to suppress FoxC2 and Snail expression, and inhibit EndMT in the treatment of PAH in the advanced stages.

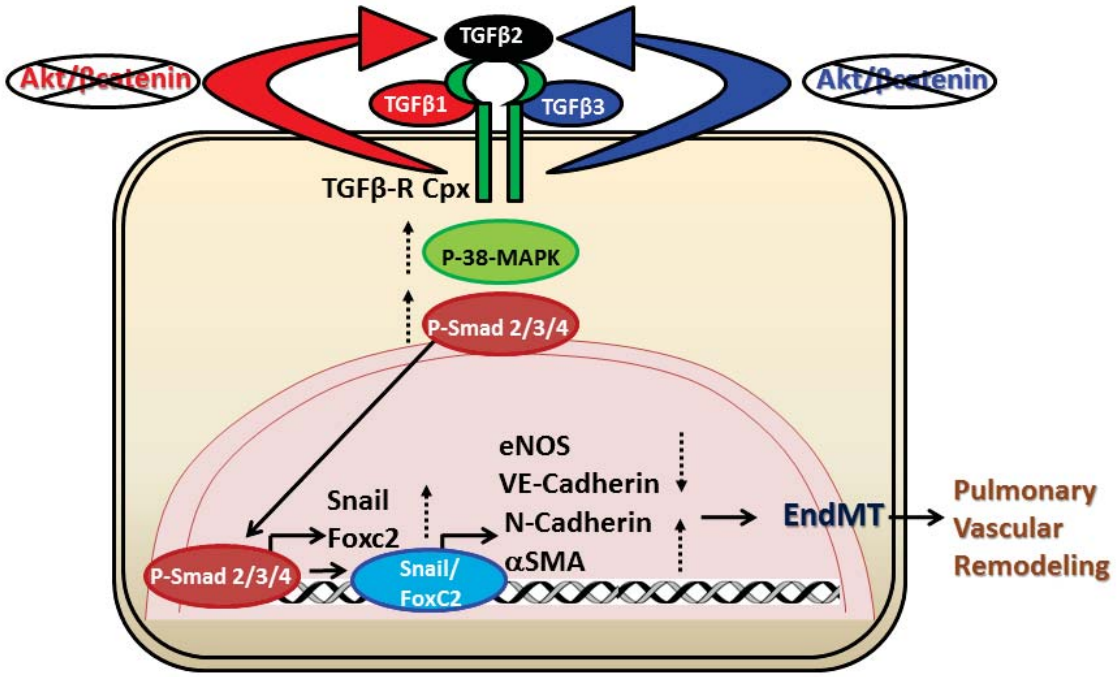


Figure 1. Akt1 mediated mechanisms promoting endothelial to mesenchymal transition.

TGFβ1-mediated Akt suppression promotes increased expression of TGFβ2, a predominant TGF-β isoform in inducing EndMT via up-regulation of FoxC2 and Snail1, and down-regulation of BMP2 and Snail2 (Slug). This results in increased expression of mesenchymal markers N-Cadherin and α-SMA and loss of endothelial marker eNOS resulting in EndMT *in-vitro* and pulmonary vascular remodeling *in-vivo*. Inhibition of β-catenin/WNT signaling by ICG-001 reverses SU-Hypoxia induced pulmonary vascular remodeling *in-vivo.*

Appendix

Targeting Src-mediated Tyr216 phosphorylation and activation of GSK-3 in prostate cancer cells inhibit prostate cancer progression *in vitro* and *in vivo*

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ABSTRACT:

Recent studies suggest a positive correlation between glycogen synthase kinase-3 (GSK-3) activation and tumor growth. Currently, it is unclear how both Akt that inhibits GSK-3 and active GSK-3 are maintained concurrently in tumor cells. We investigated the role of GSK-3 and the existence of an Akt-resistant pathway for GSK-3 activation in prostate cancer cells. Our data show that Src, a non-receptor tyrosine kinase is responsible for ^{Y216}GSK-3 phosphorylation leading to its activation even when Akt is active. Experiments involving mouse embryonic fibroblasts lacking cSrc, Yes and Fyn, as well as Src activity modulation in prostate cancer cells with constitutively active (CA-Src) and dominant negative Src (DN-Src) plasmids demonstrated the integral role of Src in ^{Y216}GSK-3 phosphorylation and activity modulation. Inhibition of GSK-3 with SB415286 in PC3 cells resulted in impaired motility, proliferation and colony formation. Treatment of PC3 cells with the Src inhibitor dasatinib reduced ^{Y216}GSK-3 phosphorylation and inhibited proliferation, invasion and micrometastasis in vitro. Dasatinib treatment of athymic nude mice resulted in impaired growth of PC3 cell tumor xenograft. Together, we provide novel insight into the Src-mediated ^{Y216}GSK-3 phosphorylation and activation in prostate cancer cells and reveal the potential benefits of targeting Src-GSK-3 axis using drugs such as dasatinib.

INTRODUCTION

Prostate cancer is the second leading cause of cancer-related death among men in the United States. Although androgen deprivation therapy is an effective treatment during the early stages of prostate cancer, due to the uncertainty in the molecular mechanisms leading to advanced stages of the disease, patients with castration-resistant prostate cancer are left with limited treatment options which includes chemotherapy, immunotherapy, or novel oral agents such as abiraterone acetate or enzalutamide.

Hyper-activation of the PI3 Kinase-Akt pathway due to PTEN mutation is one of the most common reasons for prostate cancer [1]. An activating E17K mutation in Akt has also been linked to the development of prostate cancer [2], demonstrating that Akt activity is indispensable for prostate cancer development. Our previous studies have demonstrated that changes in Akt activity results in the modulation of prostate cancer cell survival, proliferation, colony formation and tumor growth [3-5] as well as micrometastasis of prostate cancer cells via inside-out activation of the cell surface integrin $\alpha_v\beta_3$, thus aiding the cellular recognition of specific extracellular matrix (ECM) proteins abundant in the vascular basement membrane and bone [6, 7]. Glycogen synthase kinase 3 (GSK-3), a serine-threonine kinase is one of the least characterized substrate of Akt in prostate cancer cells, whose activity is inhibited by Akt via phosphorylation at serine 9 and 21 in GSK-3α and GSK-3β isoforms, respectively [8, 9].

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p70 S6-kinase mediates the cooperation between Akt1 and Mek1 pathways in fibroblast-mediated extracellular matrix remodeling

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ABSTRACT

Previous studies have demonstrated both synergistic and opposing effects of Akt and Mek1/2 in various cell functions and disease states. Furthermore, Akt has been reported to inhibit and activate cRaf/Mek pathway, suggesting that their mutual interaction and cooperation may be cell type, stimuli and/or context specific. While PI3-kinase/Akt and cRaf/Mek pathways have been implicated in the regulation of extracellular matrix (ECM) remodeling, mutual interactions between these two pathways and their specific contributions to the events leading to ECM synthesis and assembly is not clear. We investigated the specific role of Akt1 and Mek1 in ECM synthesis and assembly by NIH 3T3 fibroblasts and how these effects were reconciled to mediate overall ECM remodeling. Our study identified that cooperation between Akt1 and Mek1 is necessary to mediate ECM synthesis. Whereas Akt1 activation resulted in Mek1 activation as evidenced by increased ERK1/2 phosphorylation, Mek1 inhibition using U0126 or DN-Mek1 resulted in enhanced Akt1 phosphorylation. Interestingly, both Akt1 and Mek1 activities were needed for the synthesis and assembly of ECM. The effect of Akt1 and Mek1 on ECM synthesis was reconciled through the activation of p70 S6-kinase via phosphorylation at T421/S424 and S411, respectively. Furthermore, Akt1 and Mek1 cooperated in mediating ECM assembly *via* activation of integrin β1. Together, we show for the first time that Akt1 and Mek1 pathways cooperate in the regulation of ECM remodeling by the fibroblasts.

1. Introduction

Extracellular matrix (ECM) is a non-cellular structural network composed of mainly proteins such as fibronectin, collagens, laminins and elastin as well as proteoglycans such as dermatan sulfate and hyaluronan [1]. Fibroblasts, the major source of ECM, also perform their assembly during tissue remodeling [2], and are precursors of pathologic myofibroblasts that mediate tissue fibrosis [3]. ECM provides the necessary scaffold to the surrounding cells to group them together and confer flexibility and strength.

Research from our laboratory has demonstrated the key role of serine–threonine kinase Akt1 (Protein kinase Bα) in ECM remodeling *in vitro* and *in vivo*. Our studies in *Akt1*$^{-/-}$ mice revealed that expression and assembly of collagen matrix in normal skin and during cutaneous wound healing, as well as the expression of laminin in the vascular basement membrane are all impaired in the absence of Akt1 gene [4,5]. Pharmacological inhibition of Akt or ablation of Akt1 gene resulted in impaired fibronectin matrix assembly *via* modulation of Rac1 and P21 activated kinase pathway [6,7]. Furthermore, secretion of fibronectin and collagen types I, II and V was also regulated by Akt1 *via* mTOR signaling in response to the pro-fibrotic factor transforming growth factor-β (TGFβ) or agents such as bleomycin [8]. Recently, we also demonstrated that, apart from the pro-fibrogenic role of Akt1 in fibroblasts, sustained activation of Akt1 leads to myofibroblast differentiation associated with enhanced expression of alpha-smooth muscle cell actin (αSMA) contractile protein through serum response factor (SRF) and myocardin-mediated pathway [9]. These studies indicated that fine tuning of Akt1 activity is necessary to maintain ECM homeostasis in tissues.

Another pathway activated during a fibrogenic response that has been implicated in ECM remodeling is the cRaf/Mek/ERK1/2 pathway [10]. However, reports on mutual interactions between cRaf/Mek and PI3 kinase/Akt pathways are highly conflicting. An early study done in HEK293 cells indicated that Akt is responsible for the direct phosphorylation of cRaf^{S259} leading to inhibition of Mek/ERK1/2 pathway [11]. Another study showed that Akt can keep cRaf in an inactive as well as an active form depending upon the phosphorylation status of another serine residue (cRaf^{S338}) by an unknown kinase that promote its interaction with adaptor protein 14-3-3 [12]. Whereas one study indicated that P21 activated kinase-1 (Pak1) is responsible for the activation of cRaf signaling in mediating oncogenic transformation in Rat-1

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RESEARCH PAPER

The Akt inhibitor, triciribine, ameliorates chronic hypoxia-induced vascular pruning and TGFβ-induced pulmonary fibrosis

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BACKGROUND AND PURPOSE

Interstitial lung disease accounts for a group of chronic and progressive disorders associated with severe pulmonary vascular remodelling, peripheral vascular rarefaction and fibrosis, thus limiting lung function. We have previously shown that Akt is necessary for myofibroblast differentiation, a critical event in organ fibrosis. However, the contributory role of the Akt-mTOR pathway in interstitial lung disease and the therapeutic benefits of targeting Akt and mTOR remain unclear.

EXPERIMENTAL APPROACH

We investigated the role of the Akt-mTOR pathway and its downstream molecular mechanisms in chronic hypoxia- and TGFβ-induced pulmonary vascular pruning and fibrosis in mice. We also determined the therapeutic benefits of the Akt inhibitor triciribine and the mTOR inhibitor rapamycin for the treatment of pulmonary fibrosis in mice.

KEY RESULTS

Akt*1*$^{-/-}$ mice were protected from chronic hypoxia-induced peripheral vascular pruning. In contrast, hyperactivation of Akt1 induced focal fibrosis similar to TGFβ-induced fibrosis. Pharmacological inhibition of Akt, but not the Akt substrate mTOR, inhibited hypoxia- and TGFβ-induced pulmonary vascular rarefaction and fibrosis. Mechanistically, we found that Akt1 modulates pulmonary remodelling via regulation of thrombospondin1 (TSP1) expression. Hypoxic Akt*1*$^{-/-}$ mice lungs expressed less TSP1. Moreover, *TSP1*$^{-/-}$ mice were resistant to adMyrAkt1-induced pulmonary fibrosis.

CONCLUSIONS AND IMPLICATIONS

Our study identified Akt1 as a novel target for the treatment of interstitial lung disease and provides preclinical data on the potential benefits of the Akt inhibitor triciribine for the treatment of interstitial lung disease.

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ORIGINAL PAPER

Candesartan stimulates reparative angiogenesis in ischemic retinopathy model: role of hemeoxygenase-1 (HO-1)

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Abstract Ischemic diseases such as stroke and proliferative retinopathy are characterized by hypoxia-driven release of angiogenic factors such as vascular endothelial growth factor (VEGF). However, revascularization of the ischemic areas is inadequate, resulting in impaired neurovascular function. We aim to examine the vascular protective effects of candesartan, an angiotensin receptor blocker, in an ischemic retinopathy mouse model. Vascular density, number of tip cells, and perfusions of capillaries were assessed. Activation of Muller glial cells and levels of peroxynitrite, VEGF, VEGFR2, inducible nitric oxide synthase, hemeoxygenase-1 (HO-1) were assessed. Proangiogenic effects of candesartan were examined in human endothelial cells (EC) that were cultured in normoxia or hypoxia and transduced with siRNA against HO-1. Candesartan (1 mg/kg) and (10 mg/kg) decreased hypoxia-induced neovascularization by 67 and 70 %, respectively. Candesartan (10 mg/kg) significantly stimulated the number of tip cells and physiological revascularization of the central retina (45 %) compared with untreated pups. The effects of candesartan coincided with reduction of hypoxia-induced Muller glial activation, iNOS expression and restoration of HO-1 expression with no significant change in VEGF levels. In vitro, silencing HO-1 expression blunted the ability of candesartan to induce VEGF expression under normoxia and VEGFR2 activation and angiogenic response under both normoxia and hypoxia. These findings suggest that candesartan improved reparative angiogenesis and hence prevented pathological angiogenesis by modulating HO-1 and iNOS levels in ischemic retinopathy. HO-1 is required for VEGFR2 activation and proangiogenic action of candesartan in EC. Candesartan, an FDA-approved drug, could be repurposed as a potential therapeutic agent for the treatment of ischemic diseases.

Keywords Reparative angiogenesis · Candesartan · Hemeoxygenase-1 · iNOS · Nitrotyrosine · Endothelial cells

Abbreviations

VEGF	Vascular endothelial growth factor
p	Postnatal day
Cand	Candesartan
ROP	Retinopathy of prematurity
DR	Diabetic retinopathy
iNOS	Inducible nitric oxide synthase
HO-1	Hemeoxygenase-1

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Genetic deletion and pharmacological inhibition of Akt1 isoform attenuates bladder cancer cell proliferation, motility and invasion

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ABSTRACT

Isoform specific expression, intracellular localization and function of Akt in bladder cancer are not known. In the current study, we identified Akt1, followed by Akt2 and Akt3 as the predominant Akt isoform in human T24 and UM-UC-3 metastatic bladder cancer cells. Whereas Akt1 is localized at the membrane, cytoplasm and nucleus, Akt2 is solely cytoplasmic and Akt3 is mostly localized in the nucleus in T24 cells. ShRNA-mediated Akt1 knockdown resulted in impaired T24 cell survival, proliferation, colony formation, migration and microinvasion. Whereas pharmacological inhibition of Akt1 resulted in impaired T24 and UM-UC-3 cell motility, viability and proliferation, effect of pharmacological inhibition by Akt2 inhibitor was limited to proliferation in T24, but not UM-UC-3 cells. Our data provide important clues on the therapeutic benefits of targeting Akt1 for bladder cancer therapy.

1. Introduction

Bladder cancer is the fourth most common cancer among men in the Western countries (Siegel et al., 2014). Lack of tools for the early detection and therapeutic strategies for metastatic bladder cancer are the major problems. Candidate genes regulating bladder cancer are still under investigation. Recent studies indicate that de-regulation of catalytic domain of PI3-Kinase (p110α), phosphatase and tensin homolog (PTEN), Akt, p53, and fibroblast growth factor receptor (FGFR) can lead to the development and growth of bladder cancer (Aveyard et al., 1999; Cairns et al., 1998; Cappellen et al., 1997; Junker et al., 2008; Knowles et al., 2009; Lopez-Knowles et al., 2006). High-grade non-invasive and invasive bladder cancers are linked with loss of PTEN and p53 activity (Ching and Hansel, 2010; Puzio-Kuter et al., 2009). Mutations or loss of PTEN has been linked to many cancers (Cairns et al., 1997; Coleman et al., 2014; Huang et al., 2014; Kim et al., 1998; Li et al., 2014; Risinger et al., 1997; Sakurada et al., 1997), including bladder cancer (Aveyard et al., 1999; Cordon-Cardo, 2008; Kanda et al., 2009) thus generating research interest in PTEN/Akt pathway in bladder cancer (Saal et al., 2007).

Akt (protein kinase B) is a serine–threonine kinase that exists in three different isoforms namely Akt1, Akt2 and Akt3 (Somanath et al., 2006). Although Akt has been demonstrated in bladder cancer (Mundhenk et al., 2011), the predominant isoform expressed in bladder cancer and its effect on oncogenic response is not yet clear. Mutation in Akt1 E17K has been reported in 2/44 (4.8%) bladder cancer cell lines and 5/184 (2.7%) bladder tumors (Askham et al., 2010). In addition, significantly higher levels of Serine-473 phosphorylated (active) Akt have been reported in primary bladder carcinoma (Harris et al., 2008; Qian et al., 2009; Wu et al., 2004). However, until today, whether Akt1 is the predominant isoform in bladder cancer cells and whether its activation is necessary for bladder cancer progression is not clear. Here, we examined isoform specific expression and subcellular localization of Akt isoforms in metastatic human T24 bladder cancer cells.

2. Material and methods

2.1. Cell Lines, reagents and antibodies

Human T24 (bladder carcinoma) cells, UM-UC-3 (bladder carcinoma) and human dermal micro-vascular endothelial cells

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Novel roles of Src in cancer cell epithelial-to-mesenchymal transition, vascular permeability, microinvasion and metastasis

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ABSTRACT

The Src-family kinases (SFKs), an intracellularly located group of non-receptor tyrosine kinases are involved in oncogenesis. The importance of SFKs has been implicated in the promotion of tumor cell motility, proliferation, inhibition of apoptosis, invasion and metastasis. Recent evidences indicate that specific effects of SFKs on epithelial-to-mesenchymal transition (EMT) as well as on endothelial and stromal cells in the tumor microenvironment can have profound effects on tumor microinvasion and metastasis. Although, having been studied extensively, these novel features of SFKs may contribute to greater understanding of benefits from Src inhibition in various types of cancers. Here we review the novel role of SFKs, particularly c-Src in mediating EMT, modulation of tumor endothelial-barrier, transendothelial migration (microinvasion) and metastasis of cancer cells, and discuss the utility of Src inhibitors in vascular normalization and cancer therapy.

Contents

1. Introduction

Src family kinases (SFKs) that include isoforms such as c-Src (Sarcoma), Blk (B-lymphoid tyrosine kinase), Fgr (Gardner-Rasheed feline sarcoma), Fyn, Frk (Fyn-related kinase), Hck (Hematopoietic cell kinase), Lck (Lymphocyte specific kinase), Lyn, Yes (Yamagichi sarcoma) and Yrk (Yes-related kinase), each with a unique domain [1] have been implicated in oncogenesis. SFKs are non-receptor tyrosine kinases (nRTKs) that act downstream of receptor tyrosine kinases (RTKs) and integrins in the regulation of various stages of tumor cell proliferation and survival [2]. Among these, c-Src is the most characterized isoform that plays a definitive role in tumor metastasis by regulating earlier stages of cell proliferation such as cell migration, adhesion, and invasion [2]. Src interacts extensively with transmembrane RTKs such as

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Cellular and Molecular Life Sciences

ORIGINAL ARTICLE

Akt1 promotes stimuli-induced endothelial-barrier protection through FoxO-mediated tight-junction protein turnover

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Abstract Vascular permeability regulated by the vascular endothelial growth factor (VEGF) through endothelial-barrier junctions is essential for inflammation. Mechanisms regulating vascular permeability remain elusive. Although 'Akt' and 'Src' have been implicated in the endothelial-barrier regulation, it is puzzling how both agents that protect and disrupt the endothelial-barrier activate these kinases to reciprocally regulate vascular permeability. To delineate the role of Akt1 in endothelial-barrier regulation, we created endothelial-specific, tamoxifen-inducible Akt1 knockout mice and stable ShRNA-mediated Akt1 knockdown in human microvascular endothelial cells. Akt1 loss leads to decreased basal and angiopoietin1-induced endothelial-barrier resistance, and enhanced VEGF-induced endothelial-barrier breakdown. Endothelial Akt1 deficiency resulted in enhanced VEGF-induced vascular leakage in mice ears, which was rescued upon re-expression with Adeno-myrAkt1. Furthermore, co-treatment with angiopoietin1 reversed VEGF-induced vascular leakage in an Akt1-dependent manner. Mechanistically, our study revealed that while VEGF-induced short-term vascular permeability is independent of Akt1, its recovery is reliant on Akt1 and FoxO-mediated claudin expression. Pharmacological inhibition of FoxO transcription factors rescued the defective endothelial barrier due to Akt1 deficiency. Here we provide novel insights on the endothelial-barrier protective role of VEGF in the long term and the importance of Akt1-FoxO signaling on tight-junction stabilization and prevention of vascular leakage through claudin expression.

Keywords VEGF · Angiopoietin-1 · Akt · VE-cadherin · Claudin · Vascular permeability

Abbreviations

VEGF	Vascular endothelial growth factor
Ang-1	Angiopoietin-1
VECad-Cre-Ak1	Vascular endothelial cadherin-cre recombinase-Akt1 knockdown
HMEC	Human microvascular endothelial cells
FoxO	Forkhead box protein O
ECIS	Electric cell-substrate impedance sensing
WT	Wild type
GFP	Green fluorescent protein
eNOS	Endothelial nitric oxide synthase

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RAPID COMMUNICATION

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Modulation of long-term endothelial-barrier integrity is conditional to the cross-talk between Akt and Src signaling

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Although numerous studies have implicated Akt and Src kinases in vascular endothelial growth factor (VEGF) and Angiopoietin-1 (Ang-1)-induced endothelial-barrier regulation, a link between these two pathways has never been demonstrated. We determined the long-term effects of Akt inhibition on Src activity and vice versa, and in turn, on the human microvascular endothelial cell (HMEC) barrier integrity at the basal level, and in response to growth factors. Our data showed that Akt1 gene knockdown increases gap formation in HMEC monolayer at the basal level. Pharmacological inhibition of Akt, but not Src resulted in exacerbated VEGF-induced vascular leakage and impaired Ang-1-induced HMEC-barrier protection in vitro at 24 hr. Whereas inhibition of Akt had no effect on VEGF-induced HMEC gap formation in the short term, inhibition of Src blunted this process. In contrast, inhibition of Akt disrupted the VEGF and Ang-1 stabilized barrier integrity in the long-term while inhibition of Src did not. Interestingly, both long-term Akt inhibition and Akt1 gene knockdown in HMECs resulted in increased Tyr416 phosphorylation of Src. Treatment of HMECs with transforming growth factor-β1 (TGFβ1) that inhibited Akt Ser473 phosphorylation in the long-term, activated Src through increased Tyr416 phosphorylation and decreased HMEC-barrier resistance. The effect of TGFβ1 on endothelial-barrier breakdown was blunted in Akt1 deficient HMEC monolayers, where endothelial-barrier resistance was already impaired compared to the control. To our knowledge, this is the first report demonstrating a direct cross-talk between Akt and Src in endothelial-barrier regulation.

KEYWORDS
Akt, endothelial-barrier, Src, vascular permeability, VE-cadherin

1 | INTRODUCTION

Vascular permeability is a complex yet a highly coordinated process that not only regulates vesicular trafficking but also integrates complex junction rearrangements, and refined cytoskeletal dynamics (Goddard & Iruela-Arispe, 2013). The endothelium plays a key role in regulating vascular integrity. Recent studies suggest that impairment of endothelial function, as observed in the presence of cardiovascular risk factors, is not only a marker but also contributes to the pathogenesis of cardiovascular diseases (Landmesser, Hornig, & Drexler, 2004). Thus, improving endothelial function is an important therapeutic target for reducing vascular diseases (Bonetti, Lerman, & Lerman, 2003; Melo et al., 2004). It is evident that Akt1 is highly involved in the vascular endothelial growth factor (VEGF)-mediated vascular permeability as the phosphorylation of Akt1 increases considerably in VEGF-stimulated endothelial cells (Chen et al., 2005). However, since Akt1 is activated by not only vascular permeability-inducing agents such as VEGF and tumor necrosis factor-α (Fairaq, Goc, Artham, Sabbineni, & Somanath, 2015; Gao et al., 2016) but also by agents that promote barrier integrity such as angiopoietin-1 (Ang-1), roundabout guidance receptor-4, and sphingosine-1-phosphate, the precise role of Akt1 in regulating vascular permeability was not clear until recently (Daly et al., 2004; De Palma, Meacci, Perrotta, Bruni, & Clementi, 2006; Somanath, Kandel, Hay, & Byzova, 2007). Studies from our laboratory have demonstrated that Akt1 is important for vascular maturation and that suppression of Akt1

Fei Gao and Harika Sabbineni contributed equally to this work.

Chapter 1

METHODS TO STUDY CELLULAR PLASTICITY *IN VITRO* AND PULMONARY VASCULAR REMODELING *IN VIVO* IN PULMONARY ARTERIAL HYPERTENSION

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ABSTRACT

Pulmonary arterial hypertension (PAH) is a deadly disease with no reliable treatment options available. Uncertainties in the molecular mechanisms and incomplete knowledge on the sequence of events leading to pulmonary arterial hypertension are the major gridlocks in the development of therapeutics for this fatal disease. Research on pulmonary arterial hypertension is suffers the lack of proper cellular and animal models. Aim of this chapter is to provide easy to follow protocols supported with clear descriptions of the working principle for each experiment along with a list of all the materials necessary for the successful execution of the experiments. Whereas experimental methods *in vitro* will deal with protocols involved in characterizing the molecular mechanisms leading to fibroblast, endothelial and vascular smooth muscle differentiation into pathological mesenchymal cells. This chapter will also discuss the animal models and drugs currently being used in various laboratories to study mechanisms leading to pulmonary arterial hypertension in rodent and larger animal models.

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Chapter 2

MECHANISMS OF VASCULAR REMODELING IN PULMONARY ARTERIAL HYPERTENSION

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ABSTRACT

Pulmonary arterial hypertension (PAH) is a progressive, fatal syndrome characterized by increased pulmonary vascular resistance that leads to right-ventricular hypertrophy and heart failure, eventually leading to premature death. The prevalence rate of PAH is approximately 25 cases/million/year and the median life expectancy from the time of diagnosis in patients with idiopathic PAH when not treated is 2.8 years. There are no definitive symptoms to identify and confirm PAH until the disease progresses to vascular remodeling and heart failure, and is often associated with poor prognosis. Pulmonary arterial hypertension is characterized by progressive pulmonary vasoconstriction, lumen obliteration of arteries and arterioles, and formation of plexiform lesions. PAH, which leads to pulmonary vascular resistance and right ventricular failure resulting in premature death, is associated with poor prognosis and there is no cure except for treating the symptoms. Vascular remodeling during PAH is the major contributing factor for the advanced stages of the disease. Pathological growth of myofibroblasts from resident fibroblasts and vascular smooth muscle cells, as well as due to endothelial-to-mesenchymal transition (EndMT) in response to transforming growth factor-β (TGFβ) have all been investigated to characterize the underlying reasons for PAH. Aim of this chapter is to describe the molecular mechanisms and cellular events regulating vascular remodeling in PAH.

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