ROLE OF AKT IN MYOFIBROBLAST DIFFERENTIATION LEADING TO PULMONARY VASCULAR REMODELING AND IDIOPATHIC PULMONARY FIBROSIS

by

MAHA MAHMOUD ABDALLA

(Under the Direction of Somanath P.R. Shenoy)

ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is an incurable, chronic and progressive lung disease with severely poor prognosis. The presence of pulmonary hypertension (PH) secondary to IPF is an independent risk factor for increased mortality. Alarmingly, there are no effective pharmacologic treatments for IPF, and lung transplantation remains the only effective option. Therefore, identification of novel therapeutic targets is critical in the hopes of devising new treatments. Persistent myofibroblasts (MFs) differentiation and excess extracellular matrix (ECM) accumulation are hallmark features of IPF. Recently, it has been shown that Akt is upregulated in IPF patients; however whether Akt is necessary for MFs remains unclear. This dissertation sought to 1) identify the role of Akt in MF differentiation leading to IPF, and 2) investigate the safety and efficacy of triciribine (TCBN), a selective Akt inhibitor, as a potential therapeutic option for IPF. The work presented in this thesis has been conducted using a combined approach of pharmacological, genetics, and functional assays. We first identified that Akt is a critical determinant of pathological MF differentiation and ECM expression as evident by loss- and gain- of function studies, both *in vitro* and *in vivo*. Mechanistically, we found that

Akt1 transcriptionally regulates αSMA synthesis, independent of mTOR. Furthermore, TCBN reversed TGFβ (pro-fibrotic cytokine) - and hypoxia-induced pulmonary fibrosis and vascular remodeling, compared to placebo and rapamycin. In contrast, rapamycin, which targets mTOR downstream of Akt, exacerbated fibrosis and vascular remodeling as evident by worsening interstitial fibrosis, micro-hemorrhage, and increased peripheral vascular rarefaction. Collectively, research efforts associated with this thesis 1) identified Akt as a novel target as it is crucial for MFs - central orchestrators of IPF; 2) determined novel anti-fibrotic and anti-remodeling properties of Triciribine; and 3) determined that rapamycin activates Akt via feedback mechanisms, which sheds light on the observed detrimental effects of everolimus, a rapamycin derivative, in IPF patients. In conclusion, our study is the first to determine the crucial role of Akt1 in IPF and associated vascular remodeling. Thus, selective Akt inhibition, not mTOR, may serve as a favorable therapeutic strategy in IPF.

INDEX WORDS: Idiopathic pulmonary fibrosis, vascular remodeling, myofibroblast, extracellular matrix, Akt, mTOR, triciribine, rapamycin, chronic hypoxia, TGFβ

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DEDICATION

I dedicate this thesis to the Almighty God, my father, Mahmoud Abdalla, my mother, Magda Hussein, and my brothers, Mohamed and Mostafa, for being my pillars of support, my source of refuge, and my guiding light.

My parents have always been my foremost role models. They have instilled in us that, no matter the obstacles and challenges, persistence is the key to achieve your dreams and aspirations. They lead by example. Not succumbing to “what’s meant to be,” at the age of 6, my father was a young entrepreneur. In his early twenties, he traveled abroad and combined his business knowledge with his background as an agriculture engineer to design innovative farming techniques, which catapulted him to be a consultant.

Determined to break cultural norm at a time where girls did not continue their education and marry at a young age, my mother fought through social and cultural challenges. Rain or shine, she was determined to attend every class. Ultimately, she went to college and became a lawyer. They have taught us that so long as you tried, took risks, embraced your failures and turned them into success, and did not settle for “what’s meant to be,” you will reach your dreams and live life with no regrets.

They have sacrificed so much to secure our future. They’ve taught us the meaning of family, discipline, independence, integrity, and ingenuity. They push us to look past any challenges that we may face as there is always a solution. “You just have to use your brain, and keep at it!” In essence, this is not just my Ph.D., but my family’s Ph.D. “If you can imagine it, you can dream it. Dream it and you can become it.”

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LIST OF ABBREVIATIONS

4E-BP1	4E binding protein 1
adTGFβ	Adenovirus transforming growth factor-β
αSMA	Alpha-smooth muscle cell actin
bFGF	Basic fibroblast growth factor
CFA	Cryptogenic fibrosing alveolitis
CMC	Carboxymethylcellulose
CTGF	Connective tissue growth factor
DMEM	Dulbecco's modified eagle's medium
DNA-PK	DNA-dependent protein kinase
ECM	Extracellular matrix
ED-A FN	Fibronectin
EMT	Epithelial to mesenchymal transition
FBS	Fetal bovine serum
FDA	Food and drug administration
FHLFs	Fibrotic human lung fibroblasts
FOXO	Forkhead family of transcription factors
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSK3	Glycogen synthase kinase 3
HLFs	Human lung fibroblasts
IIP	Idiopathic interstitial pneumonia
ILK1	Integrin-linked kinase 1
IPF	Idiopathic pulmonary fibrosis
MF	Myofibroblast
MAPK	Mitogen activated protein kinase
MEFs	Mouse embryonic fibroblasts
MPAP	Mean pulmonary artery pressure
mTOR	Mammalian target of rapamycin

NIH 3T3	Mouse embryonic fibroblasts cell line
p70S6K1	p70 ribosomal protein S6 kinase 1
PAH	Pulmonary arterial hypertension
PDK1	Phosphoinositide-dependent kinase 1
PH	Pleckstrin homology
PI3K	Phosphatidylinositol-3 kinase
PIP2	Phosphatidylinositol-4,5-biphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKB (Akt)	Protein kinase B
PP2A	Protein phosphatase 2A
PRAS40	Proline-rich Akt substrate of 40 kDa
Rapa	Rapamycin
Ras	Rat sarcoma virus
RBD	Ras binding domain
Src	Steroid receptor co-activator
SRF	Serum response factor
TCBN	Triciribine
TGFβ	Transforming growth factor-β
TSC	Tuberous sclerosis protein
TSP1	Thrombospondin-1
UIP	Usual interstitial pneumonia
VSMCs	Vascular smooth muscle cell

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

IDIOPATHIC PULMONARY FIBROSIS

Idiopathic pulmonary fibrosis (IPF) is a progressive condition with severely poor prognosis. It is by definition a disease of unknown causes. It is categorized as a subgroup of idiopathic interstitial pneumonias (IIPs), and represents the most common and lethal form compared to other lung diseases [1, 2]. It is distinguished from other diseases by distinct histological hallmark feature of fibroblastic foci formation. Fibroblastic foci are characterized by aberrant fibroblast accumulation and activation into myofibroblasts, and excessive matrix deposition and remodeling leading to aberrant hypertrophic scarring of the lung tissue. The consequent loss of alveolar function and destruction of normal lung architecture leads to respiratory failure [1, 3-5].

There is no gold standard diagnostic test for IPF, where it remains a diagnosis of exclusion. Lung biopsy is one of the best diagnostic methods revealing a pattern that has been termed “usual interstitial pneumonia” (UIP). This was first recognized by Dr. Averil Liebow and colleagues of Yale University [6]. Furthermore, the pathogenesis of IPF remains elusive resulting in a lack of effective pharmacological options and treatments that have been shown to be either ineffective or worsen outcome in some patients. Lung transplant is the only effective approach and is limited to a select number of patients. Despite extensive research, the major reason for difficulties in identifying effective therapeutics is due to evolving concepts in IPF pathogenesis [1, 3, 4, 6-8].

Historical Perspective

In 1866, Dr. Austin Flint reported a condition termed “chronic pneumonitis” observed in one patient and described the rapid deterioration in health, the bulbous form of the fingertips, and noted that this condition terminated fatally [9]. This was probably the first recorded case of IPF, also formerly known as *cryptogenic fibrosing alveolitis* (CFA).

It was not until 1944 that the topic of chronic interstitial pneumonia and the pathogenesis of IPF were revisited after case reports of four patients with IPF, also coined the Hamman-Rich Syndrome. These descriptions were published by Drs. Louis Hamman and Arnold Rich who described the clinical, radiographic, and pathologic features of four patients who succumbed to pulmonary insufficiency between 1931 and 1943 at Johns Hopkins Hospital [10, 11].

They described that the duration of the disease ranged from one to six months and described that at autopsy the sections showed “diffuse thickening of the alveolar walls, and there were also small scars which had obliterated the architecture entirely.” They also noted pathogenic mediators and implicated the presence of “wandering cells” including fibroblasts and presence of collagen fibers. For the next, approximately, five decades, most clinicians associated this syndrome with IPF. However, the fundamental reason there has been a lot of controversies surrounding this syndrome and diagnosis of IPF is the duration of this disease (the first three cases were seen in the 1930s exhibited symptoms for fairly short duration of one to two months, the fourth case was described 10 years later and the patient complained of symptoms of four months duration)[10, 11]. Furthermore, many have faced challenges in trying to understand,

diagnose, and correlate different histopathologic features and symptoms to this syndrome. It was not until the mid to late 1960s, also known as “modern era of interstitial lung disease,” that interstitial pneumonia was classified into five categories based on histopathologic features by Liebow and Carrington which included usual, bronchiolitis, desquamative, lymphoid, and giant cell interstitial pneumonias. During this era, Dr. Scadding coined the term “alveolitis” and along with Liebow and colleagues became the first to correlate the histologic pattern of honeycomb with poor prognosis in UIP. Finally, after 46 years, the Hamman-Rich Syndrome is now reclassified as an acute interstitial pneumonia rather than the previous misclassification of UIP or IPF. Then, in 2002 the American Thoracic Society, reclassified interstitial pneumonia and added a new category IIP and categorized IPF as a subgroup [1, 6, 12]

The most influential period that catapulted the pursuit to understand the molecular and overall pathogenesis of IPF was generated in 1970s and 80s. Dr. Ron Crystal and colleagues proposed the “inflammation theory” where IPF began as alveolitis and progressed to interstitial fibrosis based on the results from samples collected from patients with interstitial lung disease at the Pulmonary Branch of the National Heart, Lung, and Blood Institute. They strongly recommended that therapeutic interventions should be targeted at the inflammatory phase rather than the fibrotic one. They further confirmed that by discussing the success of corticosteroid in patients under the study of Dr. Carrington and colleagues [6, 12].

However, this study is limited by the fact that it was not a randomized, placebo-controlled study, and patients enrolled had early stage IPF. Furthermore, patients with advanced IPF and/or UIP failed to respond to corticosteroid therapy. As for the

hypothesis, it has since fallen out of favor for multiple reasons. Mainly, patients with early stage IPF do not exhibit evidence of significant interstitial or alveolar inflammation. Furthermore, Fleischman and colleagues (1971) and Adamson and Bowden (1984) were the first to show that PF exhibits spatial heterogeneity and varying degrees of inflammation. More importantly, Adamson and Bowden suggested that inflammation and lung fibrosis are not invariably linked and demonstrated an association between fibrosis and enhanced synthesis of extracellular matrix (ECM) proteins, collagen and elastin [13, 14]. Later, Munger and colleagues confirmed that fibrosis and inflammation are two independent events [15]. Moreover, the presence of fibrotic zones, termed fibroblastic foci which is composed of excess myofibroblast accumulation and ECM deposition, has been identified as a major histo-pathological hallmark and a chief diagnostic criterion of IPF and correlates with poor prognosis [16, 17]. Collectively, there has been a shift in our understanding of IPF pathogenesis and that abnormal wound healing and fibrosis constitute primary events in addition to chronic inflammation [3, 8].

Epidemiology

IPF has the worst mortality and represents the most common and lethal form of interstitial lung diseases. It is associated with high mortality of 50% within 3 to 5 years after diagnosis. In cases of acute exacerbations, ineffective therapeutics, or IPF complicated with pulmonary arterial hypertension, the mortality rate has been reported at 80% after diagnosis. Furthermore, it has worse survival rate compared to other cancers including breast, prostate, and colon cancers, with median survival rate of 2 to 3 years [2, 5, 18].

Due to the lack of uniform definition and classification of IPF over the years, defining an exact prevalence and incidence rate has been a challenge. Estimated to affect 5 million people worldwide, it is prevalent in approximately 80,000 people in the United States (U.S). The incidence was estimated to be 10.7 cases per 100,000 in men and 7.4 cases per 100,000 in women in the U.S. Another study estimated the incidence to be between 6.8 and 16.3 per 100,000 people in U.S. A study from the United Kingdom estimated the incidence between 4.6 and 7.4 per 100,000 and estimated an annual increase of 11% between 1991 and 2003; it was not attributed to aging [1]. Those at risk include smokers, those exposed to environmental and occupational factors such as asbestos, and those with infections. IPF is more common in men where it is attributed with rapid progression and worse survival than women. IPF is also common in patients between the ages of 40 to 70 years. Mortality is greater among Caucasians than African American or Hispanics, and the risk of death increases with age [1, 5].

Diagnosis

Since the definition of IPF has been refined, the diagnostic criteria have changed several times in the past decade. However, there is no gold standard diagnostic method; it remains an exclusion diagnosis. Even more challenging, the clinical manifestations in IPF are general and can often be misdiagnosed for other pulmonary and heart conditions. IPF patients often present with progressive dyspnea, dry-hacking cough, fatigue and malaise, chest pain, cyanosis, and digital clubbing [1].

The diagnosis of IPF requires 1) the exclusion of other causes of pulmonary disease based on chest X-ray and patient history including medical, social, and occupational history, (2) the presence of UIP pattern, specifically, honeycombing changes (which is a cyst-like space surrounded by fibrotic tissue predominately in lower lobes) which can be detected in high-resolution computed tomography (HRCT), and (3) the presence of fibroblasts foci (areas of highly myofibroblasts accumulation with positive alpha smooth muscle like actin (αSMA) staining which is detected in surgical lung biopsy [1, 8, 19]. This histological characteristic has led to the new era of myofibroblasts and abnormal wound healing as a driving force in IPF [20, 21].

COMPLICATION: PULMONARY ARTERIAL HYPERTENSION

The presence of pulmonary arterial hypertension (PH) secondary to IPF is an independent risk factor for increased mortality [1]. It is prevalent in 32% to 85% of IPF patients. PH is a rapidly progressive, incurable and fatal disease that is characterized by progressive vascular remodeling and proliferation leading to severe vascular occlusion resulting in right ventricular-heart failure. PH is defined as mean pulmonary arterial pressure (MPAP) of $\geq$ 25 mm HG and is associated with marked loss of peripheral pulmonary arteries on pulmonary angiogram. It is associated with poor prognosis that is usually fatal within 3 years post diagnosis. Despite the improvements in its management, mortality rate remains high; 5-year mortality rate of 50%, that is worsened secondary to IPF [22, 23]. The diagnosis of PH in IPF patients poses a great challenge as there is no gold standard to diagnose it and clinical manifestations usually resemble those of other pulmonary and heart diseases. However, the most reliable diagnosis is right heart catheterization. Although there have been major strides in understanding its

pathogenesis and symptomatic therapies, there is still a need for an effective therapy to either prevent or reverse PH progression [23, 24].

The pathogenesis of PH is complex and multifactorial and remains largely undefined. A hallmark of PH is plexiform lesions (PLs) characterized by pulmonary vascular remodeling with features of medial hypertrophy, intimal fibrosis, adventitial proliferation of SMCs and SMC-like cells (myofibroblasts) and thickening leading to marked occlusion of pulmonary arteries (PAs) [25, 26]. Histological features show marked vascular remodeling as evident by peripulmonary artery adventitial thickening and muscularization and hypertrophy of smooth muscle cells in distal pulmonary arterioles. A potent contributor to vascular remodeling is the persistent differentiation of myofibroblasts and accumulation in all three layers (intima, media, and adventitia) [26-29]. Furthermore, vascular regression usually occurs in areas of dense fibrosis in IPF lung [24, 30]. Similar to IPF, there is a lack of curative pharmacologic options [31]

IPF TREATMENT

Currently, there is a lack of FDA approved pharmacologic treatments for IPF with proven beneficial effects on survival [1, 5]. Conventional therapies such as corticosteroids were the first line for more than four decades, but are of unproven efficacy and are associated with significant toxicities and worsened outcomes in most IPF patients [32-34]. Other therapies including azathioprine [35, 36] and cyclophosphamide [37] provide only marginal and temporary benefit or no benefit in most patient. Notably, conventional therapeutics (anti-inflammatories) were based on studies that had major limitations such as lack of randomized placebo-controls,

variations in diagnoses and classifications, and variations in disease severity. Thus, the nature of the disease being investigated was critiqued. Recent trials with novel drugs such as etanercept, imatinib, interferon-gamma, and acetylcysteine have shown no significant effect on disease progression or survival and in some studies, have even worsened outcome especially when combined with prednisone [5]. Noteworthy, clinical trials investigating the safety and efficacy of pirfenidone, an anti-fibrotic, anti-inflammatory, and anti-oxidant agent that targets TGFβ, have demonstrated promising results leading to its approval in Japan, Europe, China, and Mexico and is pending approval in the United States [139-142]. Recently, a Phase 3 randomized placebo-controlled trial involving 555 patients with IPF demonstrated the efficacy of pirfenidone as evident by reduced disease progression, improved lung function, exercise tolerance and progression free survival [139]. Furthermore, nintedanib, a multi-target tyrosine kinase that modulates vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) signaling, has also shown beneficial effects in IPF [143-144]. In INPULSIS trials (phase 3, randomized, double-blind, placebo controlled trials involving 1066 patients), nintedanib treated IPF patients showed significantly improved forced vital capacity over the 52-week trial period [144].

Lung transplantation remains the only non-medicinal therapy with proven effect on survival in patients with IPF refractory to medical therapy, severe functional impairment, or oxygen dependency [1]. Lung transplantation reduced the risk of death by 75% and improved quality of life. The 5-year survival rate is between 40% and 60% [38, 39]. Unfortunately, there are major limitations such as high mortality rate on the waiting list. Waiting on transplant list may take up to 3 years due to shortage of donor

organs and many IPF patients succumb to the disease while awaiting transplantation [40].

PATHOGENESIS

Despite extensive research and improvement in our knowledge, IPF and PH pathogenesis remains elusive, controversial, and failed to provide a curative therapeutics to this deadly diseases since it was first reported in 1860s [7, 28].

One of the fundamental hypotheses in IPF pathogenesis is the deregulated response to lung injury in which the alveolar epithelium undergoes pathological deregulation by myofibroblasts, ultimately leading to fibrosis [8, 21]. Physiological reconstruction of connective tissue following lung injury is composed of distinct phases (1) activation of coagulation pathways and fibrin and ECM deposition, (2) inflammatory cell migration to the site of injury and release cytokines and chemokines (TGFβ and CTGF among others), (3) recruits fibroblasts migration and activation into myofibroblasts to secrete ECM, (4) resolution by termination of stress response signal which, although unclear, occurs in part by massive apoptosis of myofibroblasts that occurs after physiological tissue repair and re-epithelialization [41].

On the other hand, in IPF, persistent myofibroblast differentiation and hypertrophic scar tissue formation is found under a layer of type II alveolar epithelial cells (AECs) leading to loss of lung elasticity and severely impaired gas exchange, ultimately severe decline in lung function. In IPF, repetitive alveolar wall injury results in abnormal re-epithelialization, continuous AEC apoptosis releases chemotactic factors and mitogens that contribute to persistent fibroblast migration and proliferation,

myofibroblast differentiation, and excessive ECM deposition leading to abnormal alveolar remodeling and scarring. In advanced stage IPF, the alveolar structure is completely replaced with proliferative fibroblast, myofibroblasts, and ECM components such as collagen and fibronectin. This ultimately leads to destruction of lung architecture and terminal dysfunction [4, 41-43].

MYOFIBROBLASTS AND THEIR ROLE IN IPF AND PH

Myofibroblasts (MFs) are the central orchestrators of this disease and its complication. MFs are instrumental in the deposition of excessive extracellular matrix, leading to hypertrophic scar tissue formation [21, 42-44]. Furthermore, they are potent contributors to the vascular remodeling that occurs in the adventitial, medial, and intimal layers of pulmonary arteries [45]. The source of MFs in lung is still debatable, but mounting evidence has demonstrated the heterogeneous origin of MFs. They can originate from resident fibroblasts, fibrocytes, VSMCs, endothelial cells, pericytes, and mesenchymal stem cells. Mounting evidence has shown that, in IPF and PH, transforming growth factor-β (TGFβ) expression is significantly up-regulated in the microenvironment and plays a major role in triggering fibrogenesis by stimulating fibroblasts to differentiate into MFs, which are more contractile and have more profibrotic potential than their originators [15, 28, 46] . MFs differentiate by altering their morphology through the formation of alpha-smooth muscle like actin (αSMA) positive stress fibers; this has also been shown in hypoxia animal models [47]. Thus, MFs exhibit a contractile VSMC-like phenotype, excess ECM protein production (collagen type I, III, IV, V, and VI, fibronectin, specifically, fibronectin splice variant ED-A), and resistance to apoptosis. These constitute key components of a vicious cycle where

ECM secretion infers MF differentiation, in part, via sheer mechanical stress or activation of latent TGFβ found in assembled fibronectin. MFs promote their continuous differentiation and excessive ECM deposition, resistance to apoptosis, in part, by upregulating survivin and PI3K/Akt pathway and lacking the fas ligand thus evading the immune response. Furthermore, MFs induce apoptosis, deregulated proliferation and ineffective migration of AECs. Collectively, this leads to the rampant formation of MFs, replacing the alveolar interstitium, which leads to impaired gas exchange and ultimately fibrosis [20, 21, 42, 44, 45].

Currently, the focus has been geared toward understanding molecular mechanisms governing αSMA synthesis in order to therapeutically modulate MF differentiation. Numerous studies have extensively characterized Rho-dependent αSMA synthesis in VSMCs and myofibroblasts, and demonstrated that Rho regulates the transcriptional activation of SRF encoding αSMA synthesis [5, 28, 48] [49]. However, the role of Akt has yet to be established in MFs.

THE ROLE OF TGFβ1 SIGNALING IN PHYSIOLOGY AND DISEASE

Transforming growth factor β1 (TGFβ1) is a key mediator in wound repair and IPF pathogenesis. TGFβ1 is a pleiotropic cytokine and a member of the TGFβ superfamily, which is a highly conserved group of cytokines, and is ubiquitously expressed by all cells and tissues. It is essential in activating a myriad of signaling cascades that modulate diverse cellular processes in embryonic development and adult tissue homeostasis (reviewed in [50]). During wound healing, TGFβ promotes epithelial apoptosis [51], epithelial-to-mesenchymal transition (EMT) [52], migration [53], ECM

production [54], fibroblast to myofibroblast differentiation [21, 55]. In IPF, TGFβ is markedly upregulated in IPF patients and murine models [56-58]. Overexpression of TGFβ induced severe persistent fibrosis [59, 60], while blocking its signaling ameliorates pulmonary fibrosis in murine models ([61, 62]. Modulating TGFβ signaling is currently under evaluation in clinical trials. Although a phase I study of GC1008, an antibody against TGFβ, has been completed in IPF patients, the results are not yet available (Clinicaltrials.gov identifier NCT00125385). A second trial, currently in recruiting phase, aims to evaluate the effects of a humanized monoclonal antibody against αvβ6 integrin, a key activator of TGFβ, in IPF patients (clinicaltrials.gov identifier NCT01371305).

THE ROLE OF PI3K/AKT PATHWAY IN PHYSIOLOGY AND DISEASE

PI3K

Phosphatidylinositol 3 kinases (PI3Ks) are a family of intracellular lipid kinases divided into three classes (I-III) according to their structure and substrate specificity. The prototypical class I PI3K is a dimeric enzyme that consists of catalytic and regulatory subunits. Class IA is mainly comprised of p110α, p110β and p110δ catalytic subunit that forms a heterodimer with p85 regulatory subunit [63, 64], PI 3-kinase is a dual specificity enzyme: autoregulation by an intrinsic protein-serine kinase activity}. Class IB consists of p110γ catalytic subunit and regulatory subunits p84 and p101. It has been shown that, in addition to the ubiquitously expressed p110α and p110β, p110δ and p110γ are functionally expressed in human lung fibroblasts and play a role in sustaining TGFβ-induced proliferation and differentiation [65], Inhibition of PI3K prevents the proliferation

and differentiation of human lung fibroblasts into myofibroblasts: the role of class I P110 isoforms}. PI3Ks can be activated by extracellular stimuli by hormones or cytokines such as TGFβ, and transmit signals from G-protein-coupled receptors as well as receptor tyrosine kinases. Intracellular proteins such as Ras and Rho family small GTPases can also activate PI3K pathway. Ras has been shown to directly activate the lipid kinase activity of p110α [66, 67], p110γ [68, 69], and p110δ [70] by mechanism including binding to N-terminal Ras binding domain (RBD) and modulating the regulatory p101 subunit. Rac and CDC42, members of The Rho family GTPases, have been shown to directly activate lipid kinase activity of p110β via its RBD [71].

Akt activation

Since its discovery as an oncogene of transforming murine leukemia virus AKT8 [72-74], Akt has emerged as one of the most important and versatile protein kinases at the core of physiological processes and disease pathogenesis. Thus, it is the most studied signaling molecule downstream of PI3K due to its regulatory role on a plethora of cellular functions. Activated PI3Ks catalyze the phosphorylation of their substrate PIP2 (phosphatidylinositol-4,5-biphosphate) into its triple-phosphorylated form PIP3 (phosphatidylinositol-3,4,5-triphosphate). PIP3, an important second messenger molecule, recruits proteins containing pleckstrin homology domains to the plasma membrane. Thus, PIP3 serves as a docking site for 3-phosphoinositide-dependent kinase 1 (PDK1) and Akt, where the former phosphorylates Akt at Thr308 leading to partial activation of Akt [75, 76].

Partial activation of Akt is sufficient to activate mTORC1 via direct phosphorylation and inactivation of tuberous sclerosis protein 2 (TSC2), and proline-rich Akt substrate of 40 kDa (PRAS40). TSC2 modulates protein translation and cell growth by negatively regulating mTORC1 when it forms a physical and functional complex with TSC1 [77-79]. TSC1-TSC2 complex inhibits p70 ribosomal protein S6 kinase 1 (p70S6K1) and activates eukaryotic initiation factor 4E binding protein 1 (4E-BP1) [80]; both are substrates of mTORC1. Phosphorylation and subsequent inactivation of TSC2 by Akt destabilizes its interaction with TSC1, thereby activating mTORC1 pathway. Similarly, PRAS40, which is a component and a substrate of mTORC1, is a negative regulator of mTORC1 activity [81-83]. Phosphorylation of PRAS40 by Akt or mTORC1 leads to its dissociation from raptor, thereby removing the inhibitory constraint on mTORC1 activity [84].

Full activation of Akt requires phosphorylation at Ser473 via autophosphorylation [85], mTORC2 [86], DNA-dependent protein kinase (DNA-PK) [87, 88], integrin-linked kinase 1 (ILK1) [89], or mitogen-activated protein kinase-activated protein kinase 2 [90]. Once active, Akt phosphorylates and modulates a complex network of substrates such as GSK-3, mTOR, TSC, FOXO as well as cross-talk with other signaling pathways such as p38 MAPK and Rac –PAK [91-97]. Subsequently, Akt regulates a plethora of cellular processes including survival, cell cycle progression, cell growth, proliferation, and migration, metabolism, and angiogenesis (**Figure 1**) [86, 98-102].

Regulating Akt activation is a complex and finely tuned process. Several groups have shown that inhibiting protein phosphatase 2A (PP2A) resulted in increased Akt phosphorylation and activation (Sato 2000; Ugi 2004; Liao 2004; Trotman 2006;

Vereshchagina 2008; Padmanabhan 2009). PP2A directly represses Akt signaling by maintaining Akt in an inactive state through preferential dephosphorylation at Thr308 and Ser473 [103-107]. Newton's group identified that Akt is a direct substrate of PH domain and leucine rich repeat protein phosphatases (PHLPP) family [108, 109]. In addition to identifying that PHLPP regulates Akt on Serine residue not Thr308, they have also identified that PHLPP1 and PHLPP2 differently regulate the phosphorylation state of specific Akt isoforms. While both can modulate Akt3, PHLPP1 regulates Akt2, and PHLPP2 regulates Akt1 [109].The lipid protein phosphatase and tensin homologue on chromosome ten (PTEN) is a potent regulator of Akt activation. PTEN indirectly inactivates Akt by dephosphorylating PIP3 into PIP2, thereby switching off PI3K/PIP3 mediated phosphorylation and activation of Akt [106, 110, 111].

Akt isoforms

Akt, also known as protein kinase B (PKB), is a multi-functional protein kinase that consists of three mammalian isoforms Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ) encoded for by three different genes AKT1, AKT2, and AKT3. Their structure is highly conserved and shares three functional domains: an amino terminal pleckstrin homology (PH) domain, a kinase domain, and a carboxy terminal regulatory domain [98].

The diversity of Akt signaling may in part be due to the isoform-specific functions. Genetic ablation of Akt1 in mice results in increased perinatal mortality, decreased body weight by 20 to 30%, growth retardation, and decreased organ size [112-114]. We have previously shown that Akt1 deficient mice exhibit vascular fragility and ECM abnormalities in the skin as well as impaired ECM remodeling [115]. Akt2 deficient mice

exhibit insulin resistance and type II diabetes like syndrome, whereas Akt1 deficient mice maintain normal glucose homeostasis [112, 116]. Conversely, Akt3 has been shown to play a critical role in postnatal brain development. Akt3 deficient mice exhibit a reduction in brain size and weight, which is partially due to decreased cell size and number, but maintain body weight and glucose homeostasis [117, 118]. This suggests all three isoforms have differential and non-redundant functions.

Interestingly, combined ablation of Akt isoforms is detrimental as evident by the embryonic lethality in Akt1/Akt3 null mice [119]. Similarly, Akt1/Akt2 ablation results in neonatal mortality, dwarfism, defects in skin, bone and skeletal muscle development, and impaired adipogenesis [120]. This suggests functional redundancy and overlap between isoforms. Interestingly, maintaining Akt1 despite combined genetic ablation of Akt2 and Akt3 (either in $Akt2^{-/-}$ /$Akt3^{-/-}$ null mice or in $Akt1^{+/-}$ $Akt2^{-/-}$ $Akt3^{-/-}$ mice) did not result in mortality or organ dysfunction [121]. This suggests that Akt1 plays a more dominant role in embryonic development and survival.

PI3K/Akt pathway in disease

The PI3K/Akt pathway is deregulated in fibrotic diseases including IPF. Akt is upregulated in fibroblastic foci of IPF lung tissue [122] and in IPF fibroblasts [123]. Increased Akt activation correlated with increased TGFβ signaling [124, 125] and deregulated regulatory pathways such as decreased PTEN function. After wound healing, PTEN activity is maintained to suppress PI3K/Akt signaling which is an effective physiologic mechanism to restrain fibroblast proliferation after injury thereby avoiding hypertrophic scar formation [126]. However, pathologic fibroblast proliferation,

differentiation, resistance to apoptosis, and increased matrix deposition correlated with Akt activation and aberrant PTEN activity [127-136]. Moreover, inhibiting PI3K or Akt activation abolished bleomycin-induced fibroblast proliferation and ECM production [137]. Interestingly, Conte and colleagues demonstrated that PI3K p110γ is upregulated in fibroblastic foci and in fibroblasts of IPF patients [138], and that PI3K plays a major role in sustaining TGFβ-mediated increase in cell proliferation and differentiation, and ECM production, independent of Akt. Compared to Akt inhibition, silencing PI3K p110α/p110γ gene expressions markedly reduced TGFβ-induced proliferation, differentiation, and collagen production without affecting Akt Ser473 phosphorylation state [65]. Thus, whether Akt plays a contributory or a causal role in mediating fibroblast activation and differentiation and IPF progression requires further examination.

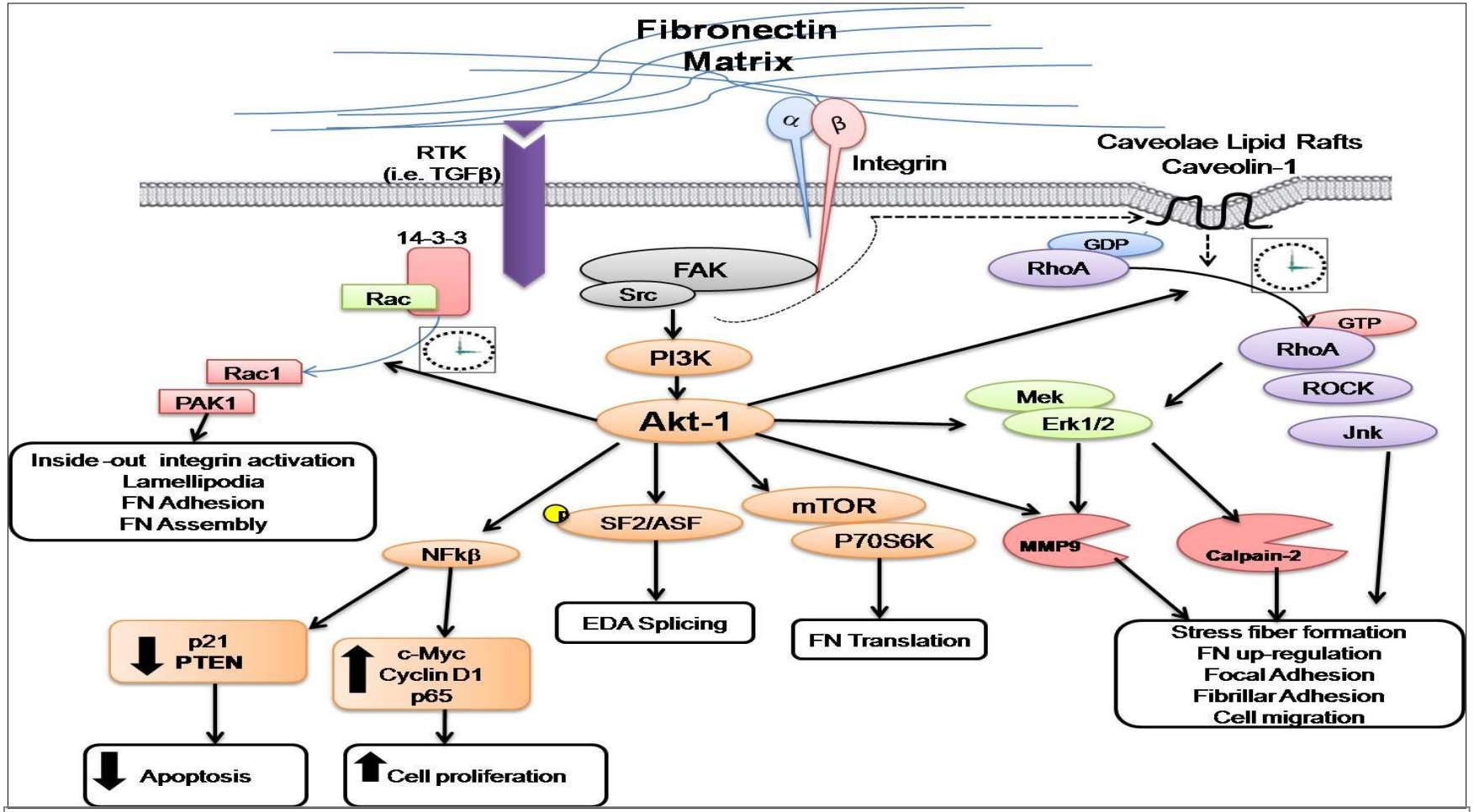

Figure 1. Akt1 mediated pathways. Adapted from Abdalla M et al. Pathways Regulating the Secretion and Assembly of Fibronectin Matrix: an Update. Book Chapter 7. *NOVA (*Nova Science Publishers); 2012; pp. 155-190.

Hypothesis and Aims of the Thesis

The overall aims of this thesis are to 1) identify the role of Akt in MF differentiation leading to IPF and secondary PH, and 2) investigate the efficacy of Triciribine, a selective Akt inhibitor, as a potential therapeutic option for IPF and PH.

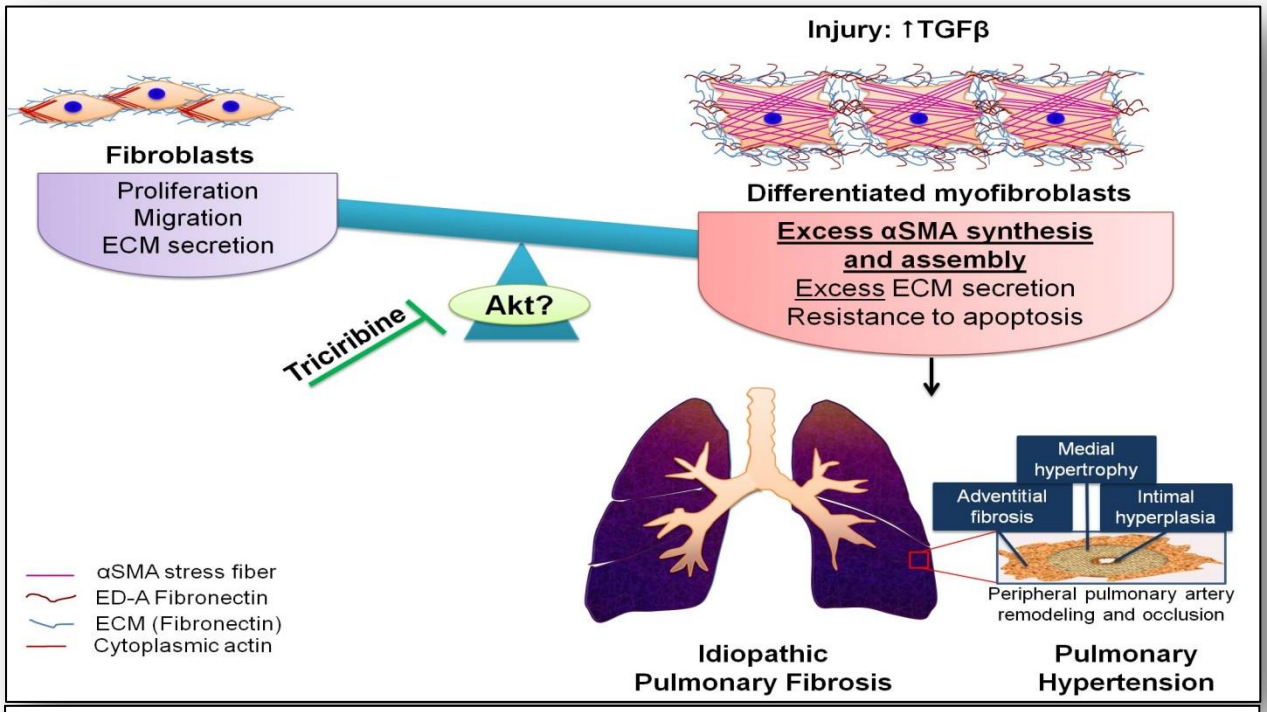

Figure 2. A schematic representation of our hypothesis

To test our hypothesis, we proposed three specific aims:

1. Test the hypothesis that Akt mediates αSMA expression in MF differentiation through transcription factor SRF *in vitro*
2. Test the hypothesis that Akt is integral for hypoxia-induced pulmonary remodeling *in vivo*
3. Test the hypothesis that Triciribine inhibits the progression of IPF in response to chronic hypoxia and adenovirus-TGFβ *in vivo*

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CHAPTER 2

AKT1 MEDIATES ALPHA-SMOOTH MUSCLE ACTIN EXPRESSION AND MYOFIBROBLAST DIFFERENTIATION VIA MYOCARDIN AND SERUM RESPONSE FACTOR

Abdalla M, Goc A, Segar L, Somanath P.R. Akt1 Mediates alpha-Smooth Muscle Actin Expression and Myofibroblast Differentiation via Myocardin and Serum Response Factor. J Bio. Chem. 2013; 288(46):33483-93

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ABSTRACT

Myofibroblast (MF) differentiation, marked by the de novo expression of smooth muscle α-actin (αSMA) stress fibers, plays a central role in wound healing and its persistence is a hallmark of fibrotic diseases. We have previously shown that Akt1 is necessary for wound healing through matrix regulation. However, the role of Akt1 in regulating MF differentiation with implications in fibrosis remains poorly defined. Here, we show that sustained activation of Akt1 was associated with a 6-fold increase in αSMA expression and assembly; an effect that is blunted in cells expressing inactive Akt1 despite TGFβ stimulation. Mechanistically, Akt1 mediated TGFβ-induced αSMA synthesis through the contractile gene transcription factors myocardin and serum response factor (SRF), independent of mammalian target of rapamycin in mouse embryonic fibroblasts and fibroblasts overexpressing active Akt1. Akt1 deficiency was associated with decreased myocardin, SRF, and αSMA expressions in vivo. Furthermore, sustained Akt1-induced αSMA synthesis markedly decreased upon RNA silencing of SRF and myocardin. In addition to its integral role in αSMA synthesis, we also show that Akt1 mediates fibronectin splice variant expression, which is required for MF differentiation, as well as total fibronectin, which generates the contractile force that promotes MF differentiation. In summary, our results constitute evidence that sustained Akt1 activation is crucial for TGFβ-induced MF formation and persistent differentiation. These findings highlight Akt1 as a novel potential therapeutic target for fibrotic diseases.

INTRODUCTION

Since its discovery in the early 1970s [1, 2], the myofibroblast (MF) has emerged as a central orchestrator of wound repair and pathologic hypertrophic scar formation characteristic of fibrotic diseases [3-6]. Differentiated from fibroblasts, MFs exhibit a contractile phenotype marked by de novo expression of α-smooth muscle cell like actin (αSMA) stress fibers [7] and are primarily responsible for excessive extracellular matrix (ECM) production, cell to matrix adhesion, and resistance to apoptosis [3, 8-10]. It is well established that accumulation of transforming growth factor β1 (TGFβ) is a major inducer of MF differentiation during wound healing and fibrosis [8, 11-13]. Interestingly, basic fibroblast growth factor (bFGF) has been shown to control the extent of TGFβ-induced effects via stimulating de-differentiation of MFs [14, 15]. This shows the intricate balance between these two cytokines in mediating physiological and pathophysiological fibrogenic responses.

Synthesis of αSMA, a principal component of MFs, is a highly regulated process that is controlled by TGFβ, splice variant ED-A fibronectin (ED-A FN), and mechanical tension [3, 5]. Numerous lines of evidence indicate that the transcription of αSMA is reliant on RhoA-mediated activation and nuclear translocation of transcription factor serum response factor (SRF) [16-24]. Furthermore, the interaction between the recently discovered myocardin, a transcription co-factor restricted to cardiac and smooth muscle cells, and SRF has been characterized in orchestrating contractile gene expression [22, 25-27]. However, defining signaling pathways that regulate this transcription network in the hopes of devising targeted therapeutics remain incompletely understood.

Research from our laboratory has established the pivotal role of protein kinase Bα (Akt1) in wound healing, ECM remodeling, and vascular maturation [28]. Furthermore, we have shown that Akt1 is instrumental for normal cellular processes including fibroblasts migration, proliferation, and cytoskeletal remodeling [29], and assembly of ECM proteins including fibronectin [30, 31]. However, the precise role of Akt in MF differentiation remains unclear. Several studies have reported a possible positive correlation between Akt and αSMA [32-35], and have established its role in anoikis resistance [11, 36]. Because Akt1 is the predominant isoform in fibroblasts, these studies suggest a role for Akt1 in MF differentiation. Collectively, this has prompted us to postulate that Akt1 modulates αSMA synthesis through myocardin and SRF leading to MF differentiation.

In the present study, our results demonstrate that Akt1 is critical for αSMA synthesis in MF differentiation. Our studies constitute evidence of a novel signaling cascade that links Akt1 to αSMA synthesis through enhanced expression of myocardin and SRF, a previously uncharacterized link in MFs. We also demonstrate the dual role of Akt1 in mediating αSMA synthesis and ED-A FN splice variant along with total fibronectin. In conclusion, our studies suggest that Akt1 is a potential therapeutic target in fibrotic diseases.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture

NIH 3T3 fibroblasts were obtained from ATCC (Manassas, VA) and stable transfected through retroviral infections using control pBabe plasmids and those expressing constitutively active CA-Akt1-GFP fusion (90 kDa) (also known as myristoylated-Akt1

(myr-Akt1), or DN-Akt1 (Akt1 K179M) vectors with puromycin resistance. Selection with antibiotic was carried out until 100% transfection efficiency was confirmed by GFP staining. To examine the role of Akt1: NIH 3T3, transfected with stable empty vector, myr-Akt1, or DN-Akt1 vector, were cultured on 6-well plates. After reaching 70% confluence, cells were subjected to serum starvation in the presence or absence of 100 pM TGFβ (a pre-determined dose [30]) for 72 h, a standardized time point that is associated with maximum MF differentiation. Cells were subjected to Western analyses and immunocytochemistry as described below. For the mechanistic pharmacologic inhibition studies: after reaching 70% confluence, NIH 3T3 and myr-Akt1 cells were treated with bFGF (20 ng/ml (29)) or TGFβ (100 pM) for 48 h. This was followed by co-treatment for 24 h (total 72 h) with inhibitors of PI3 kinase (25 μM LY294002), Akt (10 nM triciribine), mammalian target of rapamycin (mTOR) (25 nM rapamycin), or SRF/Rho (1 μM CCG1423). Cells were subjected to Western analyses as described below.

Antibodies

Anti-αSMA (catalog number SAB2500963) and anti-fibronectin (catalog number F6140) antibodies were purchased from Sigma. GAPDH (catalog number 2118), phospho-Akt (Ser-473) (catalog number 9271), and anti-SRF (catalog number 5147) antibodies were purchased from Cell Signaling (Boston, MA). Anti-myocardin (catalog number MAB 4028) antibodies were purchased from R&D Systems (Minneapolis, MN). Anti-ED-A-fibronectin (catalog number 6328) and anti-αSMA (catalog number 5694) antibodies were purchased from Abcam (Cambridge, MA).

Western Blot Analysis

Cell lysates were prepared using lysis buffer (20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 3 mM EGTA, 5 mM EDTA, phosphatase inhibitors (10 mM sodium pyrophosphate, 5 mM sodium orthovanadate, 5 mM sodium fluoride, and 10 μMokadaic acid), protease inhibitor mixture (Roche Diagnostics) and 1 mM PMSF). SDS-PAGE and Western blotting were performed as described previously [30].

siRNA Transfection

For myocardin siRNA (100 nM) and SRF siRNA (150 nM), a pool of two target-specific siRNAs (Qiagen) were designed to knockdown mouse myocardin and SRF gene expression, respectively. A non-silencing oligonucleotide sequence (non-silencing siRNA) that does not recognize any known homology to mammalian genes was obtained as a negative control. NIH 3T3 and myr-Akt1 fibroblasts were transfected with siRNAs using a FuGENE transfection kit (Qiagen) for 24–48 h with TGFβ (100 pM) treatment for 72 h (supplemental Fig. S1). Cells were subjected to Western analyses and immunocytochemistry to detect αSMA expression and assembly, respectively.

Three-dimensional Collagen Gel Contraction Assay

Prior to preparing collagen gels as described below, fibroblasts were detached by 0.05% trypsin in 0.53 mM EDTA and suspended in 10 ml of serum-free DMEM containing soybean trypsin inhibitor. The cell number was then counted with Coulter Counter. Collagen gels were prepared according to the manufacturer's protocol (Cytoskeleton, CO) by mixing rat tail tendon collagen, distilled water, 4× DMEM, and cells. The final concentration was 1× DMEM, 0.75 mg/ml of collagen, and fibroblasts

were present at 3 × 105 cells/ml. Following this, 500 µl of the mixture was cast into each well of a 24-well culture plate. The solution was then allowed to polymerize for 1 h. After polymerization, the gels were allowed to remain attached to the plates for 48 h to form stress in the presence of DMEM without FBS, then gels were gently released from the plates to mimic free-floating. The optimum concentrations of bFGF, TGFβ, and triciribine were standardized in the laboratory. The area of each gel was measured daily and imaged. Data are expressed as the percentage of area compared with the initial gel area.

Immunocytochemistry

Immunofluorescence staining was performed as described previously (29). Briefly, NIH 3T3, transfected with stable empty vector, myr-Akt1, or DN-Akt1 vector, were plated on 8-well chamber slides. After reaching 70% confluence, cells were subjected to serum starvation in the presence or absence of TGFα for 72 h. Next, cells were fixed with 4% paraformaldehyde in 1× PBS followed by permeabilization with 0.1% Triton X-100 in 1× PBS. The nonspecific staining was blocked with 2% BSA for 1 h at room temperature. The fixed and permeabilized cells were incubated with primary anti-αSMA antibody (Abcam) (dilution 1:1000) overnight at 4 °C and washed. Secondary Alexa Fluor 488-labeled antibody was applied for 1 h followed by standardized dilution of Alexa Fluor 555-labeled phalloidin (Invitrogen) for 40 min. The slides were mounted with Vectashield (Vector Laboratories, PA), and imaged by a Zeiss fluorescent microscope.

Animals

All experiments were performed with approval by the Charlie Norwood Veterans Affairs Medical Center Institutional Animal Care and Use Committees. Akt1−/−mice were

generated as previously described (28) and were maintained in the C57BL/6 background. Sex and age-matched wild-type and Akt1−/− were randomized to normobaric hypoxia (10% O2) (Biospherix, New York) or room air. To establish the hypoxic environment, the chamber was flushed with nitrogen. The chamber was opened once per week for no more than 1 h to clean the cages and replenish food and water supplies. After 14 days, mice were euthanized; lungs were isolated and subjected to Western analyses.

Statistical Analysis

All data are presented as mean ± S.D. To determine significant differences between treatment and control values, we used the Student's two-tailed t test. The significance was set at 0.05 levels (marked with symbols wherever data are statistically significant).

RESULTS

Akt1 Inactivation Abolishes TGFβ-induced Myofibroblast Differentiation

We first determined that 72 h is the optimal time for TGFβ-induced MF differentiation in NIH 3T3 fibroblasts, as measured by a 5-fold increase in αSMA expression. To examine whether Akt1 activation is required for αSMA expression, the marker for MF differentiation, fibroblasts transfected with constitutively active Akt1 (myr-Akt1) and inactive dominant-negative-Akt1 (DN-Akt1; Akt1 K179M), respectively, were serum starved and treated with control PBS or TGFβ (100 pM) for 72 h. The results showed that despite the absence of TGFβ stimulation, sustained hyperactivation of Akt1 significantly enhanced αSMA expression (~6-fold) and assembly compared with control fibroblasts; this effect was further amplified with TGFβ stimulation (Fig. 1, A and B). In

contrast, inactivation of Ak1 blunted the stimulatory effects of TGFβ on αSMA expression and assembly (Fig. 1, A and B). Thus, the absence of Akt1 impedes TGFβ-induced αSMA expression, implying that Akt1 is an important modulator of MF differentiation.

Akt1-mediated Dynamic Switch between Fibroblast and MFs Is Stimuli Dependent

Because physiological modulators of fibroblasts such as bFGF also activate Akt1 (Fig. 2A), we examined the effect of bFGF on αSMA expression and MF differentiation. To do this, we subjected control and myr-Akt1 expressing fibroblasts to serum starvation and treated the fibroblasts with control PBS, TGFβ (100 pM), or bFGF (20 ng/ml). In contrast to the stimulatory effect of TGFβ, bFGF was associated with diminished αSMA expression in control and myr-Akt1 expressing NIH 3T3 fibroblasts (Fig. 2B). This suggested a molecular see-saw of bFGF and TGFβ, clearly demonstrating a reciprocal regulation of physiological and pathological fibrogenic events by these two cytokines via differential regulation of Akt1 activity (Fig. 2, A and B).

This differential regulation of αSMA expression by bFGF and TGFβ prompted us to investigate whether Akt1 is involved in the de-differentiation effect of bFGF. Because maximum MF differentiation occurred at 72 h, to study the de-differentiation effect, we treated control and myr-Akt1 NIH 3T3 fibroblasts with bFGF (or control PBS) for 24 h after 72-h TGFβ stimulation (total 96 h). Although treatment with bFGF partially, but significantly, inhibited TGFβ-induced αSMA expression in fibroblasts (Fig. 2C), it did not show any discernible de-differentiation in myr-Akt1 expressing cells (Fig. 2D). Interestingly, although bFGF treatment increased Akt phosphorylation in NIH 3T3 cells, in combination with TGFβ, it decreased the TGFβ-induced Akt phosphorylation (Fig. 2,

C and E). Furthermore, although myr-Akt1 expressing cells were resistant to bFGF-induced de-differentiation, a decrease in endogenous Akt phosphorylation was observed in these cells (Fig. 2, D and F), thus indicating that the resistance to bFGF treatment in these cells was purely due to the expression of myr-Akt1. Together, these data indicate the need for sustained Akt1 activation in fibroblasts to drive αSMA synthesis and MF differentiation. This supports our previous findings that, whereas transient activation of Akt1 in response to bFGF mainly regulates fibroblast proliferation, matrix adhesion, and assembly (29), sustained Akt1 activation promotes TGFβ-induced MF differentiation.

Akt1 Modulates TGFβ-induced MF Differentiation through SRF

Once we identified Akt1 as a key modulator of αSMA expression, we sought to define and characterize the mechanisms by which Akt1 mediates TGFβ-induced αSMA expression and MF differentiation. Because studies have shown that the transcription factor SRF is integral for αSMA synthesis, we explored whether SRF is also a potential target of the PI3K/Akt/mTOR pathway during MF differentiation. To investigate this, we utilized inhibitors of PI3K (25 μMLY294002), Akt (10 nM triciribine), mTOR (25 nM rapamycin), and Rho signaling-responsive SRF (1 μM CCG1423) (Calbiochem) in control and myr-Akt1 expressing fibroblasts stimulated with TGFβ, and determined αSMA and SRF expressions, and Akt phosphorylation.

TGFβ-induced αSMA expression was correlated with elevated SRF expression and phosphorylation of Akt in NIH 3T3 cells (Fig. 3, A–D) and myr-Akt1 cells (Fig. 3,E–H). Although inhibiting PI3K resulted in a moderate decrease in αSMA, SRF, and Akt phosphorylation compared with TGFβ-treated NIH 3T3 cells, targeting Akt abolished

TGFβ-induced αSMA expression and was associated with a marked decrease in SRF expression and Akt phosphorylation (Fig. 3, A–D). TGFβ-stimulated myr-Akt1 expressing fibroblasts while exhibiting resistance upon PI3K inhibition, inhibiting Akt resulted in a significant decrease in αSMA and SRF expression, as well as Akt phosphorylation compared with TGFβ-treated myr-Akt1 fibroblasts (Fig. 3, E–H). Inhibiting RhoA produced a modest but significant inhibition of αSMA that was associated with a significant reduction of SRF expression and Akt phosphorylation compared with TGFβ-treated fibroblasts in NIH 3T3 (Fig. 3, A–D) and myr-Akt cells (Fig. 3, E–H). However, a significant effect of mTOR inhibitor rapamycin on αSMA expression was not observed (Fig. 3, A, B, E, and F). Interestingly, in bFGF-stimulated cells, inhibiting mTOR promoted αSMA and SRF expressions, implying that mTOR is a key factor in maintaining fibroblast stability under bFGF and suppress its differentiation to MFs (Fig. 4, A–H). Taken together, these results show that TGFβ-induced αSMA expression is mediated by the PI3K/Akt/SRF pathway, independent of mTOR.

Akt1 Regulates αSMA Synthesis through SRF and Myocardin

Because SRF is ubiquitous, not contractile gene specific, and because myocardin is a critical transcriptional co-activator of smooth muscle cell genes [37], we considered whether the regulatory effect of Akt1 on αSMA expression is mediated through myocardin in conjunction with SRF. We first evaluated the expression of myocardin and SRF in relationship to TGFβ-induced αSMA expression during MF differentiation. The results show that at 72 h, αSMA expression correlates with a significant increase in myocardin and SRF levels (∼6- and ∼3-fold, respectively) in NIH 3T3 cells (Fig. 5, A–D). Interestingly, in the absence of TGFβ stimulation, myr-Akt1-induced αSMA

expression correlated with a 3.5-fold increase in myocardin and a 1.8-fold increase in SRF expressions, compared with control fibroblasts (Fig. 5, E–H).

We argued that if Akt1 acts at least in part through myocardin and SRF, then silencing either protein should result in reduced αSMA. To test this, we performed gene knockdown experiments with myocardin siRNA and SRF siRNA. NIH 3T3 and myr-Akt1 fibroblasts were transfected with control vector, myocardin, or SRF siRNA (siMyoc and siSRF, respectively) for 48 h in the absence or presence of TGFβ; cells were pretreated with TGFβ to promote MF differentiation. Although sustained activation of Akt1 resulted in increased αSMA expression and assembly, knockdown of myocardin and SRF significantly reduced αSMA levels in myr-Akt1 expressing fibroblasts both in the absence (Fig. 6, B and E) and presence of TGFβ (Fig. 6, D and F). To validate our in vitro observations, we utilized a chronic hypoxia model as it has been shown to induce fibroblast to MF differentiation in animal models thus playing a major role in fibrotic response [38, 39]. Hence, as a proof of concept and further confirm the role of Akt1 in αSMA synthesis in vivo, we subjected wild-type (WT) and Akt1 null (Akt1−/−) mice to continuous hypoxia exposure for 14 days and lung tissues were subjected for Western analysis. As expected, Akt1−/− mice had significantly lower αSMA, myocardin, and SRF expressions in the lung tissue compared with WT mice (Fig. 6,G and H). Taken together, our data demonstrate that Akt1 plays a crucial permissive role in αSMA synthesis mediated, in part, by regulating myocardin and SRF expressions.

Akt1 Plays a Dual Role as a Modulator of TGFβ-induced αSMA and Fibronectin

Because both MF differentiation and excess ECM secretion constitute key hallmark events of deregulated tissue remodeling that occurs in fibrotic conditions such as

idiopathic pulmonary fibrosis and cirrhosis [3, 9, 33, 40, 41] and having seen that Akt1 is crucial for αSMA synthesis, we asked whether it could be involved in pathologic ECM regulation as well. We have previously shown that Akt1 regulates the synthesis of ECM proteins such as fibronectin via activation of mTOR pathway [30]. Fibronectin splice variant ED-A FN, which is not typically expressed in undifferentiated fibroblasts, is mandatory for TGFβ-induced αSMA synthesis and MF differentiation [3]. Furthermore, total fibronectin, which is secreted excessively by differentiated MFs, also exerts mechanical tension on MFs as part of a positive feedback loop [3]. Therefore, to determine whether Akt1 is involved in expression of the ED-A FN splice variant in the regulation of MF differentiation, we subjected control and myr-Akt1 expressing fibroblasts to TGFβ for 48 h and co-treated with inhibitors of Akt1 and mTOR for an additional 24 h. Expression of αSMA, ED-A FN, total fibronectin and phosphorylated Akt was determined. Inhibiting Akt significantly reduced the stimulatory effect of TGFβ on αSMA, ED-A FN, and fibronectin expressions. However, treatment with the mTOR inhibitor rapamycin only inhibited total fibronectin expression with no discernible decrease in αSMA expression in both control NIH 3T3 (Fig. 7, A–D) and myr-Akt1 expressing fibroblasts (Fig. 7, A and E-G). It is noteworthy that rapamycin reduced ED-A FN expression only in myr-Akt1 cells implying that under physiological conditions mTOR does not regulate its expression. Herein, we demonstrate that whereas both Akt1 and mTOR are needed for total fibronectin synthesis [30], Akt1 also modulates the expression of specialized ED-A FN, which sheds light on the mechanisms underlying the dynamic reciprocal mechanical interactions between cells and the ECM during MF differentiation.

Targeted Akt Inhibition Reverses TGFβ-mediated Contraction of Fibroblast-populated Collagen Lattices

To validate the observation with regards to the functional characteristics of myofibroblasts, we performed a collagen gel contraction assay and examined the degree of contraction of a three-dimensional collagen lattice, an accepted model system of MF contractility [42]. We compared the de-differentiation effect of inhibiting Akt using triciribine to bFGF in TGFβ-stimulated gels. Control and bFGF-treated lattice exhibited a slight contraction as a result of physiological proliferation of the fibroblasts, but the effects were not significant. At 96 h, the marked TGFβ-induced contraction, whereas moderately reduced by bFGF treatment, was markedly ameliorated upon Akt inhibition (Fig. 8, A and B). It is noteworthy that compared with the contractility baseline at 72 h, bFGF had no discernible effect on gel contraction (Fig. 8C) suggesting that TGFβ-mediated Akt activation is necessary for the contractile force generated by MFs. Collectively, these morphological, biochemical, and functional data confirm a role of Akt1 in mediating, at least in part, TGFβ effects on MF differentiation.

DISCUSSION

In the present study, we provide the first conclusive evidence on the significance of sustained Akt1 activation in persistent MF differentiation with potential pathophysiological implications in tissue fibrosis. First, whereas sustained activation of Akt1 mimicked TGFβ stimulation and induced a ~6-fold increase in αSMA, inactivation of Akt1 blunted TGFβ-induced αSMA expression and assembly. Second, Akt1 mediates αSMA synthesis, in part, through modulation of myocardin and SRF expression, independent of mTOR, a novel and previously uncharacterized link in MFs. Third, in

addition to its control on total fibronectin, Akt1 induces ED-A FN expression, which is mandatory for MF differentiation. Fourth, TGFβ-mediated Akt1 activation is necessary for the functional contractile force generated by MFs. Collectively, our study identifies the critical role of Akt1 and defines novel mechanisms by which it mediates MF differentiation, and sheds light on the dynamic mechanical interactions between the MF and ECM. Together, this creates a deregulated continuous positive feedback loop resulting in persistent TGFβ-induced MF differentiation with implication in progression of fibrotic diseases (supplemental Fig. S2).

Several lines of evidence have identified and characterized multiple inter-cellular networks dictating MF differentiation. However, defining key signaling pathways leading to MF differentiation with the hope of devising targeted therapeutics remains elusive. Thus far, extensive research has been geared toward RhoA-mediated αSMA synthesis in smooth muscle cells and MFs. Literature also suggests a correlation between Akt activation and αSMA expression during MF differentiation [32-35]. Additionally, it has been shown that deficiency in phosphatase and tensin homologue deleted on chromosome 10 that normally inactivate Akt resulted in a markedly high proliferation rate and increase in αSMA and collagen expressions [43]. This led us to investigate the role of Akt, Akt1 in particular, as a mediator of persistent MF differentiation. We show that inactivation of Akt1 abolished TGFβ-induced αSMA expression. Noteworthy, by conducting our studies in NIH 3T3 fibroblasts treated with TGFβ, fibroblasts expressing myr-Akt1 with sustained (constitutively active) Akt1 activity, fibroblasts expressing DN-Akt1 (inactive), and Akt1 knock-out mice, we confirm our findings and demonstrate the

clinical relevance of the association between Akt1 and MF differentiation with implications in fibrotic diseases.

RhoA-dependent signaling has been shown to involve SRF, which plays a major role in αSMA synthesis [4, 16-24, 26, 44, 45]. Notably, SRF, a ubiquitous transcription factor, is not smooth muscle-specific. Furthermore, Olson and colleagues [27] discovered that myocardin, the transcription co-activator of SRF, is a critical master activator of αSMA gene expression. Although fibroblasts are not reported to constitutively express myocardin, which is assumed to be specific to cardiac and smooth muscle cells, forced expression of myocardin has been demonstrated to drive the expression of αSMA in fibroblasts [46]. Our studies showed that the expression of both SRF and myocardin were up-regulated during TGFβ-induced MF differentiation in NIH 3T3 fibroblasts. Interestingly, we observed a positive correlation between Akt1 activity and expression levels of SRF and myocardin. To further delineate this potential molecular cross-talk between Akt1 and SRF-myocardin complex in αSMA synthesis and MF differentiation, we utilized an siRNA-mediated gene knockdown approach to target myocardin and SRF. Our studies revealed that in un-stimulated myr-Akt1 fibroblasts, myocardin and SRF knockdown resulted in ~50% attenuation of αSMA synthesis and MF differentiation. Additionally, genetic ablation of Akt1 correlated with decreased SRF and myocardin in vivo, which further confirms this association. Earlier, our experiments utilizing the RhoA/SRF inhibitor CCG-1423 exhibited ~50% decrease in MF differentiation, which led us to postulate that Akt1 and RhoA, two parallel signaling pathways might reconcile their effects in modulating TGFβ-induced αSMA synthesis. The results from the myocardin and SRF siRNA studies lend support to our hypothesis

that both Akt1 and RhoA cooperate in the regulation of αSMA synthesis, the former regulating the expression of SRF and myocardin, and the latter leading to phosphorylation and nuclear translocation of the SRF-myocardin complex. Further investigation into this signaling network will likely shed light on the intricate dynamic network governing this phenotypic behavior.

Another intriguing finding from our studies is the paradoxical utilization of Akt1 in modulating αSMA synthesis. We have previously demonstrated that Akt1 promotes bFGF-induced migration and proliferation [29]. Indeed, Greenberg et al. [47] have demonstrated that bFGF negatively regulates TGFβ-induced αSMA synthesis thus maintaining fibroblast phenotype and promoting MF de-differentiation. The interesting finding that sustained Akt1 activation is sufficient to drive a marked increase in αSMA expression and resistance to bFGF-induced de-differentiation reflects the preferential effect of Akt1 in mediating cell differentiation as opposed to proliferation. The differential utilization of Akt1, in addition to being dictated by growth factors, appears to also be dependent on the strength and duration of the stimuli and, importantly, the cooperation of Akt1 with RhoA in response to TGFβ, but not with bFGF, thereby highlighting the importance of Akt1 in physiologic regulation and, importantly, pathologic differentiation deregulation.

We have previously shown that the Akt1-mTOR pathway regulates FN translation and ECM secretion and assembly [29, 30]. Recent studies have suggested that Akt can modulate alternative splicing of FN extra domain III (ED-A FN), which plays a major role in promoting MF differentiation [48-50]. We found that unlike Akt1, targeting mTOR did not infer discernible effects on TGFβ-induced αSMA synthesis. This prompted us to

investigate their effects on total FN and ED-A FN in MFs. Our data show that, in addition to its profound effects on αSMA expression and assembly, targeting Akt markedly decreased both ED-A FN and total FN, which further highlights the pro-differentiation potential of the Akt1 pathway. Interestingly, targeting mTOR did not influence ED-A FN (or did so marginally), which suggests that mTOR primarily regulates total FN as part of ECM regulation not MF differentiation per se. However, in the presence of robust sustained activation of Akt1 along with TGFβ stimulation, rapamycin treatment did infer some effect on ED-A FN in addition to total FN in myr-Akt1 fibroblasts. The results from NIH 3T3 studies are in line with recent reports that suggested that rapamycin, whereas primarily blocks FN translation via regulation of S6K, can enhance the activity of Akt1 via a positive feedback loop [48, 51, 52]. It has been suggested that elevated basal levels of Akt can directly mediate alternative splicing of ED-A FN [48, 51]. However, our findings in myr-Akt1 fibroblasts that rapamycin modestly affects both ED-A FN and FN raise the possibility that constitutive activation of Akt1 may, partly, engage mTOR in the translational regulation of ED-A FN. Further studies are warranted to confirm this hypothesis.

In conclusion, we identify Akt1 as an integral modulator of αSMA synthesis, and found it to be mediated, in part, through interaction with myocardin and SRF, independent of mTOR activity. Moreover, we shed light on the dual and functional role of Akt1 in regulating ECM fibronectin as well as ED-A FN splice variant driving persistent MF differentiation and contractile force generation. We also characterize the paradoxical use of Akt1 in differentially mediating physiological and pathological effects in response

to bFGF and TGFβ up-regulation, respectively. Collectively, these findings highlight the importance of Akt1 as a novel therapeutic target for fibrotic diseases.

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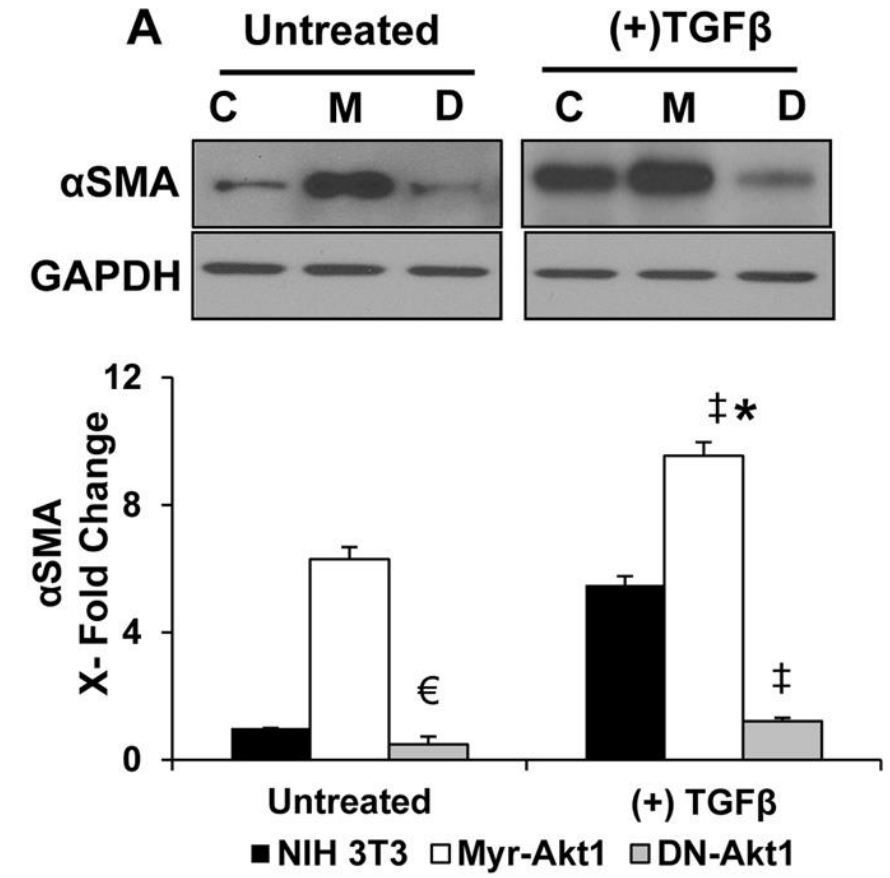
FIGURE 1.

Akt1 is critical for αSMA expression in response to TGFβ. *A,* control vector (pBabe-Puro), myr-Akt1, and DN-Akt1 expressing NIH 3T3 fibroblasts grown to 80% confluence were subjected to serum-free medium in the presence and absence of 100 pM TGFβ for 72 h. Lysates were prepared and subjected to Western analyses with antibodies against αSMA (*n* = 3) (*C,* control NIH 3T3; *M,* myr-Akt1; *D,* DN-Akt1) (▲, *p* = 0.0001 and €, *p* = 0.02 compared with control untreated NIH 3T3; *, compared with myr-Akt1 control; ‡, compared with TGFβ-treated NIH 3T3 cells). *B,* cells, subjected to serum-free medium in the presence and absence of 100 pM TGFβ for 72 h, were fixed using 4% paraformaldehyde and stained using anti-αSMA antibodies. Fluorescent images of αSMA assembly were visualized using a fluorescence microscope and photographed. *Scale bar,* 50 μM.


A
Untreated
(+)TGFβ
C
M
D
C
M
D
αSMA
GAPDH
αSMA
X- Fold Change
12
8
4
0
‡*
€
‡
Untreated
(+) TGFβ
NIH 3T3
Myr-Akt1
DN-Akt1

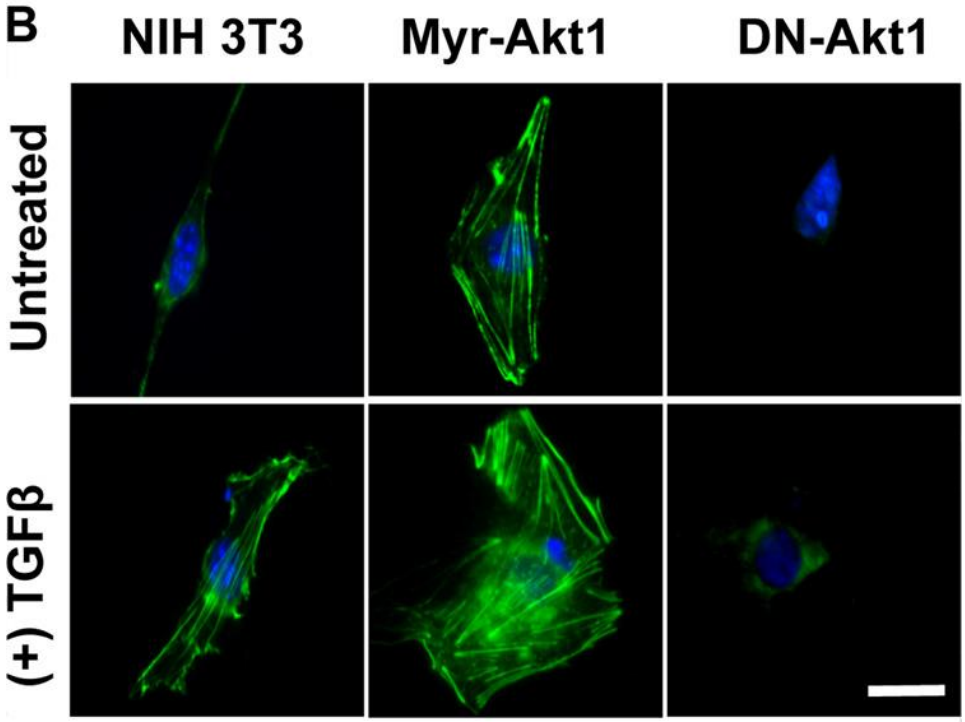

B
NIH 3T3
Myr-Akt1
DN-Akt1
Untreated
(+) TGFβ

FIGURE 2.

Akt1 is differentially utilized by TGFβ and bFGF in fibroblast to MF differentiation. *A,* a schematic representation of the hypothesis that the stimuli-dependent dynamic switch between fibroblasts (*bFGF*) and MFs (*TGF*β) is mediated by Akt1. Control vector (*B*) and myr-Akt1 (*C*) (*M*) expressing fibroblasts were subjected to serum starvation alone or plus treatment with 20 ng/ml of bFGF or 100 pM TGFβ for 72 h. Lysates were prepared and subjected to Western analyses with antibodies against αSMA (*n* = 3) (*C*, control NIH 3T3; *M,* myr-Akt1; *D,* DN-Akt1) (*, compared with myr-Akt1 control; #, compared with control; ♦, bFGF compared with TGFβ). *C–F*, to examine the de-differentiation potential, control and Myr-Akt1 expressing NIH 3T3 cells were treated with TGFβ for 72 h plus bFGF (for an additional 24 h) for a total of 96 h. Lysates were prepared and subjected to Western analyses with antibodies against αSMA and phospho-Ser-473 Akt (*n* = 3).

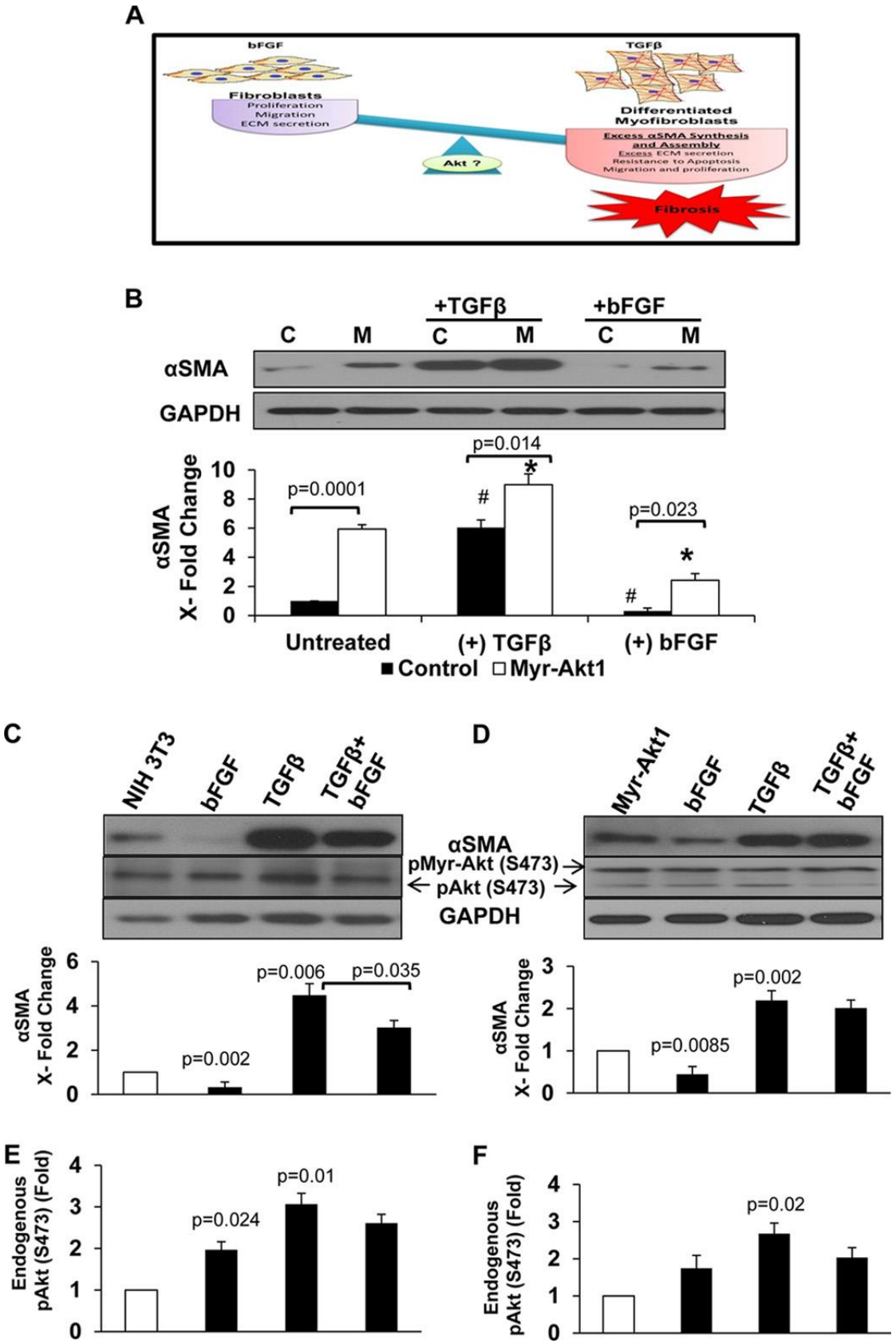

A
bFGF
Fibroblasts
Proliferation
Migration
ECM secretion
TGFβ
Differentiated
Myofibroblasts
Excess αSMA Synthesis
and Assembly
Excess ECM secretion
Resistance to Apoptosis
Migration and proliferation
Akt ?
Fibrosis
B
+TGFβ
+bFGF
C
M
C
M
C
M
αSMA
GAPDH
αSMA
X- Fold Change
p=0.0001
p=0.014
p=0.023
Untreated
(+) TGFβ
(+) bFGF
Control
Myr-Akt1
C
NIH 3T3
bFGF
TGFβ
TGFβ+
bFGF
αSMA
pMyr-Akt (S473)
pAkt (S473)
GAPDH
αSMA
X- Fold Change
p=0.002
p=0.006
p=0.035
D
Myr-Akt1
bFGF
TGFβ
TGFβ+
bFGF
αSMA
X- Fold Change
p=0.0085
p=0.002
E
Endogenous
pAkt (S473) (Fold)
p=0.024
p=0.01
F
Endogenous
pAkt (S473) (Fold)
p=0.02

FIGURE 3.

TGFβ-induced αSMA expression is Akt1-dependent through SRF, independent of mTOR. NIH 3T3 fibroblasts (*A*–*D*) and myr-Akt1 expressing fibroblasts (*E*–*H*) grown to 70% confluence were treated with TGFβ for 72 h in the absence of FBS. To examine pathways governing MF differentiation, inhibitors were applied before significant differentiation occurred: cells were treated with TGFβ for 48 h and then co-treated with inhibitors for an additional 24 h targeting the PI3K/Akt/mTOR pathway and responsive SRF pathway (LY294002, triciribine (*TCBN*), rapamycin, and CCG1423, respectively). Lysates were prepared and subjected to Western analyses with antibodies against αSMA (*B* and *F*), SRF (*C* and *G*), and pAkt (Ser-473) (*D* and *H*) (n = 4). *, compared with TGFβ; ‡, compared with control untreated cells.

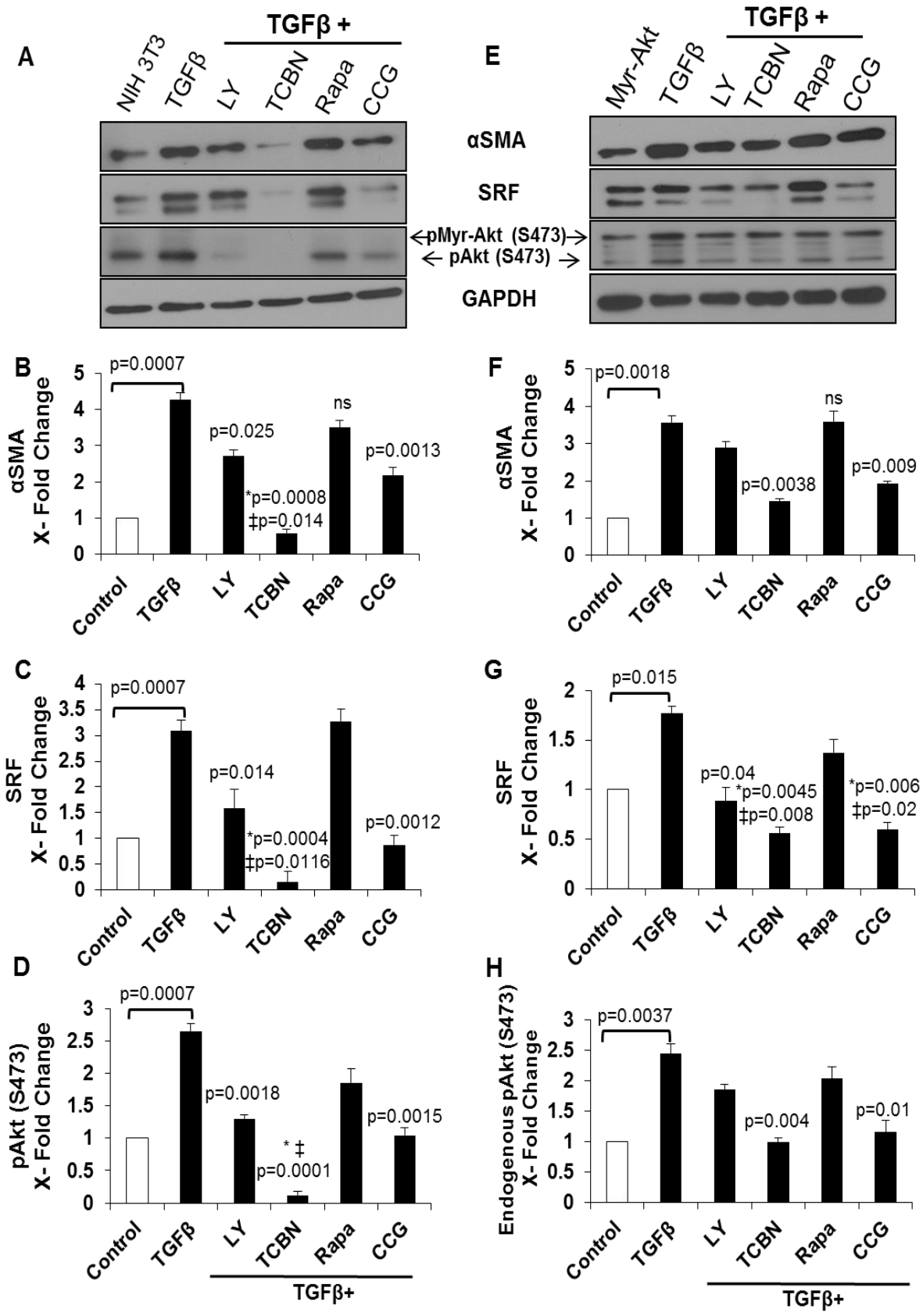

A
NIH 3T3
TGFβ
TGFβ +
LY
TCBN
Rapa
CCG
αSMA
SRF
pMyr-Akt (S473)
pAkt (S473)
GAPDH
E
Myr-Akt
TGFβ
TGFβ +
LY
TCBN
Rapa
CCG
B
αSMA X- Fold Change
p=0.0007
p=0.025
ns
p=0.0013
*p=0.0008
‡p=0.014
Control
TGFβ
LY
TCBN
Rapa
CCG
F
αSMA X- Fold Change
p=0.0018
ns
p=0.0038
p=0.009
C
SRF X- Fold Change
p=0.0007
p=0.014
*p=0.0004
‡p=0.0116
p=0.0012
G
SRF X- Fold Change
p=0.015
p=0.04
*p=0.0045
‡p=0.008
*p=0.006
‡p=0.02
D
pAkt (S473) X- Fold Change
p=0.0007
p=0.0018
* ‡
p=0.0001
p=0.0015
TGFβ+
H
Endogenous pAkt (S473) X- Fold Change
p=0.0037
p=0.004
p=0.01
TGFβ+

FIGURE 4.

bFGF-induced αSMA down-regulation is mTOR dependent. NIH 3T3 fibroblasts (*A*–*D*) and myr-Akt1 expressing fibroblasts (*E*–*H*) grown to 70% confluence were treated with bFGF for 72 h in the absence of FBS. To examine pathways governing bFGF-mediated effects on MF differentiation, cells were treated with bFGF for 48 h and then co-treated with inhibitors for an additional 24 h targeting the PI3K/Akt/mTOR pathway and responsive SRF pathway (LY294002, triciribine (*TCBN*), rapamycin, and CCG1423, respectively). Lysates were prepared and subjected to Western analyses with antibodies against αSMA (*B* and *F*), SRF (*C* and *G*), and pAkt (Ser-473) (*D* and *H*) (n = 4). *, compared with bFGF; ‡, compared with control untreated cells.

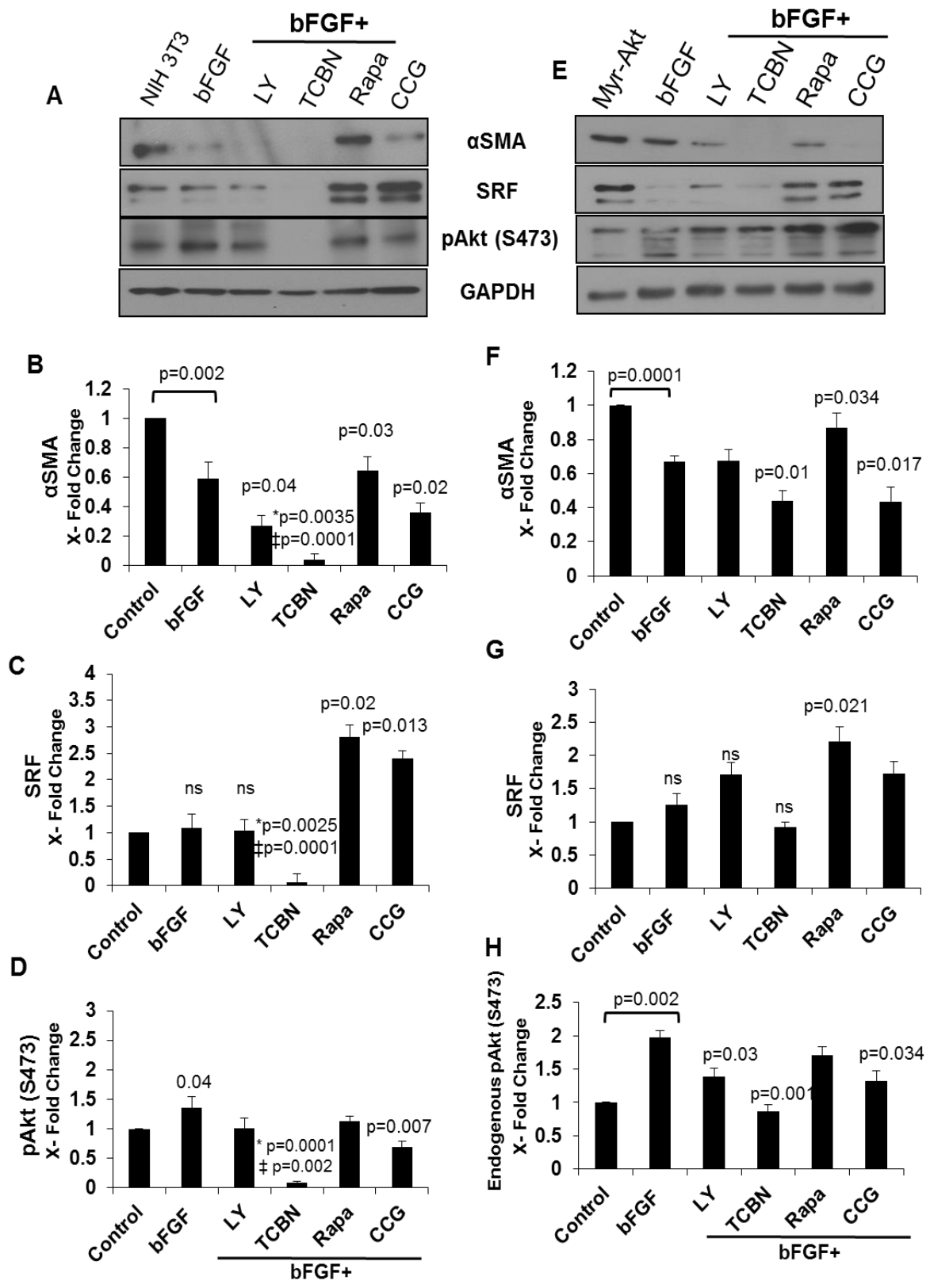

A
NIH 3T3
bFGF
bFGF+
LY
TCBN
Rapa
CCG
αSMA
SRF
pAkt (S473)
GAPDH
E
Myr-Akt
B
αSMA
X- Fold Change
p=0.002
p=0.04
*p=0.0035
‡p=0.0001
p=0.03
p=0.02
Control
F
p=0.0001
p=0.01
p=0.034
p=0.017
C
SRF
X- Fold Change
ns
*p=0.0025
‡p=0.0001
p=0.02
p=0.013
G
p=0.021
D
pAkt (S473)
X- Fold Change
0.04
* p=0.0001
‡ p=0.002
p=0.007
H
Endogenous pAkt (S473)
X- Fold Change
p=0.002
p=0.03
p=0.001
p=0.034

FIGURE 5.

Akt1 induces MF differentiation through enhanced expression of myocardin and SRF. NIH 3T3 fibroblasts treated with TGFβ for 72 h (*A*–*D*) and myr-Akt1 fibroblasts (*E*–*H*) were subjected to Western analysis for SMA, myocardin, and SRF expression (n = 4).

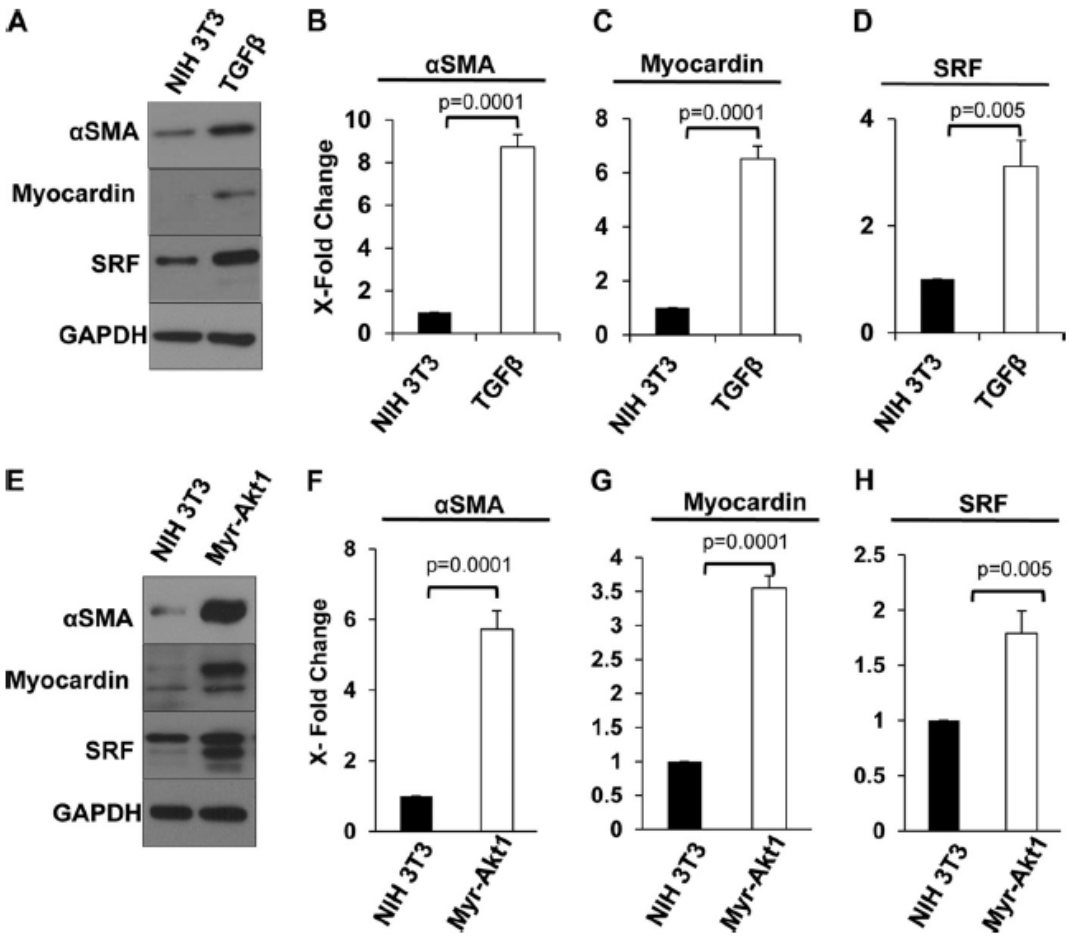

A
NIH 3T3
TGFβ
αSMA
Myocardin
SRF
GAPDH
B
αSMA
p=0.0001
X-Fold Change
10
8
6
4
2
0
NIH 3T3
TGFβ
C
Myocardin
p=0.0001
8
6
4
2
0
NIH 3T3
TGFβ
D
SRF
p=0.005
4
2
0
NIH 3T3
TGFβ
E
NIH 3T3
Myr-Akt1
αSMA
Myocardin
SRF
GAPDH
F
αSMA
p=0.0001
X- Fold Change
8
6
4
2
0
NIH 3T3
Myr-Akt1
G
Myocardin
p=0.0001
4
3.5
3
2.5
2
1.5
1
0.5
0
NIH 3T3
Myr-Akt1
H
SRF
p=0.005
2.5
2
1.5
1
0.5
0
NIH 3T3
Myr-Akt1

FIGURE 6.

siRNA-mediated knockdown of myocardin and SRF ablates Akt1-induced SMA expression and MF differentiation. Control NIH 3T3 fibroblasts (*A*and *C*) and myr-Akt1 fibroblasts (*B* and *D*) were transfected with control, myocardin, or SRF siRNA (*siMyoc,* 100 nM and *siSRF*, 150 nM), and 24 to 48 h later, cells were treated with 100 pM TGFβ for 72 h. Lysates were prepared and subjected to Western analyses, quantification was normalized to control (*, $p < 0.001$, $p < 0.01$) ($n = 3$). *E* and *F,* cells were fixed using 4% paraformaldehyde and stained using anti-αSMA antibodies. Fluorescent images of αSMA assembly were visualized using a fluorescence microscope and photographed. *Scale bar*, 50 μM. *G* and *H*, as a proof of concept, wild type (WT) Akt null (*Akt1*$^{-/-}$) mice were subjected to chronic hypoxia (a well-established model of pulmonary hypertension that induces marked MF differentiation). After 14 days of continuous exposure to 10% oxygen, mice were euthanized and whole lungs collected, homogenized, and analyzed using Western blotting for αSMA, myocardin, SRF, and Akt1 expressions. Data were normalized to GAPDH ($n = 4–5$).

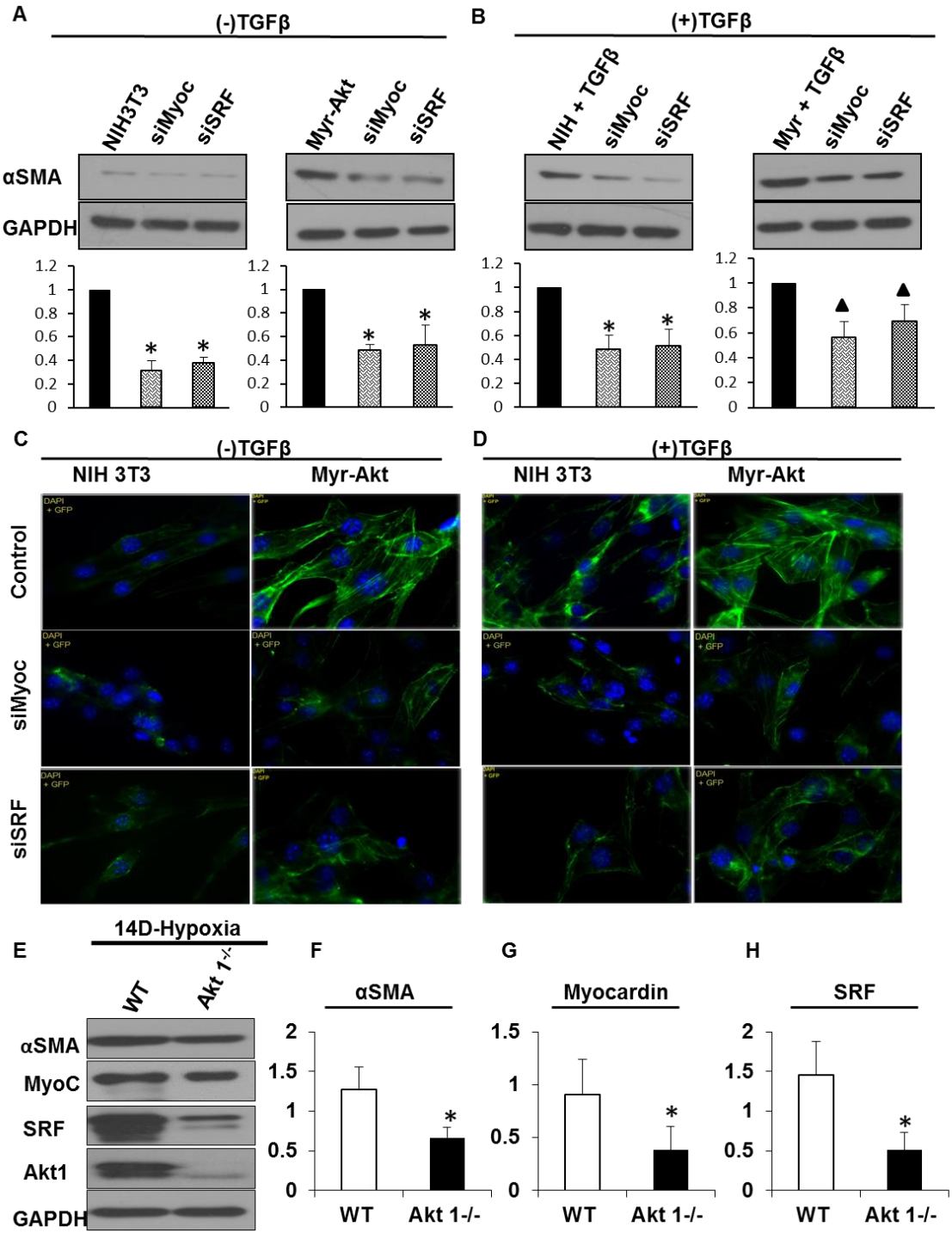

A
(-)TGFβ
NIH3T3
siMyoc
siSRF
Myr-Akt
siMyoc
siSRF
αSMA
GAPDH
B
(+)TGFβ
NIH + TGFβ
siMyoc
siSRF
Myr + TGFβ
siMyoc
siSRF
C
(-)TGFβ
NIH 3T3
Myr-Akt
Control
siMyoc
siSRF
D
(+)TGFβ
NIH 3T3
Myr-Akt
E
14D-Hypoxia
WT
Akt 1-/-
αSMA
MyoC
SRF
Akt1
GAPDH
F
αSMA
WT
Akt 1-/-
G
Myocardin
WT
Akt 1-/-
H
SRF
WT
Akt 1-/-

FIGURE 7.

Akt1-mediated expression αSMA and ED-A FN in response to TGFβ is independent of mTOR activity. Control NIH 3T3 (*A*–*D*) and myr-Akt1 expressing fibroblasts (*E*–*H*) grown to 70% confluence were subjected to serum-free medium and treated with TGFβ for 72 or 48 h and then co-treated with inhibitors for an additional 24 h targeting the Akt/mTOR pathway (triciribine (*TCBN*) and rapamycin, respectively). Lysates were prepared and subjected to Western analyses with antibodies against αSMA, ED-A FN, total fibronectin, and pAkt(Ser-473) (*n* = 3). *, compared with TGFβ; ‡, compared with control untreated cells.

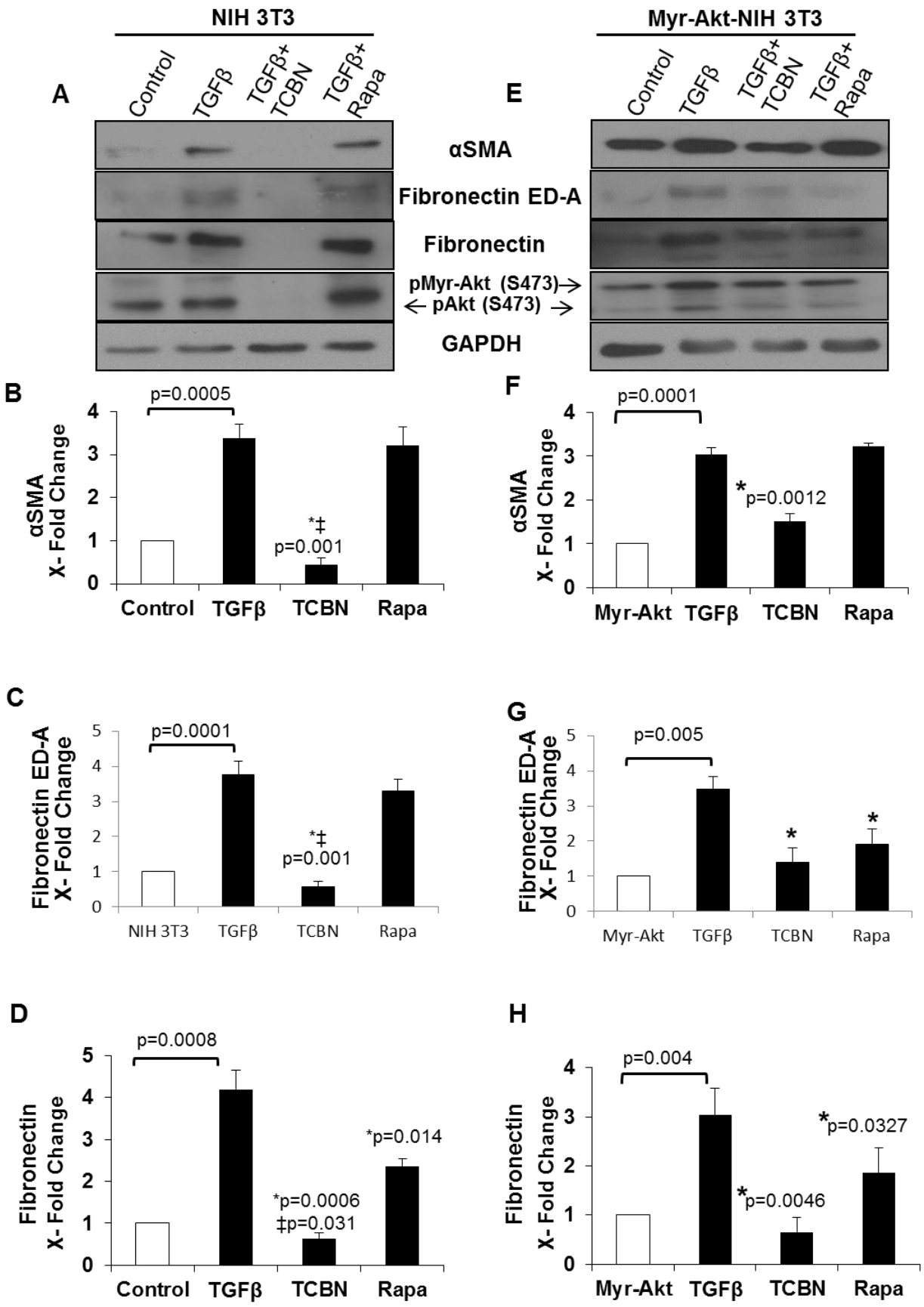
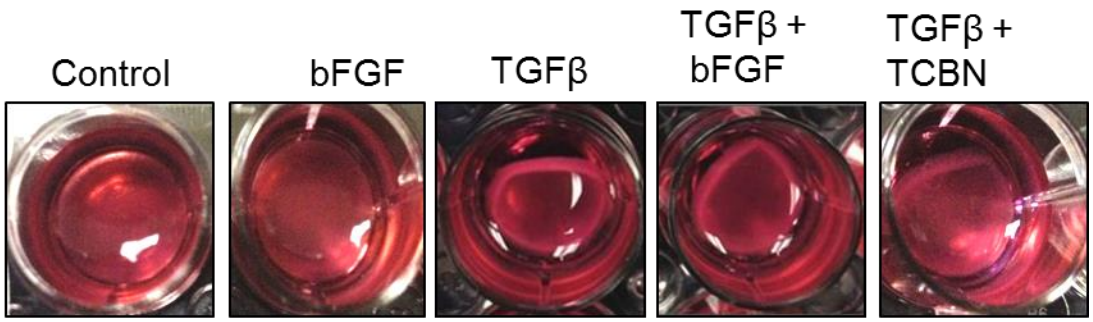

A
NIH 3T3
Control
TGFβ
TGFβ+ TCBN
TGFβ+ Rapa
αSMA
Fibronectin ED-A
Fibronectin
pMyr-Akt (S473)→
← pAkt (S473) →
GAPDH
E
Myr-Akt-NIH 3T3
Control
TGFβ
TGFβ+ TCBN
TGFβ+ Rapa
B
αSMA X- Fold Change
p=0.0005
*‡ p=0.001
Control
TGFβ
TCBN
Rapa
F
αSMA X- Fold Change
p=0.0001
*p=0.0012
Myr-Akt
TGFβ
TCBN
Rapa
C
Fibronectin ED-A X- Fold Change
p=0.0001
*‡ p=0.001
NIH 3T3
TGFβ
TCBN
Rapa
G
Fibronectin ED-A X- Fold Change
p=0.005
*
*
Myr-Akt
TGFβ
TCBN
Rapa
D
Fibronectin X- Fold Change
p=0.0008
*p=0.014
*p=0.0006
‡p=0.031
Control
TGFβ
TCBN
Rapa
H
Fibronectin X- Fold Change
p=0.004
*p=0.0327
*p=0.0046
Myr-Akt
TGFβ
TCBN
Rapa

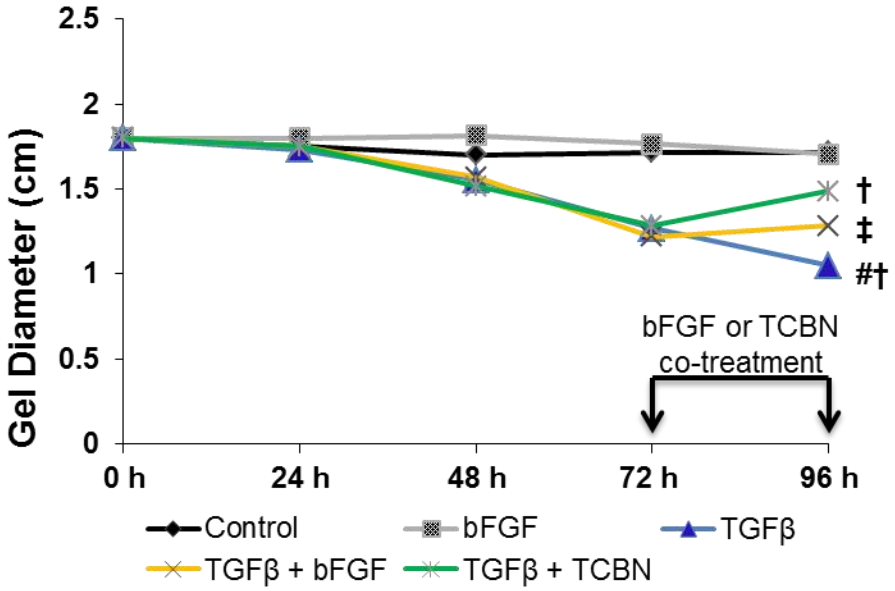
FIGURE 8.

Akt inhibition reverses TGFβ-mediated contraction of fibroblast-populated collagen lattices. *A,* after seeding NIH 3T3 fibroblasts in neutralized collagen solution, cells were serum starved and incubated with bFGF or TGFβ for 96 h or TGFβ for 72 h, and co-treated with bFGF or triciribine (*TCBN*) for an additional 24 h (total 96 h) to examine the de-differentiation effect. Degree of gel contraction was assessed daily. *B,* degree of contraction was compared at 96 h between groups. *C*, degree of de-differentiation/relaxation compared with 72 h (n = 3). #, compared with control; †, p = 0.0001 compared with TGFβ; ‡, p = 0.001 compared with TGFβ.

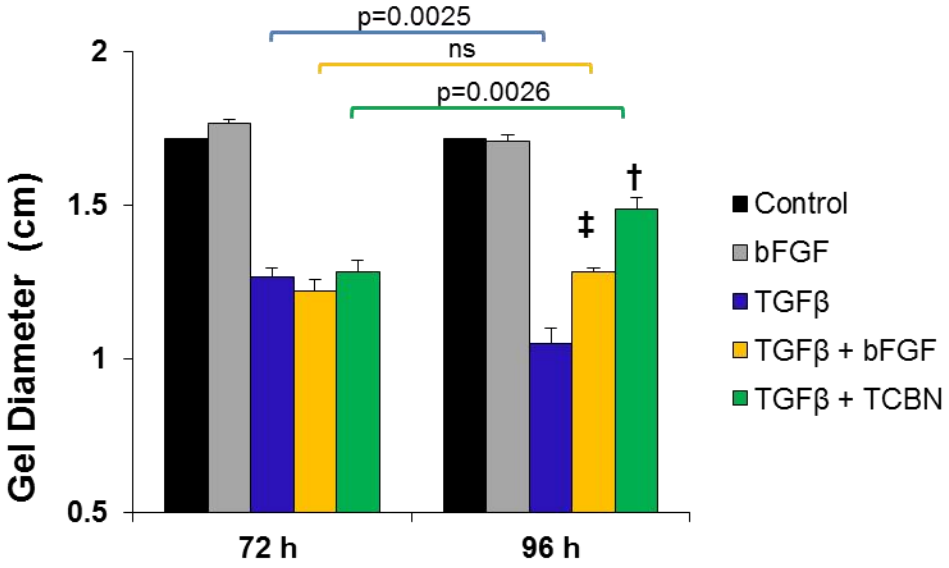
A


Control
bFGF
TGFβ
TGFβ + bFGF
TGFβ + TCBN

B


Gel Diameter (cm)
2.5
2
1.5
1
0.5
0
0 h
24 h
48 h
72 h
96 h
†
‡
#†
bFGF or TCBN
co-treatment
Control
bFGF
TGFβ
TGFβ + bFGF
TGFβ + TCBN

C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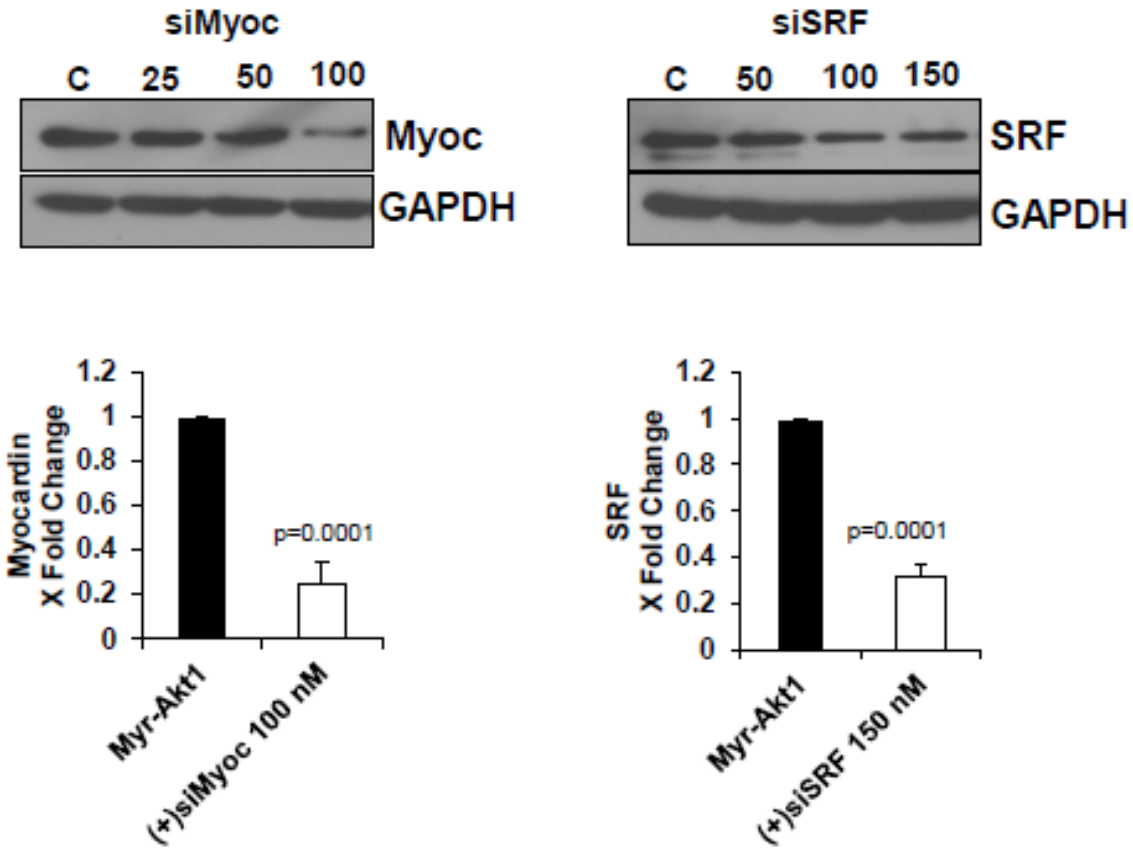
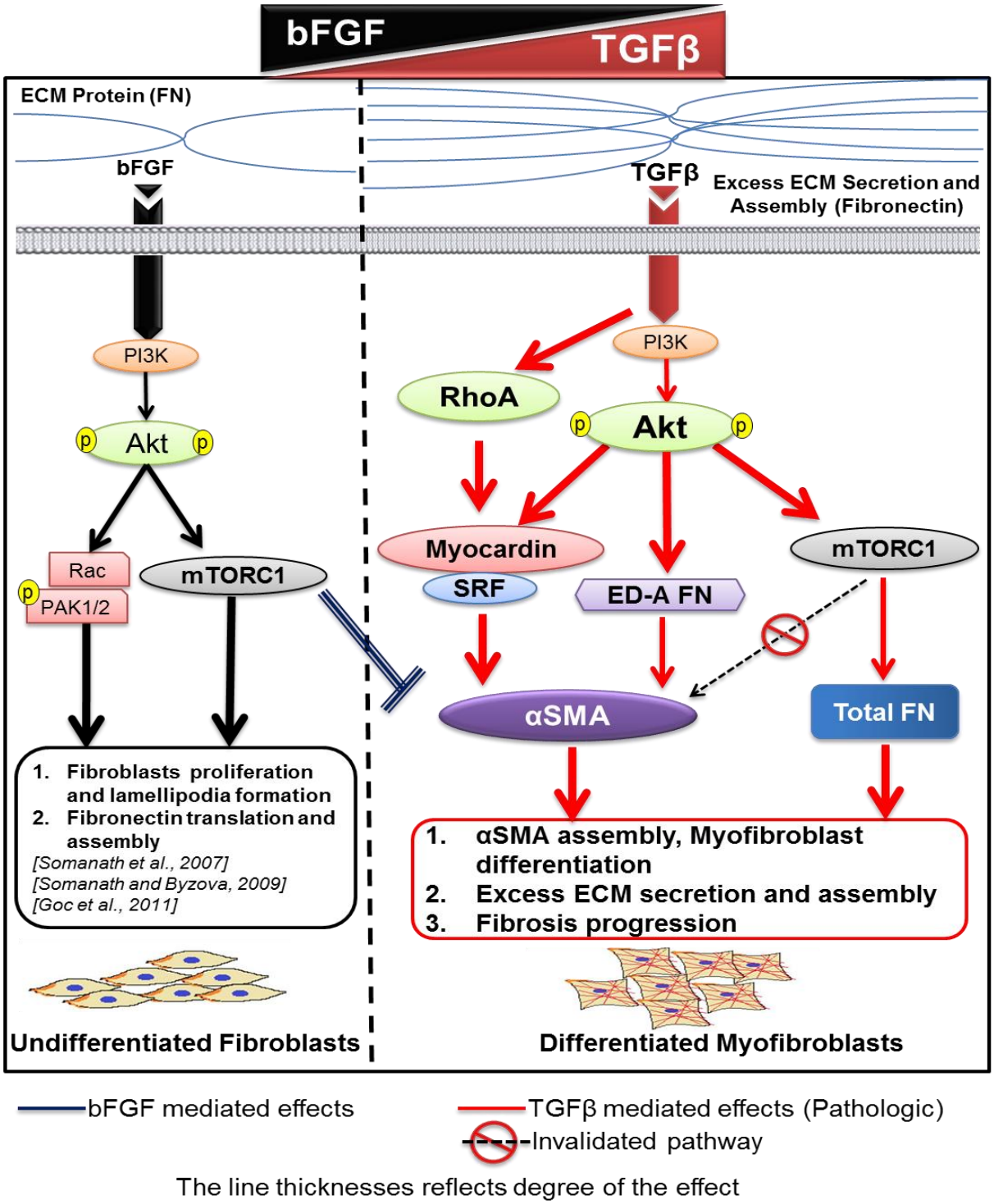
TGFβ
TGFβ + bFGF
TGFβ + TCBN

Supplemental Figure 1: Degree of gene silencing of SRF and Myocradin in myr-Akt1 NIH 3T3. MyrAkt1 expressing cells, which expresses higher concentrations of SRF and Myocardin were utilized for the standardization of SiRNAs. Optimum knockdown was observed at 100 nM of SiMyocardin and 150 nM of SiSRF. Approximately 70% knockdown was obtained with each of the SiRNAs. Upper panel shows representative Western blots and the lower panel shows densitometry OD values (n=3).



Supplemental Figure 2. Schematic representation of the working hypothesis



CHAPTER 3

TRICIRIBINE, A SELECTIVE AKT INHIBITOR, AMELIORATES TGFβ- AND HYPOXIA-INDUCED PULMONARY FIBROSIS AND VASCULAR REMODELING

Abdalla M, Prakash R, Ergul A, Pruitt A, Fagan S.C., Somanath P.R. Triciribine, a Selective Akt Inhibitor, Ameliorates TGFβ- and Hypoxia Induced Pulmonary Fibrosis and Vascular Remodeling. 2014 (To be submitted to Circulation Research)

ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is an incurable, chronic and progressive disease with severely poor prognosis and often leads to pulmonary hypertension (PH). Myofibroblast (MFs), marked by de novo expression of αSMA stress fibers, are central orchestrators of IPF and associated vascular remodeling. We have previously shown that Akt1 is necessary for MF differentiation. Recently, it has been shown that Akt, a major survival protein, is upregulated in IPF patients; however its role remains unclear. Here we investigated 1) whether Akt isoform 1 mediates the pathogenesis of IPF; and 2) the efficacy of Triciribine (TCBN), a selective Akt inhibitor as a potential therapeutic option for IPF compared to the mTOR inhibitor rapamycin in two rodent models of the disease and in IPF fibroblasts. Our results show that Akt1 deficient mice are protected from hypoxia induced pulmonary fibrosis and remodeling. In contrast, hyperactivation of Akt1 induced severe focal fibrosis. TCBN reversed adTGFβ- and hypoxia-induced IPF and vascular remodeling, respectively, compared to Rapamycin. Mice treated with TCBN had markedly lower tissue dense infiltration and fibrosis, lower adventitial and medial remodeling, lower αSMA and ECM expression. Conversely, Rapamycin exacerbated IPF, pulmonary vascular remodeling and right ventricular remodeling and was associated with exacerbated hepatotoxicity. Mechanistically, rapamycin induced Akt activation and was associated with elevated thrombospondin-1 expression. Collectively, we identify Akt1 as a novel target as it is crucial in mediating IPF and associated vascular remodeling. Thus, targeting Akt, not mTOR, may serve as a favorable therapeutic strategy in advanced stage IPF.

INTRODUCTION

Idiopathic Pulmonary fibrosis (IPF) is an incurable, chronic and progressive lung disease with severely poor prognosis and limited therapeutic options [1]. Lung transplantation remains the only non-medicinal therapy with proven efficacy on survival in IPF patients [2, 3]. Thus, identification of novel therapeutic targets is critical in the hopes of devising new treatments. IPF is characterized by proliferating myofibroblasts and excess deposition of extracellular matrix (ECM) proteins leading to progressive hypertrophic scar formation [4]. Most IPF patients develop secondary pulmonary hypertension, which is an independent risk factor for increased mortality in IPF and is characterized by obliterative vascular remodeling [1, 5]. It has been shown that IPF patients exhibit hyperactivation of Akt in IPF lung [6]. However, whether Akt is critical for the pathogenesis of IPF and associated vascular complication remains unclear.

Recently, we have established the crucial role of Akt1 in myofibroblast differentiation [7]. We have also demonstrated that Akt1 is necessary for, ECM regulation [7, 8] and vascular integrity [9]. Furthermore, it has been shown that mice deficient in phosphatase and tensin homologue (PTEN), which normally inactivates Akt pathway, developed fibroproliferative response consistent with fibrosis [6]. Since several features of IPF resemble deregulated Akt-mediated processes, we sought to investigate the contributory role of Akt, Akt1 in particular, in IPF and associated vascular remodeling. Recent clinical report on everolimus, a rapamycin derivative that targets mTOR downstream of Akt, exacerbated IPF and was associated with worsened outcome [10]. Thus, we also set out to investigate the therapeutic implications of targeting Akt using the selective inhibitor Triciribine [11] compared to targeting mTOR using rapamycin.

Herein, we report three major novel findings on the role of Akt1 in IPF pathogenesis and as a therapeutic target to manage the disease. The results of our study 1) identified Akt as a novel target as it is crucial for hypoxia-induced pulmonary fibrosis and vascular remodeling, and its hyperactivation induced severe focal pulmonary fibrosis; 2) determined novel anti-fibrotic and anti-remodeling properties of Triciribine. Thus, it can potentially be a therapeutic option for IPF; 3) determined that rapamycin induces severe pulmonary and hepatic toxicities through feedback activation of Akt1/TSP1 axis. This also sheds light on the observed detrimental effects of everolimus, a rapamycin derivative, in IPF patients.

EXPERIMENTAL PROCEDURES

Antibodies

Anti-αSMA (cat# SAB2500963) and anti-fibronectin (cat#F6140) antibodies were purchased from Sigma (St Louis, MO). GAPDH (cat#2118), phospho-Akt (s473) (cat# 9271), phosphoT389-p70S6K (cat#9205), phosphoT37/46-4E-BP1 (CAT#2855), and anti-SRF (cat# 5147) antibodies were purchased from Cell signaling (Boston, MA). Anti-myocardin (cat# MAB 4028) antibodies were purchased from R&D (Minneapolis, MN). Collagens type I and III antibodies were purchased from Rockland (Gilbertsville, PA). Anti-ED-A fibronectin (cat# 6328), anti-Collagen VI (ab6588), and anti- αSMA (cat#5694) antibodies were purchased from Abcam (Cambridge, MA).

Cell Culture

Normal and fibrotic human lung fibroblasts (HLFs) were purchased from Lonza. Normal HLFs were cultured on a 6-well plate. After reaching 70% of confluence, cells were

subjected to serum starvation in the presence or absence of 100 pM TGFβ (a pre-determined dose (Goc et al, 201)) for 48 h. This was followed by co-treatment for 24 h (total 72 h) with inhibitors of PI3Kinase (25 μM LY294002), Akt (10 nM triciribine), mTOR (25 nM rapamycin), or SRF/Rho (1 μM CCG1423). Cells were subjected to western analyses as described below. For fibrotic HLFs, cells were cultured in serum starvation in a 6-well plate and treated with inhibitors of Akt (10 nM triciribine) or mTOR (25 nM rapamycin) for 24 h. Cells were subjected to western analyses as described below

Western blot analysis

Cell lysates were prepared using lysis buffer (20mM Tris- HCl, pH7.4; 1% Triton X-100, 3mM EGTA, 5mM EDTA, phosphatase inhibitors [10mMsodium pyrophosphate, 5mM sodium orthovanadate, 5mM sodium fluoride, and 10μM okadaic acid], protease inhibitor cocktail [Roche Diagnostics, Basel,Switzerland] and 1mM PMSF). SDS-PAGE and western blotting were performed.

Animals

All experiments were performed with approval by the Charlie Norwood VAMC Institutional Animal Care and Use Committees. $Akt1^{-/-}$ mice were generated as previously described [9] and were maintained in the C57BL/6 background. Age-matched wild-type and $Akt1^{-/-}$ were utilized in two models of the disease as described below.

Animal models

To study the degree of pulmonary fibrosis, WT mice were subjected to intra-tracheal adenovirus control or TGFβ at day 0. After 7 days, saline, TCBN, or Rapa was

administered IP daily for 3 additional days (n=6-8 mice/group). To assess fibrosis accompanied with vascular remodeling, mice were subjected to normoxia or hypoxia (10% O2) (Biospherix, New York, NY) for 14 days, followed by IP administration of saline, TCBN, or Rapa for 7 days under the same oxygen conditions. TCBN was dissolved in carboxymethylcellulose and PEG-400, and Rapamycin in 100% Ethanol. Both were then diluted (1:1000) in PBS. Total daily IP injection is 100 ul (TCBN: 0.5 mg/kg/day; Rapa: 1.5 mg/kg/day). Pharmacological inhibitors were administered daily while the mice were maintained in hypoxia chamber to minimize exposure to air and spontaneous reversal of vascular remodeling. To establish the hypoxic environment, the chamber was flushed with nitrogen. The chamber was opened once per week for no more than 1 hour to clean the cages and replenish food and water supplies. After 21 days, mice were euthanized; lungs were isolated and subjected to analyses (n=6-8 mice/group).

We conducted a drug-drug comparison of TCBN vs Rapa and drug-placebo comparison to provide a more comprehensive and clinically relevant study. Following completion of these studies, mice were euthanized by C1-C2 cervical dislocation. After lung isolation, the left lung was subjected to histology for H&E, Mason Trichrome, and immuno-fluorescence staining, and the right lung was subjected to western analyses.

Pulmonary vascular casting

Mice were anesthetized with ketamine/xylazine, then microfil casting agent (Flowtech, 1:2 dilution + 3.2% curing agent) was infused into the pulmonary artery through the right ventricle. Lungs were fixed in 4% paraformaldehyde overnight, and then cleared with

ethanol-methylsalicylate per the manufacturer's instructions. Peripheral vasculatures were imaged using light microscopy. (n=4-8 mice/group; 6 groups/disease model)

Fibrosis Score – Quantitative and semi-quantitative (Ashcroft Score) methods

IPF Classification: quantitative evaluation of fibrotic changes (Ashcroft scale) was obtained as follows, 1) the severity of the fibrotic changes in each lung section was given as the mean score from the observed microscopic fields; 2) 8-10 fields/lung section were quantified, and each field was assessed individually for severity and allotted a score from 0 (normal) to 8 (total fibrosis) (6-8 mice/group); 3) In ImageJ, the threshold (severity score) for each field were averaged and are presented as the average for each lung section; 4) the mean of their individual scores (both based on threshold and Ashcroft Scale) was taken as the fibrotic score

Histologic and immunohistochemical assessments

Left lung lobe, heart, and liver tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 μm thickness. The severity of pulmonary fibrosis was scored (0–8 scale) in lung sections stained for collagen with Masson’s trichrome stains using the grading system described by Ashcroft et al. (Ashcroft et al. 1988). The sections were stained with H&E to determine inflammatory cell infiltration. Immunostaining for αSMA and fibronectin was performed and validated by western analyses. Furthermore, for vascular remodeling studies, sections from each animal were stained with hematoxylin and eosin and Masson’s trichrome and examined by digital photomicroscopy at various magnifications to determine the severity of the disease. We analyzed 10 to 15 peripheral pulmonary vessels/lung/mouse/group and determined medial wall/lumen diameter ratio. Immunohistochemical/

immunofluorescence for αSMA and fibronectin were confirmed with western analyses. We have measured the right ventricular wall thickness in heart section (average of 8-10 measurements/animal/group. Finally, liver sections we assessed semiquantitatively using the threshold/ImageJ (as described above in Fibrosis Score section) and the percent area in 8-10 sections/liver section/mouse/group was assessed.

Statistical analysis

Data are presented as means ± SD. We performed all the analyses using two-way ANOVA on the ranks of the data were used to compare control, insult (hypoxia or adTGFβ), and control/insult plus TCBN or Rapamycin for all variables. T-tests with a two-tailed distribution were used to compare control and Akt1 deficient mice on all variables. Statistical significance was determined at alpha=0.05.

RESULTS

Akt1$^{-/-}$ mice are resistant to chronic hypoxia-induced pulmonary remodeling

We previously showed that Akt1 is necessary for myofibroblast differentiation, ECM assembly, and maintaining vascular integrity. Since several of these events are deregulated in IPF, we hypothesized that Akt1 modulates fibrogenesis leading to pulmonary fibrosis and secondary vascular remodeling.

To investigate whether Akt signaling is a requisite for the development of pulmonary fibrosis and vascular remodeling, we examined the patterns of morphological changes of Akt1 knockout mice subjected to hypoxia over a period of 2 weeks compared to *WT* mice. Interestingly, normoxic Akt1 deficient mice exhibited morphological features similar to 14 d-hypoxic *WT* mice (Figure 1 A - C). We postulated that this could be due

to a compensatory response possibly through Akt2. Indeed, Akt2 expression was significantly increased in Akt1 deficient mice compared to *WT* mice (Supplemental Figure 1).

We first examined the course of morphological alteration due to hypoxia over a period of 7 days and 14 days. Wild type mice subjected to hypoxia developed patterns consistent with disease progression as evident by patchy fibrotic lesions and mild vessel luminal narrowing and medial thickening at day 7 that progressed to diffuse fibrosis, increased collagen deposition and marked vascular remodeling at day 14 (Figure 1A). In contrast, hypoxic Akt1$^{-/-}$ mice showed improved histopathological patterns consistent with disease regression as evident by decreased fibrotic lesions, collagen deposition, and vascular remodeling at days 7 and 14 (Figure 1A).

We then evaluated the degree of tissue fibrosis and vascular remodeling of 14d-hypoxic Akt1$^{-/-}$ compared to *WT* mice. Despite chronic hypoxia exposure, Akt1$^{-/-}$ mice had minimal fibronectin accumulation in the interstitial space, which was confirmed with western analyses and indicates resistance to tissue fibrosis (Figure 1B and D). Furthermore, 14d-hypoxic Akt1$^{-/-}$ mice had normal appearing pulmonary arterioles as evident by the absence of medial thickening and luminal narrowing, decreased αSMA assembly, and decreased fibronectin deposition (Figure 1C).

Several lines of evidence have demonstrated that the matricellular protein thrombospondin-1 (TSP1), a potent activator of TGFβ, is an important mediator of tissue fibrosis, matrix assembly, and vascular remodeling and pruning. We have previously shown that Akt1 directly regulates the expression of TSP1. Accordingly, we hypothesized that the protective effects observed in hypoxic Akt1 deficient mice is due

to decreased TSP1 expression. Indeed, western analyses revealed that the increased TSP1 expression in hypoxic *WT* mice is blunted in 14D-hypoxic Akt1$^{-/-}$ mice (Figure 1E). Collectively, our results demonstrate that the absence of Akt1 impedes chronic hypoxia-induced pulmonary fibrosis and remodeling through TSP1.

Sustained activation of Akt1 induces severe focal pulmonary fibrosis

To further confirm the causal role of Akt1 in pulmonary fibrosis, we subjected WT mice to a two-hit intratracheal administration of adenovirus gene transfer of control vector, TGFβ (major pro-fibrotic cytokine in IPF), or constitutively active Akt1 (myr-Akt1), as described in Methods. We found that sustained hyperactivation of Akt1 induced severe focal fibrosis with honeycomb formation similar to that observed in TGFβ treated mice (Figure 1F). Collectively, by utilizing two rodent models of the disease, our data demonstrate that Akt1 is necessary for the onset and progression of pulmonary fibrosis and associated remodeling.

Triciribine, a selective Akt inhibitor, attenuates myofibroblast differentiation in TGFβ-stimulated human lung fibroblasts

Once we identified Akt1 as a critical mediator of pulmonary fibrosis, we set out to investigate the therapeutic potential of targeting Akt pathway in myofibroblasts (MFs), which are the central orchestrators of IPF. Persistent myofibroblasts (MFs) differentiation, marked by *de novo* expression of αSMA, leads to progressive deposition of excess ECM; both events are cardinal features in IPF. We previously showed that Akt1 is necessary for MF differentiation and persistence in mouse embryonic fibroblasts (Abdalla et al, 2013); herein we aimed to delineate its role in lung specific fibroblasts isolated from normal subjects and IPF patients. We first determined that 72 h is the

optimal time for TGFβ-induced MF differentiation in primary normal human lung fibroblasts (HLFs), as measured by increased αSMA expression, and was associated with elevated fibronectin expression and Akt phosphorylation (Supplemental Figure 2 A-D). We and others have shown that αSMA synthesis is transcriptionally regulated by serum response factor (SRF) and its co-activator, myocardin. Thus, to determine the role of Akt pathway on αSMA synthesis, we subjected normal HLFs to serum starvation in the presence or absence of TGFβ stimulation for 48 h, and co-treated for an additional 24 h with inhibitors of PI3K (25 μM LY294002), Akt (10 nM triciribine (TCBN)), mTOR (25 nM rapamycin) and Rho signaling-responsive SRF (1 μM CCG1423). As expected, TGFβ stimulation induced a significant increase in αSMA expression compared to unstimulated HLFs, indicating MFs differentiation; this was associated with increased myocardin and SRF expressions (Figure 2A-D). The stimulatory effects of TGFβ significantly decreased upon inhibiting PI3K and Rho-responsive SRF. Notably, while targeting Akt using TCBN abolished αSMA-myocardin-SRF expressions, targeting mTOR using rapamycin did not induce any discernible effect on their expressions (Figure 2A-D). Together, our results demonstrate that αSMA synthesis and MF differentiation is mediated by PI3K/Akt pathway, independent of mTOR. Importantly, the observed difference between TCBN and rapamycin suggests that rapamycin may have unfavorable therapeutic consequences.

Triciribine, not rapamycin, downregulates both MF differentiation and fibronectin in IPF fibroblasts

In view of clinical reports that the rapamycin derivative, everolimus, exacerbated disease progression in IPF patients along with our findings that MF differentiation is

mediated by unrestrained Akt signaling independent of mTOR, prompted us to conduct a drug-drug comparison in fibrotic human lung fibroblasts (FHLFs) isolated from IPF patient. We subjected FHLFs to serum starvation and treated with 10 nM TCBN or 25 nM rapamycin for 24 h, and analyzed αSMA, fibronectin, and TSP1 expression levels. Targeting Akt using TCBN significantly reduced αSMA, fibronectin, and TSP1 expressions in FHLFs (Figure 2 E-H). We confirmed further confirmed the regulatory role of Akt on TSP1 by conducting a gene silencing study in FHLFs and TGFβ-stimulated mouse embryonic fibroblasts. Inactivating Akt1 resulted in decreased TSP1 gene expression in both cell types (Supplemental Figure 2 E).

In contrast, rapamycin did not modulate their expressions (Figure 2E-H). This supports our previous studies and demonstrates that although rapamycin targets ECM proteins, MF differentiation still occurs via unopposed transcriptional activation of αSMA. Consequently, MFs produce excess ECM resulting in persistent differentiation and abnormal ECM accumulation. On the other hand, TCBN exhibits dual role by targeting both MF differentiation and the ECM. This suggests that targeting Akt, not mTOR, may serve as a favorable therapeutic strategy in IPF.

Triciribine ameliorates TGFβ1-induced pulmonary fibrosis, while rapamycin induces severe structural abnormalities in vivo

The distinct opposing effects of TCBN and rapamycin observed *in vitro*, led us to investigate their therapeutic implications *in vivo* utilizing disease mouse model of adenovirus TGFβ-induced PF. It is well established that TGFβ1 is a pro-fibrotic master cytokine in IPF. In addition to the canonical pathway, TGFβ also elicits signaling responses through non-canonical activation of Akt signaling. Notably, Akt has been

shown to be hyperactivated in fibrotic foci in IPF lung. Therefore, we hypothesized that TCBN may effectively manage pulmonary fibrosis by disrupting TGFβ/Akt signaling, compare to rapamycin. To test our hypothesis, we established an experimental severe focal pulmonary fibrosis model induced by TGFβ1 overexpression using intratracheal adenoviral gene transfer. Mice were subjected to a one time intratracheal administration of either control empty vector or adenovirus TGFβ1 (adTGFβ), then, on day 7, both control and adTGFβ mice were treated daily for 3 days (total study duration: 10 days) with PBS (saline), TCBN (0.5 mg/kg/day), or rapamycin (Rapa; 1.5 mg/kg/day). Noteworthy, a recent study reported that DMSO exerts anti-fibrotic properties. Thus, to exclude false positive results, TCBN was dissolved in carboxymethylcellulose (CMC) and PEG-400 solution and Rapa in 100% ethanol. Both were then diluted in PBS (1:1000). To ensure that the solvents do not affect the outcome of our studies, mice were injected IP for 3 days with CMC/PEG and 100% ethanol both diluted in PBS (1:1000). Examination of lung sections from both groups showed normal histology similar to control group (data not shown).

AdTGFβ administration induced marked destruction of normal pulmonary architecture that correlated with an Ashcroft fibrosis score of ~7.6, and resulted in heterogeneous pattern (both spatial and temporal) consistent with advanced stage IPF (Figure 3A-C). TGFβ induced substantial interstitial fibrosis, extensive deposition of fibrillar collagen, loss of alveolar parenchyma, microscopic honeycomb foci, as indicated by H&E and Masson's trichrome staining, and % fibrosed area. This was also associated with marked increase in fibronectin and αSMA assembly as demonstrated by immunofluorescence staining (Figure 3 A-F). Strikingly, TCBN administration reversed

the adTGFβ-induced pathological structural changes, and correlated with an Ashcroft fibrosis score of ~1.2 (categorized as minimal fibrosis) (Figure 3F). This is evident by markedly decreased fibrotic lesions, deposition of collagen fibers, fibronectin, and αSMA (Figure 3A-D).

The functional role for TCBN in the blockade of adTGFβ-induced pulmonary fibrosis was further demonstrated at the protein levels by western blot analysis. TCBN significantly decreased the stimulatory effects of adTGFβ on αSMA and its transcription factors (Figure 4A), TSP1 and other ECM protein expression (collagen I, III, and VI, and fibronectin splice variant (ED-A FN)) (Figure 4B). These effects were associated with decreased phosphorylation of Akt, P70S6K1, and 4EBP1 (Figure 4C and D). Noteworthy, TCBN increased the phosphorylation of βCatenin (Figure 4C and D). This suggests that in addition to targeting the ECM and the transcriptional regulation of αSMA, flagging the pro-fibrotic βCatenin signaling for degradation may constitute another mechanism by which TCBN exerts its anti-fibrotic effects.

In contrast, although rapamycin treatment correlated with decreased fibrotic lesions and improved Ashcroft fibrosis score of ~5 (Figure 3B and F), it induced thickening of the alveolar space, diffuse alveolar microhemorrhage, capillary congestion, and dense inflammatory infiltrates as evident by H&E and Masson's trichrome staining (Figure 3A and B). Notably, even in the absence of adTGFβ, control mice treated with rapamycin, exhibited similar pulmonary toxicity effects (Figure 3A-F). Western analyses revealed that although rapamycin markedly decreased collagen I expression, it did not modulate collagen III, VI, or ED-A FN (Figure 4B). Importantly, rapamycin alone induced increased TSP1 expression to the same extent as adTGFβ; this effect was amplified in

adTGFβ stimulated mice. This further supports the notion of unopposed Akt activation in response to rapamycin. Additionally, no discernible effect was observed on αSMA and its transcription factors (Figure 4A). Although rapamycin significantly decreased mTOR pathway signaling, it did not modulate the stimulatory effects of adTGFβ on Akt phosphorylation (Figure 4C). Even in the absence of adTGFβ, Akt phosphorylation is increased with rapamycin treatment (Figure 4C). This suggests that rapamycin activates a pro-fibrotic feedback loop as a result of switching off the inhibitory effect of S6K1 on TGFβ signaling. This is further amplified by the accumulation and activation of the pro-fibrotic βCatenin pathway (Figure 4D). Collectively, our results reveal novel anti-fibrotic properties of TCBN in pulmonary fibrosis marked by upregulated TGFβ signaling *in vivo,* whereas rapamycin attains a potentially detrimental pulmonary toxicity profile through feedback activation of Akt/TSP1 axis.

Triciribine ameliorates chronic hypoxia-induced pulmonary vascular remodeling, while rapamycin induces severe structural abnormalities in vivo

Since IPF is often accompanied by vascular remodeling, which contributes to the development of secondary complications such as pulmonary hypertension, we sought to investigate the therapeutic effects of TCBN compared to rapamycin in modulating these vascular complications. To do this, we utilized the well-established model of chronic hypoxia-induced pulmonary vascular remodeling. Mice were subjected to 21 days of hypoxia (10% oxygen) or normoxia, and treated from days 14 to 21 with PBS (Saline), TCBN (0.5 mg/kg/day), or Rapa (1.5 mg/kg/day) IP daily under the same conditions (normoxia or continuous hypoxia). Chronic hypoxia induced marked vascular remodeling as evident by increased medial thickness of peripheral pulmonary arterioles

(Figure 5D and E), which correlated with increased collagen, fibronectin, and αSMA deposition as demonstrated by H&E, Masson's trichrome, and immunofluorescence staining (Figure 5 B-D). This was also confirmed at the protein level where hypoxia increased the levels of αSMA and its transcription factors (SRF and myocardin) (Figure 5F), and ECM proteins (Figure 5G). TCBN treatment reversed vascular remodeling as it markedly reduced the stimulatory effects of hypoxia as evident by immunohistochemistry and western analyses (Figure 5A-G). On the other hand, rapamycin exacerbated hypoxia-induced pulmonary vascular remodeling and resulted in alveolar hemorrhage and congestion (Figure 5A-G). Noteworthy, under normoxic conditions, rapamycin alone induced dense inflammatory infiltration and vascular remodeling (5A-D). Collectively, these findings reveal novel anti-remodeling effects of TCBN, whereas rapamycin is likely to exacerbate vascular remodeling.

Triciribine reverses hypoxia-induced right ventricular remodeling and hepatotoxicity

The hypoxia-induced pulmonary vasculopathies often lead to right ventricular hypertrophy. Thus, we next determined the effects of TCBN compared to rapamycin on right ventricular wall thickness in mice subjected to 21 d hypoxia or normoxia. Under normoxic conditions, neither TCBN nor rapamycin induced any discernible effects on wall thickness (Figure 6A and B). Hypoxia induced a modest but significant increase in right ventricular wall thickness compared to normoxic mice, which was reversed by TCBN treatment (Figure 6 A and B). However, rapamycin treatment was associated with enlarged heart (Supplemental Figure 3) and increased right-sided wall thickness (Figure 6A and B).

Next, we assessed the safety profile of both drugs in the liver of mice subjected to chronic hypoxia. Compared to TCBN, chronic rapamycin treatment was associated with significantly enlarged liver weight (Supplemental Figure 3). Liver histology revealed that TCBN improved hypoxia-induced hepatic injury. In contrast, rapamcyin resulted in pronounced liver toxicity. This is evident by marked degeneration and atrophy of hepatic cords and sinusoidal dilation (Figure 6 C and D).

Triciribine prevents hypoxia- and adTGFβ- induced vascular rarefaction, while rapamycin exacerbates them

The above findings prompted us to examine the aberrant vascularization that occurs during pulmonary fibrosis, and determine the effects of TCBN and rapamycin on the vascular tree. To do this, we utilized arterial casting to visualize the vascular tree of mice subjected hypoxia or normoxia. Compared to the diffuse vascular blush observed in normoxic mouse lung, hypoxia resulted in severe vascular pruning as demonstrated by binary images and high magnification of peripheral vessels (Figure 7A and C). TCBN prevented progressive pruning of the vasculature, which supports our previous finding that TCBN alleviates vessel occlusion (Figure 7A and C). In contrast, rapamycin treatment was associated with severely reduced vascular density and marked small-vessel pruning as evident by high magnification images. Noteworthy, while TCBN did not modulate vascular density in normoxic mice, rapamycin was associated with decreased vascular filling and increased vessel pruning (Figure 7A and C).

To further confirm the above findings, we studied the vascular bed of adTGFβ-induced pulmonary fibrosis. To do this, we subjected mice to either adenovirus control vector or adTGFβ and treated with TCBN, Rapa, or saline from days 3 to 7; on day 8, lungs were

casted and isolated. To assess the drug response, we also isolated lungs of adTGFβ mice on day 4 (24 hours post saline treatment). AdTGFβ markedly reduced pulmonary vascularization and was associated with severe pruning of small vessels on day 8 compared to control (Figure 7B and D) and to 4d adTGFβ (Supplemental Figure 4).TCBN treatment maintained the vasculature (Figure 7B and D and Supplemental Figure 4), whereas rapamycin treated mice showed blunting of the vasculature (Figure 7B and D). Collectively, these results demonstrate the therapeutic efficacy of TCBN in reversing the progression of pulmonary fibrosis and remodeling, whereas rapamycin is likely to exacerbate disease progression and the development of secondary complications.

DISCUSSION

Although it has been shown that Akt is hyperactivated in fibroblastic foci of IPF lung, a causal link has not been established. Here, we report several novel observations: (1) Akt1 is a crucial mediator of pulmonary fibrosis and vascular remodeling; (2) the specific Akt inhibitor Triciribine is a novel therapeutic option to reverse/halt progressive TGFβ- and hypoxia- induced pulmonary fibrosis and vascular remodeling and rarefaction; (3) targeting mTOR using rapamycin induces Akt/TSP1 activation and is associated with severe side effect profile consisting of pulmonary toxicity, exacerbated vascular and cardiac remodeling, and severe hepatotoxicity; this sheds light on the observed detrimental effects of everolimus, a rapamycin derivative, in IPF patients.

The pathologic hallmarks of IPF include persistent myofibroblasts differentiation and excess matrix deposition. Additionally, vascular remodeling and occlusion often takes

place during tissue fibrosis. We and others have shown that Akt is necessary for MF differentiation, ECM secretion and assembly, apoptosis, fibroblast migration and proliferation, and vascular integrity [8, 12-16]. Since events in IPF and vascular complication are consistent with deregulated Akt-mediated cellular and molecular processes, we investigated the contributory role of Akt in IPF. In addition to utilized normal and fibrotic human lung fibroblasts, we also utilized two animal models: chronic hypoxia- [17] and adenovirus TGFβ- [18] induced severe pulmonary fibrosis.

The results showed that Akt1 deficient mice are protected from hypoxia-induced vascular remodeling and fibrosis as evident by markedly reduced interstitial collagen, fibronectin, and αSMA deposition, reduced luminal space and medial thickening of pulmonary arterioles. Interestingly, it has been shown that thrombospondin1 deficiency protect against hypoxia-induced pulmonary hypertension [19]. This prompted to postulate that the observed decrease in ECM and overall fibrotic response is due to decreased TSP1 activation. Indeed, we found that hypoxic Akt1 deficient mice had blunted TSP1 expression which correlated with fibronectin expression. Similarly, silencing Akt1 activation in TGFβ-stimulated mouse embryonic fibroblasts, and in fibrotic human lung fibroblasts resulted in decreased TSP1 gene expression. This supports our previous finding that Akt1 control TSP1 [12] and corroborates previous reports on the regulatory role of TSP1 on fibronectin and the ECM [20-22]. Finally, we found that sustained hyperactivation of Akt1 resulted in focal severe fibrosis that resembled adTGFβ-induced pulmonary fibrosis consistent with IPF. Collectively, our results provide direct evidence on the causal role of Akt1 in disease onset and progression in pulmonary fibrosis.

Next, we set out to investigate the therapeutic implication of targeting Akt pathway using the pharmacological agent Triciribine (TCBN). Currently in phase trials in various cancers, the role of TCBN in fibrosis is unknown [11]. The observation that fibrotic human lung fibroblasts isolated from IPF patient were resistant to mTOR inhibition using rapamycin as evident by the lack of discernible effect on αSMA, fibronectin, and TSP1 expression, supports our previous finding that MF differentiation (potent producers of ECM) is Akt-dependent, independent of mTOR [7]. Thus, this prompted us to conduct a drug- drug study and investigate the efficacy and safety of TCBN and Rapamycin in adTGFβ- and hypoxia-induced pulmonary fibrosis and remodeling.

Our results identify novel anti-fibrotic and anti-remodeling properties of the specific Akt inhibitor Triciribine as evident by markedly reduced fibrotic lesions, myofibroblast and matrix accumulation, decreased medial thickening of pulmonary arterioles, and reduced vascular pruning, and right ventricular thickening. Mechanistically, we found that in addition to the transcriptional regulation of αSMA synthesis, TCBN also mediates its effects by targeting Akt/TSP1 axis, mTOR pathway, and increased phosphorylation of βCatenin (induces pro-fibrotic gene transcription [23]) flagging it for degradation. As for safety profile, TCBN markedly improved the hypoxia-induced cardiac remodeling and hepatic injury,

In contrast, rapamycin exacerbated the progression of pulmonary fibrosis and vascular remodeling and was associated with marked increase in alveolar congestion and micro-hemorrhage, severe pulmonary vascular rarefaction, right ventricular thickening and hepatotoxicity. The effects of rapamycin in fibrosis appear is controversial. Although studies have previously shown anti-fibrotic and anti-remodeling effects of rapamycin in

murine models [24-26], this could be attributed to the use of the dissolving agent DMSO, which is a potent anti-fibrotic compund [27], intermittent exposure to hypoxia which has been shown to reverse the vascular remodeling [28], or utilizing a high dose rapamycin that exceeds the maximum tolerated dose in humans. However, our results are consistent with clinical reports of pulmonary toxicities associated with rapamycin derivatives, sirolimus and everolimus [29-33]. Importantly, this finding sheds light on adverse effects observed of everolimus in IPF patients [10].

Mechanistically, we found that the adverse effects of rapamycin are attributed, at least in part, to increased Akt activation due to blocking the inhibitory effect of S6K1 on signal transduction, this also correlated with increased TSP1 expression. This corroborates previous reports of the rapamycin-induced activation of Akt [34-37]. Increased TSP1 has been shown to promote vascular rarefaction [38], and rapamycin has been reported to increase TSP1 activation [39]. Thus, this supports our results that observed rapamycin-induced severe vascular remodeling and pruning is mediated, at least, in part by TSP1 activation.

We also found that rapamycin does not transcriptionally regulate αSMA synthesis leading to MF differentiation and ECM production. This supports our previous studies [7] and demonstrates that although rapamycin targets ECM protein translation [8], MF differentiation still occurs via unopposed transcriptional activation of αSMA. Consequently, MFs produce excess ECM resulting in persistent differentiation and abnormal ECM accumulation leading to IPF progression. On the other hand, TCBN exhibits dual role by targeting both MF differentiation and the ECM. This suggests that targeting Akt, not mTOR, may serve as a favorable therapeutic strategy in IPF.

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FIGURE 1.

Akt1 deficiency protects against hypoxia-induced pulmonary fibrosis and remodeling, while its hyperactivation induces severe focal pulmonary fibrosis. (A) Masson's trichrome stained section of the pulmonary interstitium and peripheral pulmonary arterioles of *WT* and Akt1$^{-/-}$ mice subjected to normoxia or chronic hypoxia for 7 and 14 days (n=3-5 mice/group). Scale bar: 200 and 50 μM. (B and C) Fibronectin, αSMA, and laminin immunofluorescence staining in pulmonary interstitium and small pulmonary arteries of normoxic and 14d-hypoxic *WT* and Akt1$^{-/-}$ mice (n=4-5 mice/group). Scale bar: 100 and 25 μM. (D) Western blot analysis of total fibronectin and thrombospondin-1 expression levels in 14d-hypoxic *WT* and Akt1$^{-/-}$ mice (n=4-5 mice/group). Relative expression values obtained by densitometric analysis was normalized to GAPDH. (F) Masson's trichrome staining of *WT* mouse lung harvested 14 days after intratracheal adenovirus gene transfer of control vector, TGFβ, or myr-Akt1 (constitutive active Akt1) (n=3 mice/group). Scale bar: 100 μM

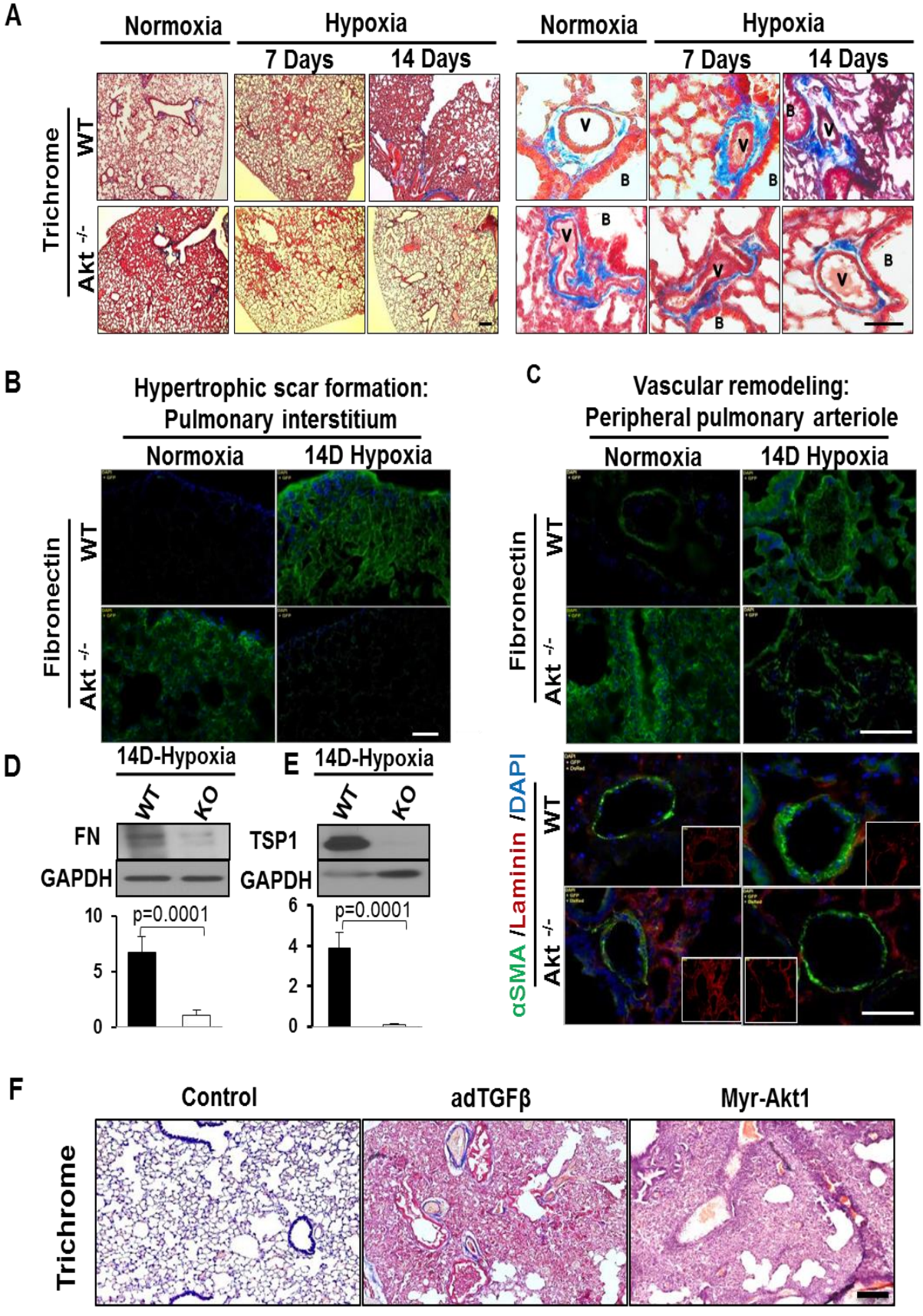

A
Normoxia
Hypoxia
7 Days
14 Days
Normoxia
Hypoxia
7 Days
14 Days
Trichrome
WT
Akt -/-
V
B
B
Hypertrophic scar formation:
Pulmonary interstitium
Normoxia
14D Hypoxia
Fibronectin
WT
Akt -/-
C
Vascular remodeling:
Peripheral pulmonary arteriole
Normoxia
14D Hypoxia
Fibronectin
WT
Akt -/-
D
14D-Hypoxia
WT
KO
FN
GAPDH
10
5
0
p=0.0001
E
14D-Hypoxia
WT
KO
TSP1
GAPDH
6
4
2
0
p=0.0001
αSMA /Laminin /DAPI
WT
Akt -/-
F
Control
adTGFβ
Myr-Akt1
Trichrome

FIGURE 2.

Targeting Akt, not mTOR, attenuates MF differentiation and fibronectin expression in human lung fibroblasts. (A-D) normal human lung fibroblasts grown to 70% confluence were treated with TGFβ for 72 h in the absence of FBS. To examine pathways governing MF differentiation, inhibitors were applied before significant differentiation occurred: cells were treated with TGFβ for 48 h and then co-treated with inhibitors for an additional 24 h targeting the PI3K/Akt/mTOR pathway and responsive SRF pathway (LY294002, triciribine (TCBN), rapamycin, and CCG1423, respectively). Lysates were prepared and subjected to western analyses with antibodies against αSMA (B), myocardin (C), SRF (D) (n = 4). *, compared with TGFβ; ‡, compared with control untreated cells. (E-H) fibrotic human lung fibroblasts grown to 70% confluence in the absence of FBS were treated TCBN or rapamycin for 24 h. Lysates were prepared and subjected to western analyses with antibodies against αSMA (F), fibronectin (G), thrombospondin-1 (H) (n = 3).

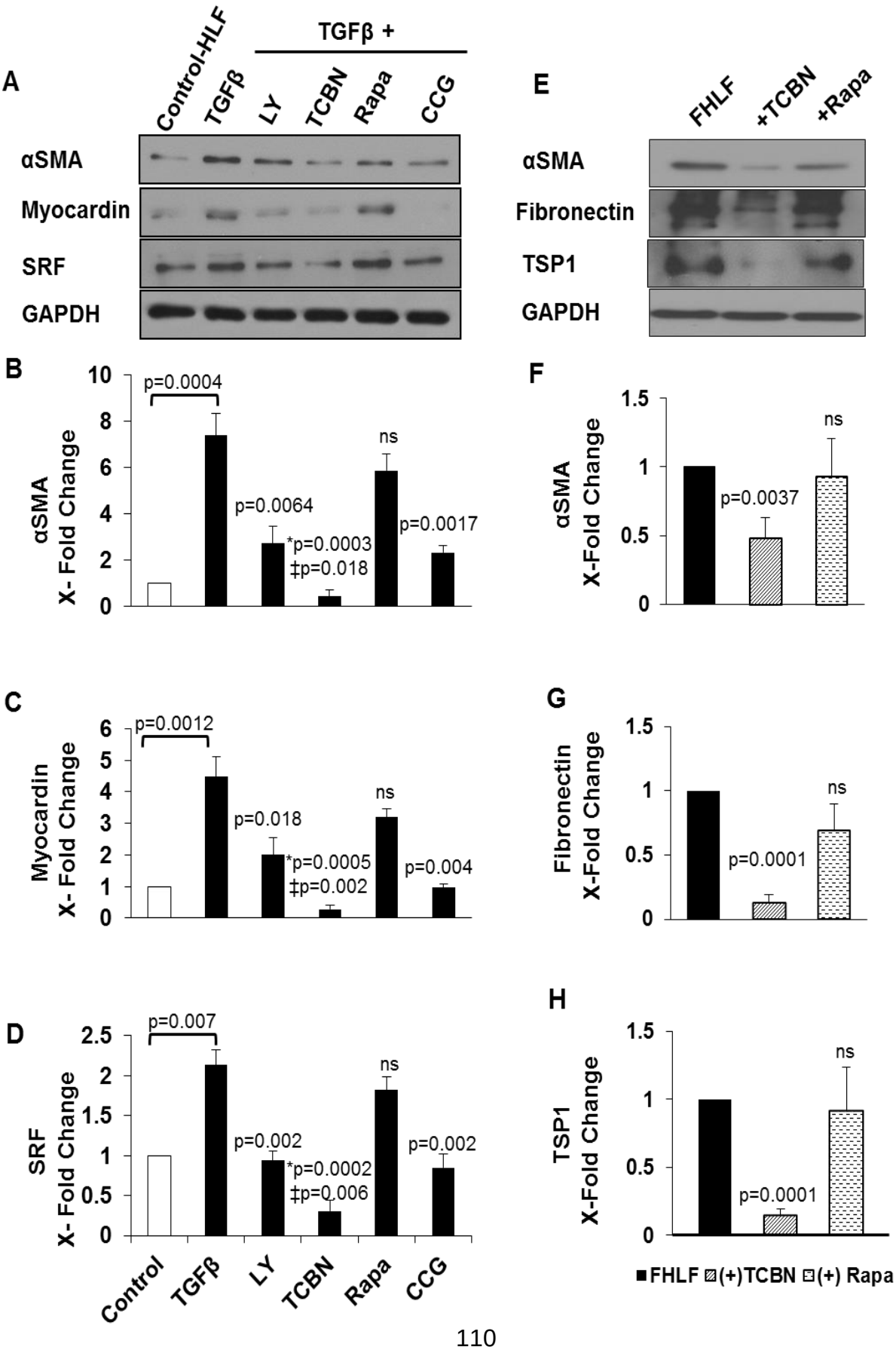
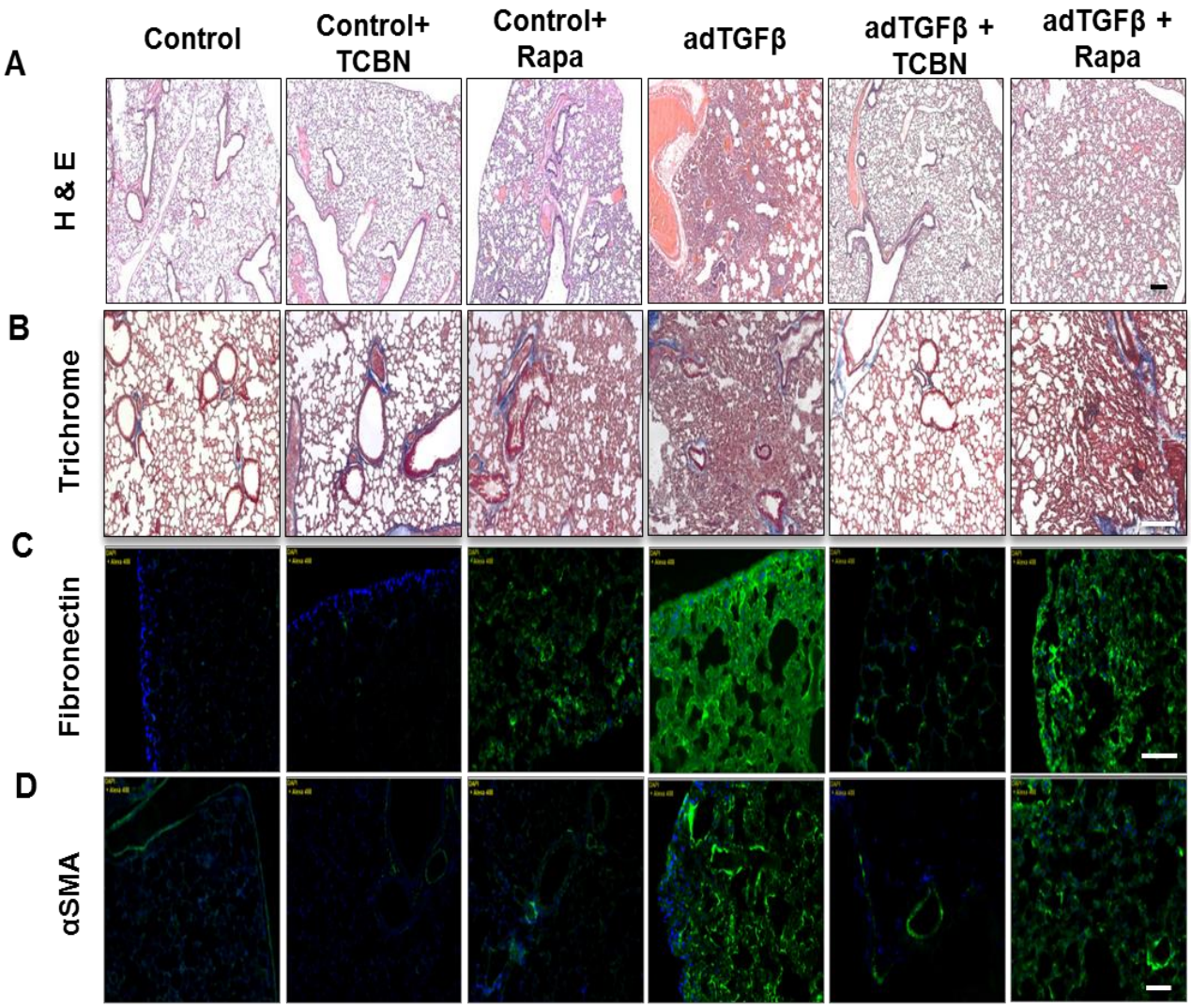
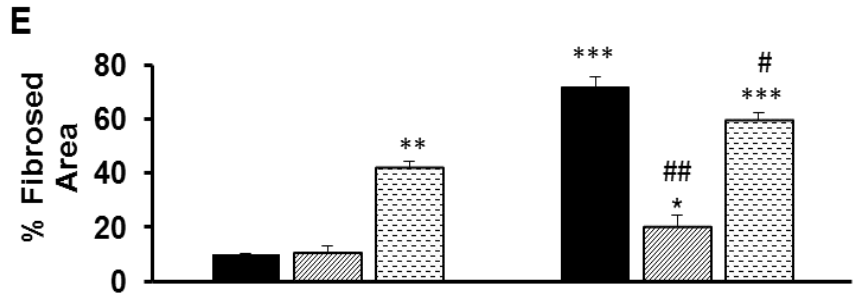

A
Control-HLF
TGFβ
TGFβ +
LY
TCBN
Rapa
CCG
αSMA
Myocardin
SRF
GAPDH
E
FHLF
+TCBN
+Rapa
αSMA
Fibronectin
TSP1
GAPDH
B
αSMA
X- Fold Change
p=0.0004
p=0.0064
*p=0.0003
‡p=0.018
ns
p=0.0017
F
αSMA
X-Fold Change
p=0.0037
ns
C
Myocardin
X- Fold Change
p=0.0012
p=0.018
*p=0.0005
‡p=0.002
ns
p=0.004
G
Fibronectin
X-Fold Change
p=0.0001
ns
D
SRF
X- Fold Change
p=0.007
p=0.002
*p=0.0002
‡p=0.006
ns
p=0.002
Control
TGFβ
LY
TCBN
Rapa
CCG
H
TSP1
X-Fold Change
p=0.0001
ns
FHLF
(+)TCBN
(+) Rapa

FIGURE 3.

Triciribine ameliorates TGFβ-induced pulmonary fibrosis *in vivo*. (A) Hematoxylin and eosin staining, (B) Masson's trichrome staining, (C) fibronectin immunofluorescence staining, and (D) αSMA immunofluorescence staining of lung sections. (E-F) to assess the degree of fibrosis we calculated percent fibrosed area (by quantifying area intensity using ImageJ of 5-10 fields/lung section/mouse/group; n=6-8 mice/group), and the Aschroft fibrosis score for grading pulmonary fibrosis. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus saline-treated control mice; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ $^{\#\#\#}P < 0.001$ versus adTGFβ-treated mice. Scale bar = (A) 500 μm, (B, C) 100 μm, (D) 200 μm.


A
Control
Control+ TCBN
Control+ Rapa
adTGFβ
adTGFβ + TCBN
adTGFβ + Rapa
H & E
B
Trichrome
C
Fibronectin
D
αSMA


E
% Fibrosed Area
80
60
40
20
0
**
***
##
*
#
***

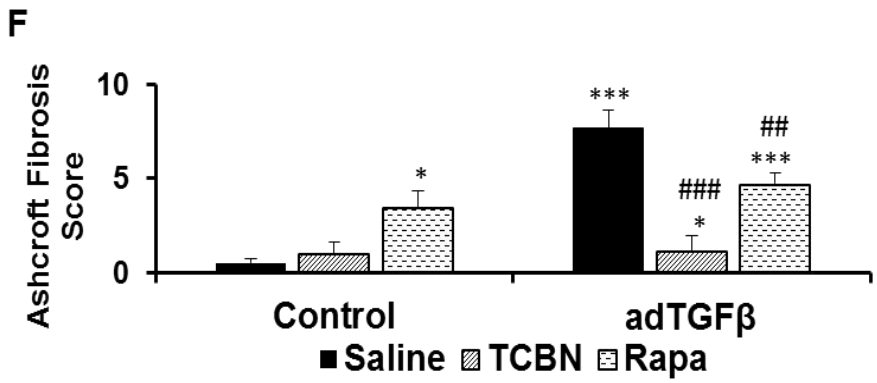

F
Ashcroft Fibrosis Score
10
5
0
*
***
###
*
##
***
Control
adTGFβ
Saline
TCBN
Rapa

FIGURE 4.

Triciribine reverses TGFβ-induced matrix protein expression and αSMA synthesis. Western analysis of (A) αSMA and its transcription factors SRF and myocardin, (B) ECM proteins including thrombospondin-1, ED-A FN, Collagen types I, III, and VI, (C) Akt mediated signaling pathways. (D) Schematic representation of proposed mechanism of TCBN compared to rapamycin. # compared with saline-treated control mice; * $p < 0.01$ compared with TGFβ-treated mice; ‡$p < 0.001$ compared with TGFβ-treated mice. (n=6-8 mice/group).

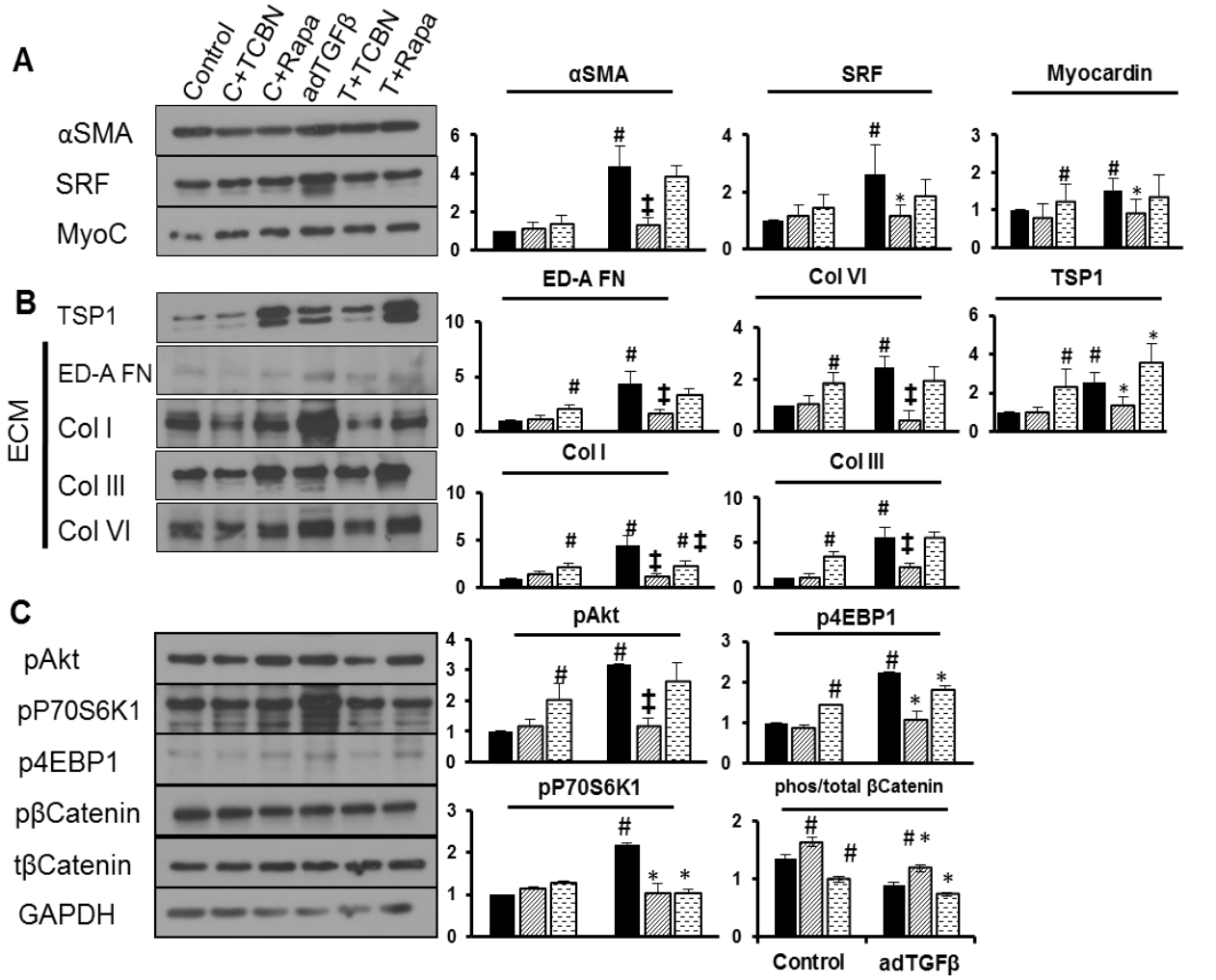

A
Control
C+TCBN
C+Rapa
adTGFβ
T+TCBN
T+Rapa
αSMA
SRF
MyoC
αSMA
SRF
Myocardin
B
TSP1
ECM
ED-A FN
Col I
Col III
Col VI
ED-A FN
Col VI
TSP1
Col I
Col III
C
pAkt
pP70S6K1
p4EBP1
pβCatenin
tβCatenin
GAPDH
pAkt
p4EBP1
pP70S6K1
phos/total βCatenin
Control
adTGFβ
D
TGFβ
TCBN
Akt
Rapa
mTORC1
GSK3β
Myocardin
SRF
p
βCatenin
βCatenin accumulation
Degradation
S6K1
4E-BP1
↑ αSMA
eIF-4e
•Nuclear translocation
•Pro-fibrotic gene transcription
ECM Translation
MF Differentiation

FIGURE 5.

Triciribine reverses hypoxia-induced pulmonary fibrosis and vascular remodeling. (A) Hematoxylin and eosin staining, (B) Masson's trichrome staining, (C) fibronectin immunofluorescence staining, and (D) αSMA immunofluorescence staining of lung sections. (E) To quantify vascular remodeling, we measured the ration of small pulmonary artery vessel wall thickness to lumen diameter (6-10 vessels/lung section/mouse/group). Western analysis of (F) αSMA and its transcription factors SRF and myocardin, (G) ECM proteins including ED-A FN, Collagen types I, III, and VI. #p< 0.01 and ##p< 0.001 compared with saline-treated normoxic mice; * p< 0.05 and ** p< 0.01 compared with hypoxic mice; ‡p < 0.001 compared with hypoxic mice. S: saline, T: TCBN, R: Rapamycin. (n=6-8 mice/group). Scale bar = (A and C) 200 μm, (B) 100 μm, (D) 50 μm.

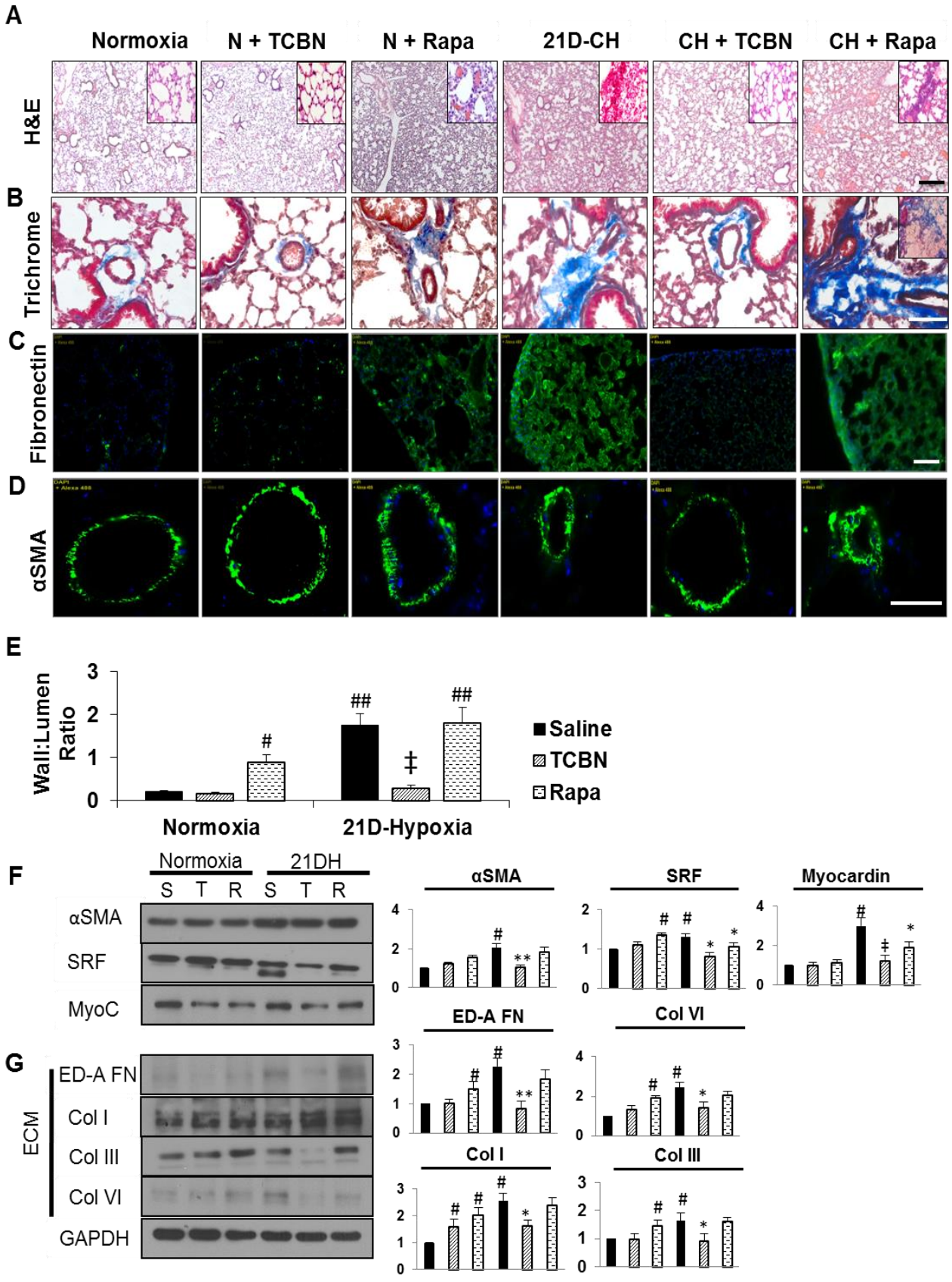
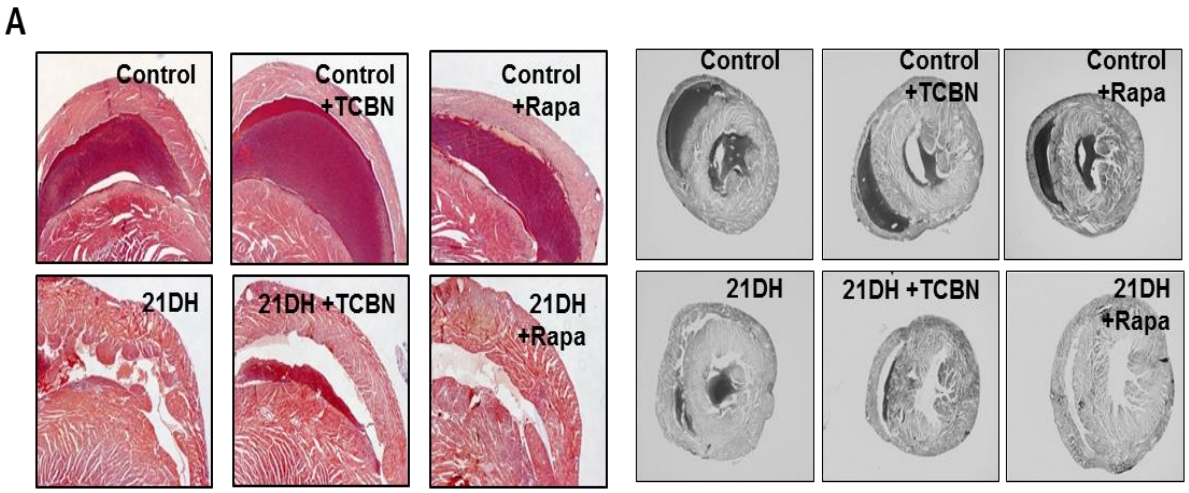
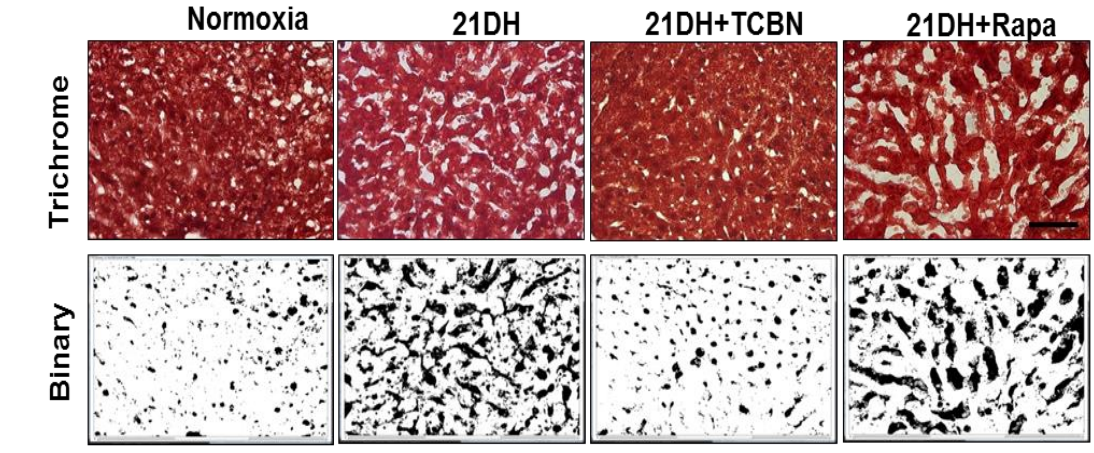

A
Normoxia
N + TCBN
N + Rapa
21D-CH
CH + TCBN
CH + Rapa
H&E
B
Trichrome
C
Fibronectin
D
αSMA
E
Wall:Lumen Ratio
Saline
TCBN
Rapa
Normoxia
21D-Hypoxia
F
Normoxia
21DH
S T R S T R
αSMA
SRF
MyoC
Myocardin
ED-A FN
Col VI
G
ECM
ED-A FN
Col I
Col III
Col VI
GAPDH
Col I
Col III

FIGURE 6.

Triciribine reverses hypoxia-induced right ventricular remodeling and hepatic injury. (A) Masson's trichrome staining of the right ventricle. (B) Quantification of wall thickness (we took 6-10 measurements/heart tissue/mouse; n=6-8 mice/group). (C) Masson's trichrome staining and binary images (using ImageJ Software) of liver sections. (D) Quantification of percent area based on binary images. We measured 6-10 fields/liver tissue/mouse; n=6-8 mice/group. ##p< 0.001 compared with saline-treated normoxic mice; ‡p < 0.001 compared with 21D-hypoxic mice. Scale bar = (A) 200 μm and (C) 100 μm.


A
Control
Control +TCBN
Control +Rapa
21DH
21DH +TCBN
21DH +Rapa
B
Right Ventricle wall thickness
800
600
400
200
0
p=0.028
p=0.0028
p=0.007
Saline
TCBN
Rapa
Normoxia
21DH
RV/LV+S Area (%)
35
30
25
20
15
10
5
0
*
#
#
*
Normoxia
21DH


C
Normoxia
21DH
21DH+TCBN
21DH+Rapa
Trichrome
Binary

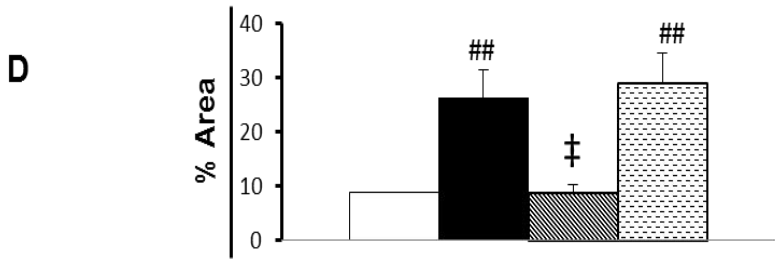
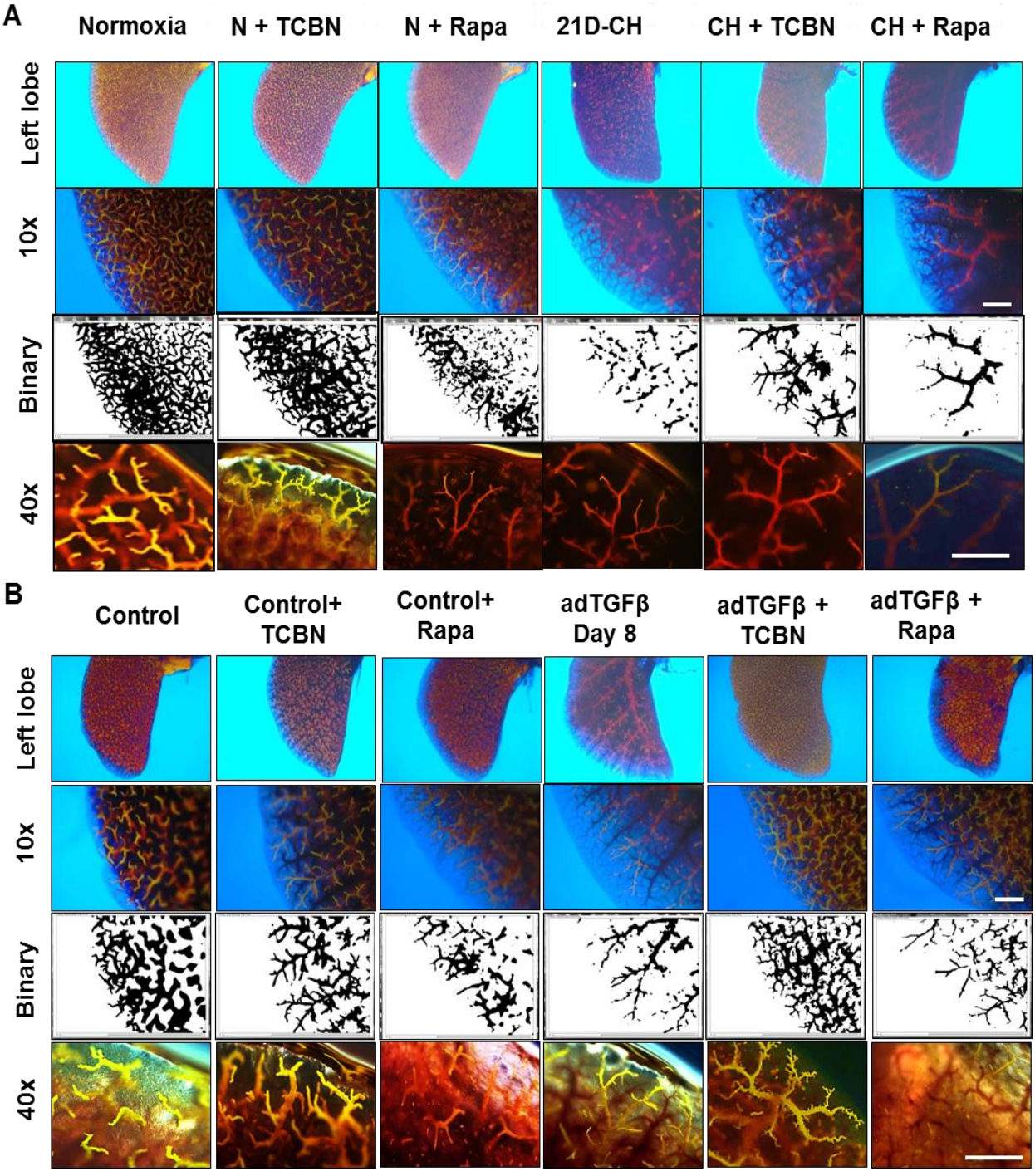

D
% Area
40
30
20
10
0
##
‡
##

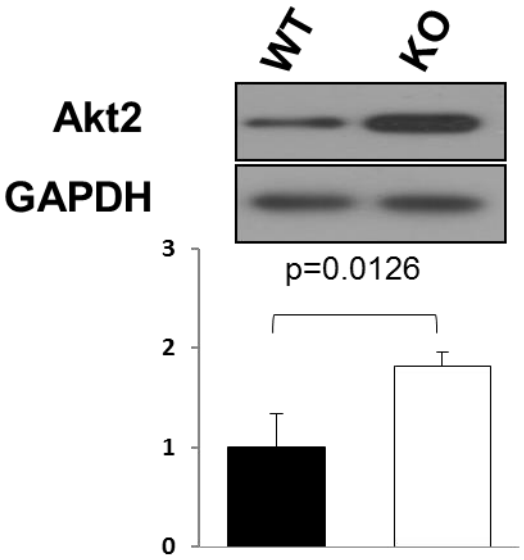
FIGURE 7.

Triciribine prevents hypoxia- and adTGFβ- induced vascular rarefaction. Representative images showing vascular branching of the left lobe after microfil casting of mice subjected to (A) normoxia or chronic hypoxia and (B) control vector or adTGFβ, and treated with saline, TCBN, or rapamycin. (C and D) percent vascular density calculated in both disease model; n=3-5 mice/group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus saline-treated normoxic mice; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ $^{\#\#\#}P < 0.001$ versus hypoxic- or adTGFβ-treated mice. Scale bar in order 500 µm, 200 µm, and 50 µm.


A
Normoxia
N + TCBN
N + Rapa
21D-CH
CH + TCBN
CH + Rapa
Left lobe
10x
Binary
40x
B
Control
Control+ TCBN
Control+ Rapa
adTGFβ Day 8
adTGFβ + TCBN
adTGFβ + Rapa
Left lobe
10x
Binary
40x
C
Vascular Density (%)
100
80
60
40
20
0
**
##
**
***
#
***
Saline
TCBN
Rapa
Normoxia
21D-Hypoxia
D
140
120
100
80
60
40
20
0
*
###
**
**
##
***
Control
adTGFβ

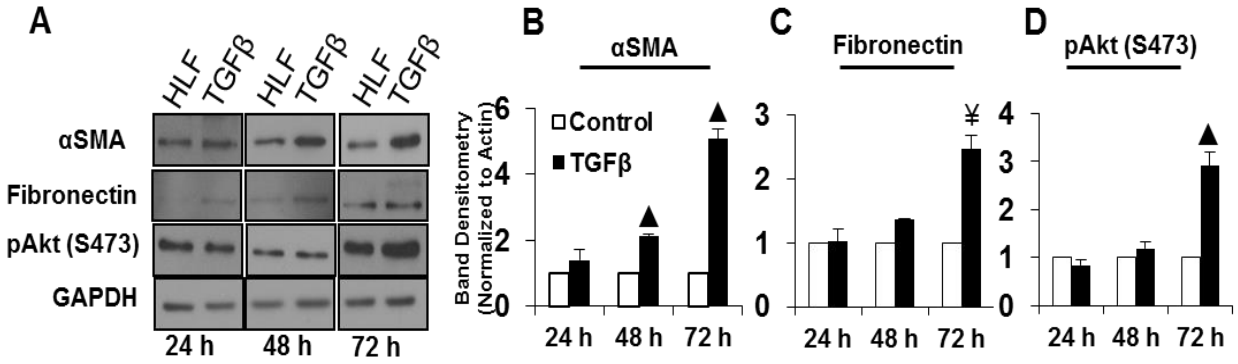
Supplemental Figure 1

Akt2 partially compensated for genetic deletion of Akt1. Western blot analysis of Akt2 in normoxic *WT* and Akt1$^{-/-}$ mice (n=4-5 mice/group). Relative expression values obtained by densitometric analysis were normalized to GAPDH.

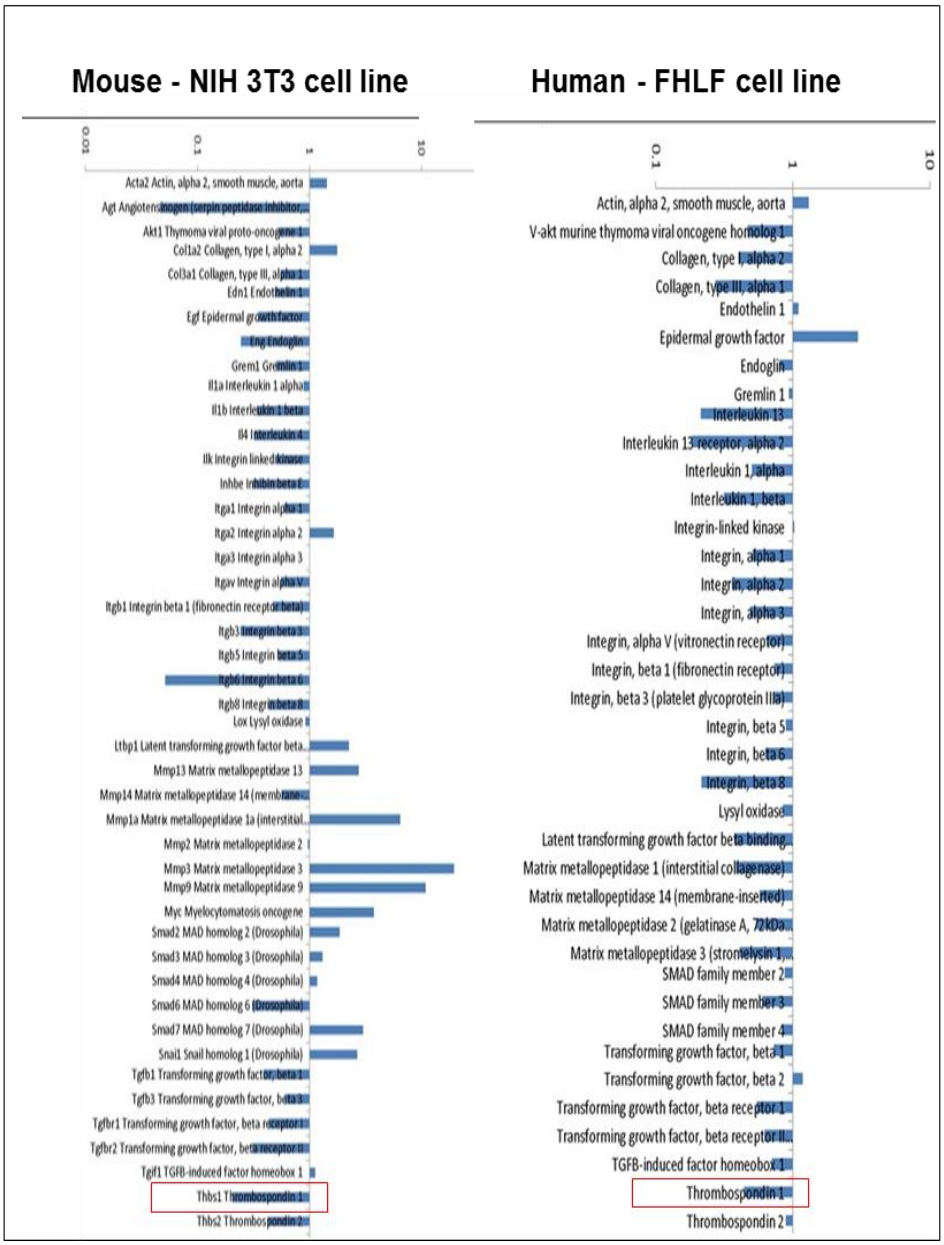

WT
KO
Akt2
GAPDH
p=0.0126
3
2
1
0

Supplemental Figure 2.

MF differentiation occurs at 72 h in TGFβ-stimulated human lung fibroblasts, and gene expression changes due to Akt1 inactivation in MFs and IPF fibroblasts. (A) Representative western blots of cells treated with 100 pM TGFβ for 24, 48, and 72 h in serum free conditions. Quantified expression levels of (B) αSMA, (C) fibronectin, and (D) phosphorylated Akt. (E) Effect of inactivating Akt on fibrotic gene expression in TGFβ-stimulated mouse embryonic fibroblasts and IPF fibroblasts.

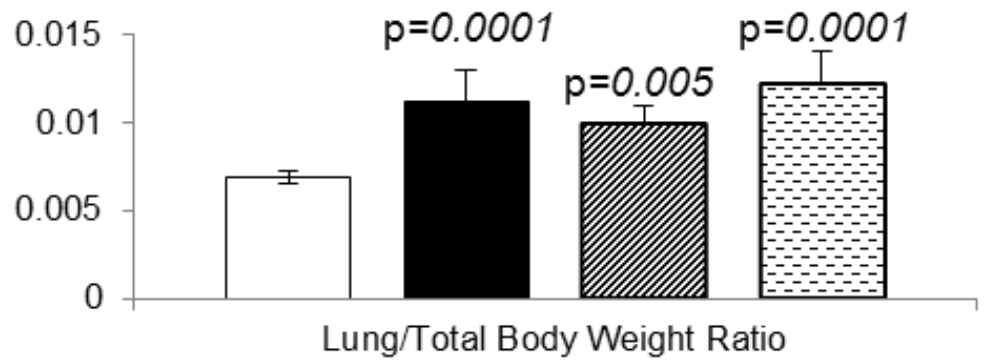

A
HLF
TGFβ
HLF
TGFβ
HLF
TGFβ
αSMA
Fibronectin
pAkt (S473)
GAPDH
24 h
48 h
72 h
B
αSMA
Band Densitometry (Normalized to Actin)
Control
TGFβ
24 h
48 h
72 h
C
Fibronectin
¥
24 h
48 h
72 h
D
pAkt (S473)
24 h
48 h
72 h

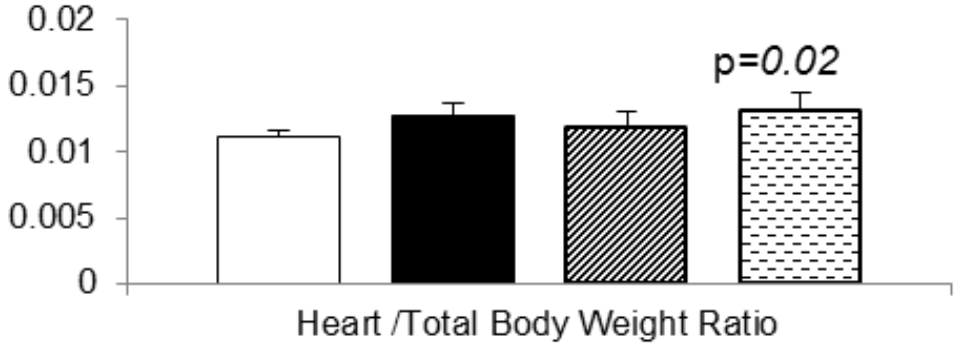
E

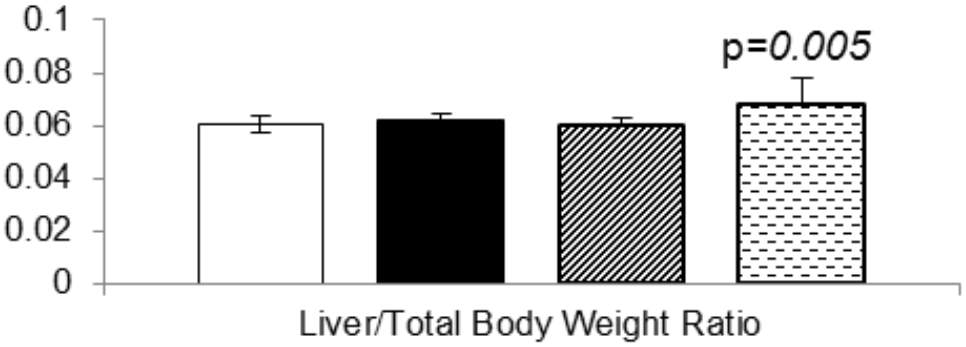

Mouse - NIH 3T3 cell line
Human - FHLF cell line
Actin, alpha 2, smooth muscle, aorta
V-akt murine thymoma viral oncogene homolog 1
Collagen, type I, alpha 2
Collagen, type III, alpha 1
Endothelin 1
Epidermal growth factor
Endoglin
Gremlin 1
Interleukin 13
Interleukin 13 receptor, alpha 2
Interleukin 1, alpha
Interleukin 1, beta
Integrin-linked kinase
Integrin, alpha 1
Integrin, alpha 2
Integrin, alpha 3
Integrin, alpha V (vitronectin receptor)
Integrin, beta 1 (fibronectin receptor)
Integrin, beta 3 (platelet glycoprotein IIIa)
Integrin, beta 5
Integrin, beta 6
Integrin, beta 8
Lysyl oxidase
Matrix metallopeptidase 1 (interstitial collagenase)
Matrix metallopeptidase 14 (membrane-inserted)
Matrix metallopeptidase 2 (gelatinase A, 72kDa)
SMAD family member 2
SMAD family member 3
SMAD family member 4
Transforming growth factor, beta 1
Transforming growth factor, beta 2
Transforming growth factor, beta receptor I
Transforming growth factor, beta receptor II
TGFB-induced factor homeobox 1
Thrombospondin 1
Thrombospondin 2

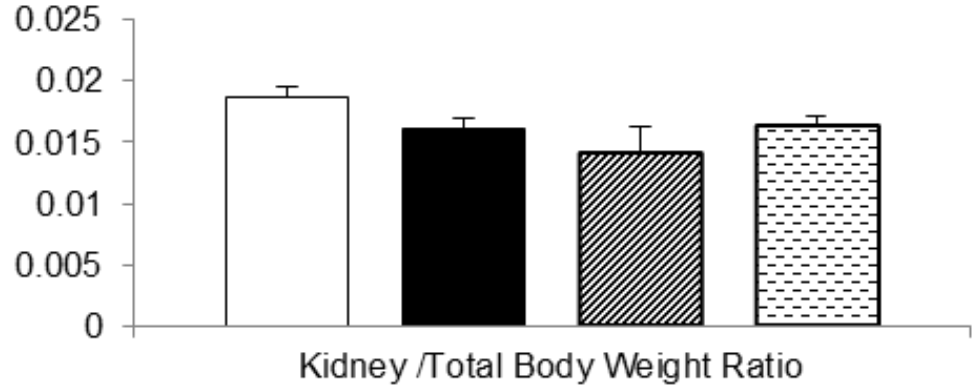
Supplemental Figure 3.

Effect of Triciribine and rapamycin on organ and body weight during hypoxia. The ratio of organ to body weight was calculated for the lung, heart, liver, and kidney (n=6-8 mice/group).





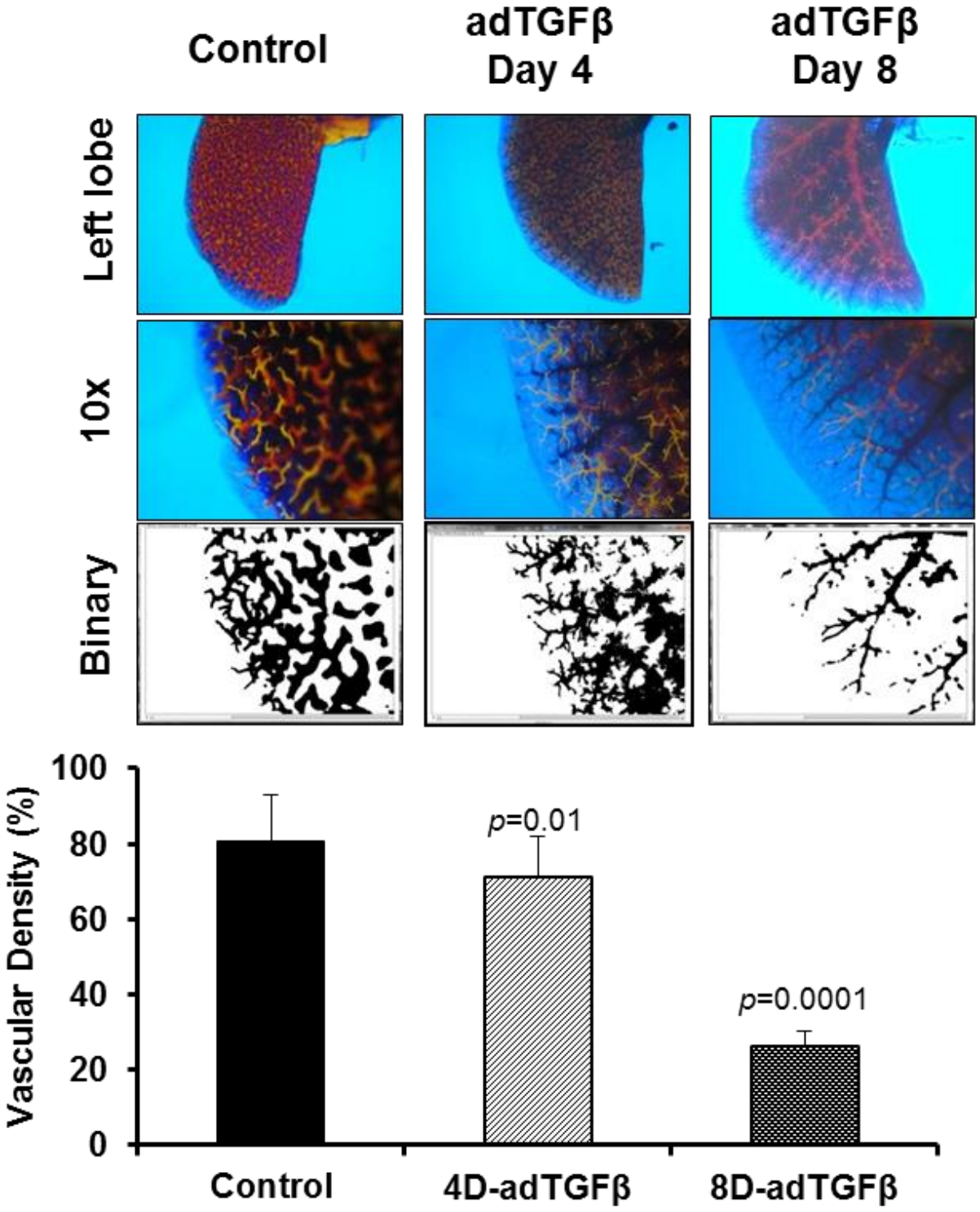




Normoxia ■21DH ▨21DH+TCBN ▣21DH+Rapa

Supplemental Figure 4

Effect of adTGFβ on the vasculature over a period of 4 and 8 days. Representative images showing vascular branching of the left lobe after microfil casting of mice subjected to control vector or adTGFβ for 4 days and 8 days.


Control
adTGFβ Day 4
adTGFβ Day 8
Left lobe
10x
Binary
Vascular Density (%)
100
80
60
40
20
0
p=0.01
p=0.0001
Control
4D-adTGFβ
8D-adTGFβ

CHAPTER 4

INTEGRATED DISCUSSION

Effective therapeutic interventions for IPF have remained limited largely due to incomplete understanding of the molecular and cellular mechanisms underlying its pathogenesis [1, 2]. Although several lines of evidence demonstrated a correlation between hyperactivation of Akt pathway and fibrosis progression, relatively little was known about the causal link between Akt and IPF. Thus, the aim of this thesis was to determine the role of Akt in persistent myofibroblast differentiation and its subsequent implications in IPF.

The main findings of this dissertation can be categorized and summarized as follows, (1) Akt1 and its the pathological role in fibrosis: (a) Akt1 is necessary for the induction and persistence of MF differentiation as it mediates αSMA synthesis, specialized and total fibronectin expression, and the contractile force generated by MFs; these effects are independent of mTOR downstream of Akt. (b) Sustained activation of Akt1 mimicked TGFβ-induced pulmonary fibrosis in vivo. Furthermore, Akt1 deficiency protects against hypoxia-induced pulmonary fibrosis and remodeling.

(2) The therapeutic effects of targeted Akt inhibition to manage IPF and associated complications: (a) Selective Akt inhibition using Triciribine (TCBN) attenuates αSMA synthesis and fibronectin expressions in fibrotic human lung fibroblasts; (b) TCBN reverses TGFβ- and hypoxia-induced pulmonary fibrosis, vascular remodeling, cardiac

hypertrophy, and halts vascular rarefaction; (C) Rapamycin, targets mTOR, exacerbated TGFβ- and hypoxia-induced pulmonary and cardiac remodeling and was associated with hepatic injury.

Fibroblastic foci are hallmark of IPF and are characterized by clusters of proliferative fibroblasts, differentiated myofibroblasts and excessive ECM accumulation [3, 4]. The myofibroblast (MF) has emerged as a central orchestrator of IPF as it is a major source of ECM proteins, thus, promoting matrix stiffness and distortion of lung architecture. MF differentiation from fibroblasts, marked by de novo expression of αSMA stress fibers, plays a central role in physiological wound repair, while its persistence and resistance to apoptosis is characteristic of deregulated tissue repair and fibrosis.

Identifying the similarities and differences between physiological and hypertrophic tissue repair is a critical aspect in understanding the pathogenesis of IPF. While the former is a temporary process terminated when MFs undergo apoptosis [5], the latter is a permanent process marked by sustained production of TGFβ1, persistent fibroblasts migration and proliferation, MF differentiation and resistance to apoptosis, excess ECM accumulation, and aberrant reepithelialization [6]. The seminal report by Selman and colleagues [1] showing that PF results from abnormal wound repair in the absence of inflammation led to the paradigm shift in our understanding of it pathogenesis. This sheds light on the clinical failure of anti-inflammatory therapies. Importantly, this highlights the need for therapeutic strategies aimed at the fibroproliferative responses to halt fibrosis progression.

It is well established that TGFβ1, a pro-fibrotic cytokine, is a potent mediator of MF differentiation and the ECM in IPF [7-9]. It exert its pro-fibrotic effects through the

canonical Smad pathway and non-canonical Akt pathway [10-14]. Additionally, studies correlated the resistance to apoptosis and deregulated ECM production, characteristic of fibrotic MFs, to the activation of PI3K/Akt pathway [15-18]. Importantly, Xia and colleagues showed that Akt is hyperactivated in the fibroblastic foci of IPF lung [19].

Research from our laboratory established the central role of Akt in the regulation of fibroblast proliferation and migration, ECM secretion and assembly, and maintaining vascular integrity [20-24]. Interestingly, the effects of deregulated Akt signaling closely resemble the pathological events in IPF. Collectively, these observations identified a gap in our knowledge and raise the question as to what role the activation of Akt signaling plays in MF differentiation and IPF progression.

Myofibroblast differentiation is characterized by de novo expression of αSMA stress fibers [25-27]. We and others have previously shown that Akt pathway translationally regulates the ECM [16, 28]; however it was unclear whether Akt is necessary for αSMA synthesis. Our results showed that sustained activation of Akt1 mimicked TGFβ stimulation and induced a ~6-fold increase in αSMA. Importantly, inactivation of Akt1 blunted TGFβ-induced αSMA expression and assembly in mouse embryonic fibroblasts, and decreased fibrotic gene expression in IPF fibroblasts. Mechanistically, we found that Akt1 mediates αSMA synthesis, in part, through modulation of the transcription factors myocardin and SRF, independent of mTOR, a novel and previously uncharacterized link in MFs.

MFs are potent producers of pro-fibrotic cytokines and the ECM including fibronectin splice variant (ED-A), which further promotes αSMA synthesis. Noteworthy, the sheer mechanical stress of the ECM on MFs promotes persistent differentiation; a positive

feedback loop [6, 27]. Our results showed that in addition to its control on total fibronectin, Akt1 induces ED-A FN expression, which is mandatory for MF differentiation. Functionally, we demonstrated that TGFβ-mediated Akt1 activation is necessary for the functional contractile force generated by MFs.

Due to lack of clear evidence of Akt isoform 1 and its role in pulmonary fibrosis, we investigated the pathologic effects of chronic hypoxia in Akt1 null and wild type mice. Chronic hypoxia is well established model of pulmonary fibrosis and associated vascular remodeling [29, 30]. Mice were subjected to 14 day chronic exposure to 10% oxygen or normoxia. Interestingly, hypoxic Akt1 deficient mice were protected from hypoxia induced fibrosis and vascular remodeling as evident by decreased fibronectin accumulation and decreased peripheral pulmonary artery wall thickness. To further verify the pathological role of Akt1, we administered constitutively active Akt1 or adenovirus TGFβ intratracheally. Remarkably, sustained activation of Akt1 induced severe focal fibrosis similar to TGFβ.

Once we established the pathological role of Akt1, we sought to identify whether targeting Akt is an effective therapeutic strategy. Our results identify novel anti-fibrotic and anti-remodeling properties of Triciribine (TCBN), a selective Akt inhibitor currently in phase trials for different types of cancer [31]. TCBN markedly attenuated αSMA synthesis and ECM expression in TGFβ-stimulated mouse embryonic and human lung fibroblasts, and in IPF fibroblasts. TCBN reversed fibrotic lesions, honeycomb, and vessel wall remodeling and preserved the vasculature.

Clinical evidence has shown that the rapamycin derivative, everolimus, worsened outcomes in IPF patients [32]. Additionally, everolimus and sirolimus have been shown

to be associated with severe pulmonary toxicities [33-37]. In agreement, our results of rapamycin treatment in two experimental models of PF demonstrated increased pulmonary toxicity, increased fibrotic lesions and dense infiltration, marked vascular thickening and rarefied vasculature, and was associated with cardiac remodeling and hepatic injury. Interestingly, these effects correlated with increased Akt activation, which supports previous reports of rapamycin-mediated feedback activation of Akt pathway [38-42]. Furthermore, Lamoullie and colleague reported that while rapamycin attenuated TGFβ-induced translational regulation on cell size, it did not modulate epithelial-to-mesenchymal transition (EMT) nor did it affect fibronectin expression and assembly [43]. This suggests that rapamycin may also exacerbate abnormal re-epithelialization and persistent differentiation, thus contributing to disease progression. However, this will require further studies to confirm this process.

Collectively, this dissertation demonstrates a causal link between Akt1 and IPF and illustrates the importance of fine-tuning Akt pathway to maintain homeostasis. Sustained hyperactivation of Akt1 induced MF differentiation and excess ECM expression *in vitro*, and severe focal pulmonary fibrosis *in vivo*. We determined that targeting Akt pathway using TCBN reverses pulmonary fibrosis and vascular remodeling. Conversely, we identified that targeting mTOR using rapamycin worsened disease progression due to feedback activation of Akt signaling. Our results offer pre-clinical evidence of the critical role of Akt in IPF pathogenesis. Therefore, targeting Akt, not mTOR, may serve as a favorable therapeutic strategy in IPF.

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CHAPTER 5

SUMMARY AND CLINICAL SIGNIFICANCE

SUMMARY

Idiopathic Pulmonary fibrosis (IPF) is an incurable, chronic and progressive lung disease with severely poor prognosis. The presence of pulmonary hypertension (PH) secondary to IPF is an independent risk factor for increased mortality. Alarmingly, there are no effective pharmacologic treatments for IPF, and lung transplantation remains the only non-medicinal therapy with proven efficacy on survival. Therefore, identification of novel therapeutic targets is critical in the hopes of devising new treatments. Persistent myofibroblasts (MFs) differentiation and excess extracellular matrix (ECM) accumulation are hallmark features of IPF. Differentiated from fibroblasts, MFs are marked by de novo expression of α-smooth muscle like actin (αSMA) stress fibers, and are potent producers of ECM proteins. Recently, it has been shown that Akt, a major survival protein, is upregulated in IPF patients; however whether Akt is necessary for MFs remains unclear. This dissertation sought to 1) identify the role of Akt in MF differentiation leading to IPF, 2) investigate the safety and efficacy of triciribine (TCBN), a selective Akt inhibitor, as a potential therapeutic option for IPF and 3) characterize the underlying mechanisms. The work presented in this thesis has been conducted using a combined approach of pharmacological, genetics, and functional assays. We first identified that Akt is a critical determinant of pathological MF differentiation and ECM expression as evident by loss- and gain- of function studies, both in vitro and in vivo.

Mechanistically, we found that Akt1 mediates αSMA through direct interaction with contractile gene transcription factors myocardin and SRF, independent of mTOR. Furthermore, in vivo, TCBN reversed TGFβ (pro-fibrotic cytokine) - and hypoxia-induced fibrosis, respectively, compared to placebo and rapamycin. Mice treated with TCBN had decreased tissue, dense infiltration and fibrosis, lower adventitial and medial remodeling, decreased vascular loss, lower αSMA and specialized ECM synthesis and assembly. Conversely, rapamycin, which targets mTOR downstream of Akt, exacerbated IPF and vascular remodeling as evident by worsening interstitial fibrosis, micro-hemorrhage, and increased peripheral vascular loss. Collectively, research efforts associated with this thesis 1) identified Akt as a novel target as it is crucial for MFs - central orchestrators of IPF; 2) determined novel anti-fibrotic properties of Triciribine. Thus, it can potentially be a therapeutic option for IPF; 3) determined that while rapamycin regulates some ECM proteins, MF differentiation still occurs via unopposed transcriptional activation of αSMA and specialized ECM proteins resulting in persistent differentiation and abnormal ECM deposition. This also sheds light on the observed detrimental effects of everolimus, a rapamycin derivative, in IPF patients.

CLINICAL SIGNIFICANCE

Importance of this problem: IPF is a progressive and incurable disease with poor prognosis for which therapeutic options are limited. PF complicated with vascular remodeling (PAH) is a predictor of poor prognosis. Lung transplant is the only effective approach. **Gap in knowledge:** The role of Akt in mediating myofibroblast differentiation remains unclear

This dissertation 1) identified a novel, and previously uncharacterized, interaction between Akt and αSMA synthesis. Our results indicate for the first time to our knowledge that Akt1 is an integral mediator of MF differentiation via modulation of αSMA synthesis and fibronectin in vitro leading to IPF progression in vivo. Mice deficient in Akt1 were protected from chronic hypoxia-induced PF and vascular remodeling; 2) Determined a previously unknown anti-fibrotic and anti-remodeling properties of Triciribine, a selective inhibitor of Akt activation currently utilized in cancer studies, and at a dose that is half of that utilized in cancer studies. Our results identify a novel interaction between Akt and thrombospondin-1 leading to IPF and secondary PH that is modulated by Triciribine; 3) we shed light the on the detrimental effects observed with rapamycin derivative in IPF patients. Our results show that rapamycin activates PI3K pathway via inhibiting the regulatory S6K-feedback loop (**Figure 1**).

Translational impact: drug repurposing of Triciribine, a potent Akt inhibitor currently utilized in cancer studies. We are the first group to demonstrate the anti-fibrotic and anti-remodeling properties of Triciribine at a dose 50% less than that utilized in in vivo cancer studies. This dose equates to 2 mg/m2 (maximum tolerated dose in patients is 40 mg/m2). Herein we also demonstrate the superior effect of Triciribine compared to

rapamycin in attenuating IPF with vascular remodeling complications. Our findings may likely impact the therapeutic approach to this fatal disease by identifying novel target leading to effective therapeutics as opposed to conventional symptomatic relief.

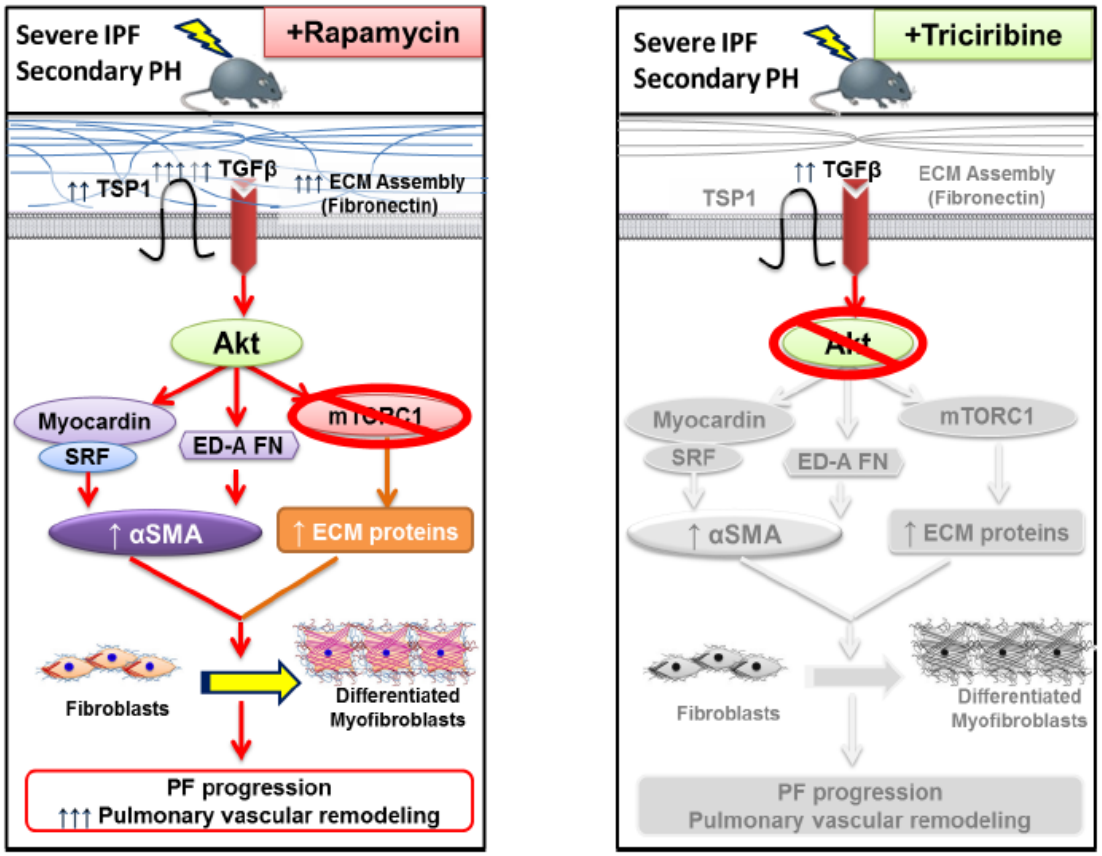

Figure 1. Schematic representation of the novel findings of this thesis. Akt is a novel target as it is crucial for mediating myofibroblasts differentiation and ECM deposition; both constitute two key components driving IPF-PH. Compared to rapamycin, Triciribine has novel anti-fibrotic and anti-remodeling properties and can potentially be an effective therapeutic option for IPF and secondary vascular remodeling.

APPENDIX

Includes 1 unpublished manuscript, 4 published manuscripts, and 2 book chapters

1. **Akt1 Mediates alpha-Smooth Muscle Actin Expression and Myofibroblast Differentiation via Myocardin and Serum Response Factor.**

   Abdalla M, Goc A, Segar L, Somanath P.R.

   J Bio. Chem. 2013; 288(46):33483-93

2. **Dasatinib Regulates TGFβ-Induced Myofibroblast Differentiation through Src Pathway: Potential Role in Pulmonary Fibrosis**

   Abdalla M, Thompson L, Burke S, Gurly E, Newsome R, Somanath P.R.

   In preparation for submission. 2014

3. **Pathways Regulating the Secretion and Assembly of Fibronectin Matrix: An Update.**

   Abdalla M, Goc A, Somanath PR.

   Fibronectin: Current Concepts in Structure, Function and Pathology. Nova Science Publishers Inc, New York; 2012; Chapter 7, pp. 155- 190

4. **Methods to Study Fibronectin Secretion and Matrix Assembly by Fibroblasts *in vitro* and *in vivo.***

   Goc A, Abdalla M, Somanath PR.

   Fibronectin: Current Concepts in Structure, Function and Pathology. Nova Science Publishers Inc, New York; Chapter 6, pp141- 154

5. **Rac1 activation driven by 14-3-3ζ dimerization promotes prostate cancer cell-matrix interactions, motility and transendothelial migration.**

   Goc A, Abdalla M, Al-Azayzih A, Somanath PR.

   PLoS One. 2012; 7(7):e40594.

6. **P21 activated kinase-1 (Pak1)promotes prostate tumor growth and microinvasion via inhibition of transforming growth Factor-βexpression and enhanced matrix metalloprotease-9 secretion.**

   Goc A, Al-Azayzih A, Abdalla M, Al-Husein B and Somanath PR.

   J Biol Chem. 2012; 288(5):3025-35

7. **Cerebrovascular Complications of Diabetes: Focus on Stroke**

   Ergul A, Kelly-Cobbs A, Abdalla M, Fagan SC.

   Journal of Endocrine, Metabolic & Immune Disorders-Drug Targets (EMID-DT).

   Jan 2012

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Akt1 Mediates α-Smooth Muscle Actin Expression and Myofibroblast Differentiation via Myocardin and Serum Response Factor*

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Background: Significance of Akt1 in myofibroblast differentiation is unknown.
Results: Akt1 mediates myofibroblast differentiation via serum response factor (SRF) and myocardin signaling leading to α-smooth muscle synthesis.
Conclusion: Akt1-myocardin-SRF signaling induces myofibroblast differentiation.
Significance: The role of Akt1 on myofibroblast differentiation highlights it as a potential target for the treatment of fibrotic diseases.

Myofibroblast (MF) differentiation, marked by the *de novo* expression of smooth muscle α-actin (αSMA) stress fibers, plays a central role in wound healing and its persistence is a hallmark of fibrotic diseases. We have previously shown that Akt1 is necessary for wound healing through matrix regulation. However, the role of Akt1 in regulating MF differentiation with implications in fibrosis remains poorly defined. Here, we show that sustained activation of Akt1 was associated with a 6-fold increase in αSMA expression and assembly; an effect that is blunted in cells expressing inactive Akt1 despite TGFβ stimulation. Mechanistically, Akt1 mediated TGFβ-induced αSMA synthesis through the contractile gene transcription factors myocardin and serum response factor (SRF), independent of mammalian target of rapamycin in mouse embryonic fibroblasts and fibroblasts overexpressing active Akt1. Akt1 deficiency was associated with decreased myocardin, SRF, and αSMA expressions *in vivo*. Furthermore, sustained Akt1-induced αSMA synthesis markedly decreased upon RNA silencing of SRF and myocardin. In addition to its integral role in αSMA synthesis, we also show that Akt1 mediates fibronectin splice variant expression, which is required for MF differentiation, as well as total fibronectin, which generates the contractile force that promotes MF differentiation. In summary, our results constitute evidence that sustained Akt1 activation is crucial for TGFβ-induced MF formation and persistent differentiation. These findings highlight Akt1 as a novel potential therapeutic target for fibrotic diseases.

Since its discovery in the early 1970s (1, 2), the myofibroblast (MF)[2] has emerged as a central orchestrator of wound repair and pathologic hypertrophic scar formation characteristic of fibrotic diseases (3–6). Differentiated from fibroblasts, MFs exhibit a contractile phenotype marked by *de novo* expression of α-smooth muscle cell like actin (αSMA) stress fibers (7) and are primarily responsible for excessive extracellular matrix (ECM) production, cell to matrix adhesion, and resistance to apoptosis (3, 8–10). It is well established that accumulation of transforming growth factor β1 (TGFβ) is a major inducer of MF differentiation during wound healing and fibrosis (8, 11–13). Interestingly, basic fibroblast growth factor (bFGF) has been shown to control the extent of TGFβ-induced effects via stimulating de-differentiation of MFs (14, 15). This shows the intricate balance between these two cytokines in mediating physiological and pathophysiological fibrogenic responses.

Synthesis of αSMA, a principal component of MFs, is a highly regulated process that is controlled by TGFβ, splice variant ED-A fibronectin (ED-A FN), and mechanical tension (3, 5). Numerous lines of evidence indicate that the transcription of αSMA is reliant on RhoA-mediated activation and nuclear translocation of transcription factor serum response factor (SRF) (16–24). Furthermore, the interaction between the recently discovered myocardin, a transcription co-factor restricted to cardiac and smooth muscle cells, and SRF has been characterized in orchestrating contractile gene expression (18, 25–27). However, defining signaling pathways that regulate this transcription network in the hopes of devising targeted therapeutics remain incompletely understood.

Research from our laboratory has established the pivotal role of protein kinase Bα (Akt1) in wound healing, ECM remodeling, and vascular maturation (28). Furthermore, we have shown

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This article contains supplemental Figs. S1 and S2.

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[2] The abbreviations used are: MF, myofibroblast; Akt, protein kinase B; αSMA, α-smooth muscle like actin; TGFβ, transforming growth factor β; bFGF, basic fibroblast growth factor; DN-Akt, dominant-negative Akt; Myoc, myocardin; myr-Akt, myristoylated (active) Akt; SRF, serum response factor; mTOR, mammalian target of rapamycin; FN, fibronectin.

Dasatinib Regulates TGFβ-Induced Myofibroblast Differentiation through Src Pathway: Potential Role in Pulmonary Fibrosis

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Abstract

Persistent myofibroblast differentiation is hallmark of fibrotic diseases including pulmonary fibrosis. Myofibroblasts are characterized by neo-expression of alpha smooth muscle actin (αSMA) and producing excess extracellular matrix (ECM) fibronectin deposition. Identifying effective anti-fibrotic therapeutic intervention remains a constant challenge. Recently studies have shown promising effects of tyrosine kinase inhibitors such as dasatinib and sorafinib on inhibiting transforming growth factor β (TGFβ) signaling, a well-established trigger of fibrosis. Here, we investigated the role of Src in mediating myofibroblast differentiation and explore the therapeutic potential of dasatinib, a multi-targeted tyrosine-kinase inhibitor currently in cancer therapy. Maximum TGFβ–induced myofibroblast differentiation occurred at 72hr as evident by marked increase in αSMA expression that was associated with increased fibronectin. Interestingly, targeted Src inhibition using PP2 mimicked dasatinib effect and attenuated myofibroblast differentiation as evident by blunted αSMA, however, modest inhibition of fibronectin was observed in NIH 3T3 and FHLFs. Mechanistically, our data suggests that dasatinib modulates αSMA synthesis through Src via the transcription factor SRF, independent of GSK pathway. Collectively, our results show that myofibroblast differentiation is mediated through Src pathway. Dasatinib could potentially be a therapeutic option in patients with IPF.

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Chapter 7

PATHWAYS REGULATING THE SECRETION AND ASSEMBLY OF FIBRONECTIN MATRIX: AN UPDATE

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ABSTRACT

Fibronectin (FN) is an essential extracellular matrix (ECM) protein that plays an important role in tissue repair both physiologically and pathophysiologically. The polymerization of FN into the matrix is a highly regulated process to ensure ECM accurately regulates various cellular functions including adhesion, differentiation, proliferation, migration and apoptosis and that it relays accurate signals intracellularly. FN, which is synthesized and secreted by different cell types, is organized into a fibrillar network through a highly regulated process termed fibrillogenesis. This process requires direct interaction with cell-surface adhesion receptors such as integrins and exertion of mechanical force to induce FN conformational change from the soluble, compact and non-functional state to the insoluble, extended and highly adhesive state. This process is mediated through the binding of the activated cell surface integrins, primarily α5β1 integrin, to the RGD (Arg-Gly-Asp) cell-binding domain on FN. In integrating the extracellular environment with the interior of the cell, integrins have a unique ability to transduce signals via "outside-in" and "inside-out" signaling. Growth factors, particularly transforming growth factor beta (TGFβ), modulate integrin activation in fibroblasts. TGFβ controls the transcription of genes encoding integrins via binding to its receptor, leading to the phosphorylation of intracellular effector proteins Smad2 and 3 and activation of downstream signaling and translocation to the nucleus. The crosstalk between integrin and TGFβ is essential for FN matrix assembly; however, the molecular mechanisms underlying FN secretion and assembly are not well known. Studies have shown that modulation of focal adhesion kinase (FAK), Rho-GTPases, protein kinase Bα (Akt1), mammalian target of rapamycin (mTOR), and MAP kinase pathways are among the regulatory mechanisms that play an integral role in FN secretion and assembly. Abnormalities in the crosstalk between integrins, growth factors and FN are characterized by excessive differentiation and ECM remodeling resulting in aberrant response to tissue injury and contributing to a variety of pathological processes.

The aim of this review is to describe the mechanisms and consequences of FN secretion and matrix assembly, and provide a brief overview of its clinical impact. First, we describe FN structure and the important features responsible for interaction with integrins and subsequent cell functioning. Next, we focus on the matrix assembly and essential mechanisms underlying this process. Finally, we discuss how FN can drive pathophysiological processes in fibrosis and cancer.

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Chapter 6

METHODS TO STUDY FIBRONECTIN SECRETION AND MATRIX ASSEMBLY BY FIBROBLASTS *IN VITRO* AND *IN VIVO*

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ABSTRACT

Fibronectin (FN) is a high molecular weight glycoprotein composed of two structurally similar monomers forming a dimer linked by a pair of disulfide bonds. It is the product of a single *FN* gene, undergoing alternative splicing leading to 20 potential isoforms. Fibronectin exists in two forms: soluble (major component of blood plasma) and insoluble (major component of the extracellular matrix). It is secreted by many different cell types including endothelial cells, keratinocytes and monocytes. However, fibroblasts are the major source of fibronectin synthesis in a variety of tissues, which not only secrete fibronectin, but also assemble the soluble dimers into an insoluble matrix enabling a remodeling process in many tissues including such aspects as cell-cell communication, cell-to-basement-membrane attachment, and regulation of inflammation as well as maintaining tissue integrity. Through regulation of cell adhesion, growth, migration, and differentiation, fibronectin plays a crucial role in various essential processes such as embryogenesis, nerve regeneration, wound healing, angiogenesis, and cancer metastasis. Altered fibronectin synthesis, secretion, degradation, and organization have been associated with a number of pathological conditions, including fibrosis and cancer. Thus, it is important to better understand the significance of this multifunctional protein in both physiological and pathological events. In this chapter, we summarize the methods used to study the synthesis and assembly of fibronectin *in vitro* and *in vivo*, and may also provide useful tools to investigate various aspects of matrix biology.

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Rac1 Activation Driven by 14-3-3ζ Dimerization Promotes Prostate Cancer Cell-Matrix Interactions, Motility and Transendothelial Migration

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Abstract

14-3-3 proteins are ubiquitously expressed dimeric adaptor proteins that have emerged as key mediators of many cell signaling pathways in multiple cell types. Its effects are mainly mediated by binding to selective phosphoserine/threonine proteins. The importance of 14-3-3 proteins in cancer have only started to become apparent and its exact role in cancer progression as well as the mechanisms by which 14-3-3 proteins mediate cancer cell function remain unknown. While protein 14-3-3σ is widely accepted as a tumor suppressor, 14-3-3ζ, β and γ isoforms have been shown to have tumor promoting effects. Despite the importance of 14-3-3 family in mediating various cell processes, the exact role and mechanism of 14-3-3ζ remain unexplored. In the current study, we investigated the role of protein 14-3-3ζ in prostate cancer cell motility and transendothelial migration using biochemical, molecular biology and electric cell-substrate impedance sensing approaches as well as cell based functional assays. Our study indicated that expression with wild-type protein 14-3-3ζ significantly enhanced Rac activity in PC3 cells. In contrast, expression of dimer-resistant mutant of protein 14-3-3ζ (DM-14-3-3) inhibited Rac activity and associated phosphorylation of p21 activated kinase-1 and 2. Expression with wild-type 14-3-3ζ or constitutively active Rac1 enhanced extracellular matrix recognition, lamellipodia formation, cell migration and trans-endothelial migration by PC3 cells. In contrast, expression with DM 14-3-3ζ or DN-Rac1 in PC3 cells significantly inhibited these cell functions. Our results demonstrate for the first time that 14-3-3ζ enhances prostate cancer cell-matrix interactions, motility and transendothelial migration *in vitro* via activation of Rac1-GTPase and is an important target for therapeutic interventions for prostate cancer.

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P21 activated kinase-1 (Pak1) promotes prostate tumor growth and microinvasion via inhibition of TGFβ expression and enhanced MMP9 secretion*

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*Running Title: Pak1 in prostate cancer

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Key words: Prostate cancer; Pak1; Pak6; microinvasion; MMP9; TGFβ.

Background: The significance of Pak1 in prostate cancer remains unclear.

Results: Pak1 knockdown impaired prostate tumor growth via increased expression of TGFβ and reduced secretion of MMP9.

Conclusions: We demonstrated that Pak1 is a more potent mediator of prostate cancer cell migration and tumor growth than Pak6, the predominant isoform in the prostate.

Significance: A novel role of Pak1 in prostate cancer is identified.

SUMMARY

P21 activated kinases (Paks) are major effectors downstream of small Rho family of GTPases. Among the six isoforms, Pak1 is the most ubiquitous and the best characterized member. Previous studies have shown that inhibition of Pak6, which is predominantly present in the prostate compared to other tissues, inhibit prostate tumor growth *in vivo*. Even though Pak1 has been identified in normal prostatic epithelial cells and cancer cells, its specific role in the development of prostate cancer remains unclear. We report here that highly invasive prostate cancer cells express significantly higher levels of Pak1 protein compared to non-invasive prostate cancer cells. Furthermore, prostate tumor tissues and prostate cancer metastasized to lungs showed higher expression of Pak1 compared to normal tissues. Interestingly, Pak6 protein expression levels did not change with the invasive/metastatic potential of the cancer cells or tumors. While inhibition of Pak1, and not Pak6, resulted in impaired PC3 cell migration, the effects of Pak1 knockdown on transendothelial migration (microinvasion), tumor growth and tumor angiogenesis was higher compared to Pak6 knockdown. Finally, gene array data revealed reduced expression of MMP9 with the ablation of either Pak1 or Pak6 gene expression in PC3 cells, whereas protein levels of TGFβ was significantly elevated with specific modulation of Pak1 activity or ablation of Pak1 gene. Our observations suggest that although some level of functional redundancy exists between Pak1 and Pak6 in prostate cancer cells, targeting Pak1 is a potential option for the management of prostate tumor growth, microinvasion and metastasis.

INTRODUCTION

P21 activated kinases (Paks) are a family of six serine-threonine kinases which are categorized into Group-I and Group-II Paks based on their mechanism of activation (1). Group-I Paks differ from their Group-II counterparts on their activation by small Rho GTPases such as Rac and cdc42 (2) as well as their specific involvement in inducing cytoskeletal changes, lamellipodia and filopodia formation in mammalian cells in the promotion of cell motility (1, 3). Group-II Paks lack the auto inhibitory domain, acidic and Pix-binding regions as well as Rac/cdc42 binding CRIB domain, which are present in all Group-I Pak isoforms (4-6).

Cerebrovascular Complications of Diabetes: Focus on Stroke

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Abstract: Cerebrovascular complications make diabetic patients 2-6 times more susceptible to a stroke event and this risk is magnified in younger individuals and in patients with hypertension and complications in other vascular beds. In addition, when patients with diabetes and hyperglycemia experience an acute ischemic stroke they are more likely to die or be severely disabled and less likely to benefit from the one FDA-approved therapy, intravenous tissue plasminogen activator. Experimental stroke models have revealed that chronic hyperglycemia leads to deficits in cerebrovascular structure and function that may explain some of the clinical observations. Increased edema, neovascularization and protease expression as well as altered vascular reactivity and tone may be involved and point to potential therapeutic targets. Further study is needed to fully understand this complex disease state and the breadth of its manifestation in the cerebrovasculature.

Keywords: Cerebral vasculature, diabetes, hemorrhage, ischemia, stroke, targets.

INTRODUCTION

The steadily increasing prevalence of obesity in developed nations has contributed to the alarming rate of diagnoses of type 2 diabetes (T2D) and prediabetes, even in children [1]. In addition, there is an equally alarming increase in the number of younger patients diagnosed with type 1 diabetes (T1D) [2-4]. Given the mortality and morbidity due to cardiovascular diseases (CVD) associated with diabetes, this increase in the incidence of diabetes will have an avalanche effect in health care. Hyperglycemia ravages all vascular beds in the human body, and in the brain, this has devastating consequences. A growing body of literature indicates that the cerebrovascular sequelae of diabetes may play an important role in the pathogenesis of cerebral complications of diabetes including stroke [5, 6] and other neurological diseases including Alzheimer's Disease and Vascular Cognitive Impairment [7, 8]. Since diabetes is the fastest growing risk factor for stroke globally, this review will focus on stroke as a complication of diabetes and readers are referred to recent reviews for the neurodegenerative complications of diabetes [8-11]. While traditionally stroke is considered a macrovascular complication of diabetes, there is increasing evidence that the microvasculature of the brain is severely affected in both forms of diabetes [7, 8]. In the following review, we will present the mechanisms and of both T1D and T2D [12]. In fact, although T2D patients make up the vast majority of diabetic stroke (97% in the Nurses' Health Study), [13] the relative risk is actually greater in T1D, with more than 4 fold higher rates of stroke at all ages of the disease [13]. This is magnified at younger ages, with T1D patients between 15 and 34 years of age having stroke rates more than 16 times that of the general population [14]. Other vascular complications, in particular, diabetic nephropathy, predicted even greater risk of up to 75 times that of the stroke rate of aged matched controls [14]. Nevertheless, most diabetic stroke occurs in T2D and most epidemiologic and prevention data reflects this bias.

A prospective population based study, with approximately 20 year follow-up, evaluated the effect of T2D alone on CVD in 13,105 subjects. The study showed an increased relative risk for developing stroke of 1.5 to 2 fold in men and 2 to 6.5 fold in women [15]. This increased risk is seen even early after diagnosis. In a short term study conducted to evaluate 5-year risk of stroke in newly treated T2D patients, the relative risk of stroke was 2.1 (95% CI, 1.8 to 2.3) in the T2D group, compared to the general population. Additionally, younger patients (30 to 44 years old) had higher relative risk for stroke compared to older patients [16].

Chapter

PATHWAYS REGULATING THE SECRETION AND ASSEMBLY OF FIBRONECTIN MATRIX: AN UPDATE

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ABSTRACT

Fibronectin (FN) is an essential extracellular matrix (ECM) protein that plays an important role in tissue repair both physiologically and pathophysiologically. The polymerization of FN into the matrix is a highly regulated process to ensure ECM accurately regulates various cellular functions including adhesion, differentiation, proliferation, migration and apoptosis and that it relays accurate signals intracellularly. FN, which is synthesized and secreted by different cell types, is organized into a fibrillar network through a highly regulated process termed fibrillogenesis. This process requires direct interaction with cell-surface adhesion receptors such as integrins and exertion of mechanical force to induce FN conformational change from the soluble, compact and non-functional state to the insoluble, extended and highly adhesive state. This process is mediated through the binding of the activated cell surface integrins, primarily α5β1 integrin, to the RGD (Arg-Gly-Asp) cell-binding domain on FN.

In integrating the extracellular environment with the interior of the cell, integrins have a unique ability to transduce signals via "outside-in" and "inside-out" signaling. Growth factors, particularly transforming growth factor beta (TGFβ), modulate integrin activation in fibroblasts. TGFβ controls the transcription of genes encoding integrins via

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binding to its receptor, leading to the phosphorylation of intracellular effector proteins Smad2 and 3 and activation of downstream signaling and translocation to the nucleus. The crosstalk between integrin and TGFβ is essential for FN matrix assembly; however, the molecular mechanisms underlying FN secretion and assembly are not well known. Studies have shown that modulation of focal adhesion kinase (FAK), Rho-GTPases, protein kinase Bα (Akt1), mammalian target of rapamycin (mTOR), and MAP kinase pathways are among the regulatory mechanisms that play an integral role in FN secretion and assembly.

Abnormalities in the crosstalk between integrins, growth factors and FN is characterized by excessive differentiation and ECM remodeling resulting in aberrant response to tissue injury and contributing to a variety of pathological processes. The aim of this review is to describe the mechanisms and consequences of FN secretion and matrix assembly, and provide a brief overview of its clinical impact. First, we describe FN structure and the important features responsible for interaction with integrins and subsequent cell functioning. Next, we focus on the matrix assembly and essential mechanisms underlying this process. Finally, we discuss how FN can drive pathophysiological processes in fibrosis and cancer.

ABBREVIATIONS

Akt	protein kinase B
ARF	ADP-ribosylation factor
Cav-1	caveolin-1
Cdc42	cell division cycle 42
EC	endothelial cell
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial mesenchymal transitions
ERK	extracellular signal regulated kinase
FAT	focal adhesion targeting,
FGF	fibroblast growth factor
FN	fibronectin
FAK	focal adhesion kinase
GAP	GTPase-activating protein
GEF	GDP–GTP exchange factor
GSK3β	glycogen synthetase kinase 3 type β,
JNK	jun N-terminal kinase
MAPK	mitogen activated protein kinase
MEF	mice embryonic fibroblast
MLC	myosin light chain
MLCP	myosin light chain phosphatase
mTOR	mammalian target of rapamycin
MMP	matrix metalloproteinase
Src	steroid receptor co-activator
PAK	p21-activated kinase
PDGF	platelet derived growth factor
PI3K	phosphatidylinositol-3 kinase

PP2A	protein phosphatase 2A
PTEN	phosphatase and tensin homologue deleted on chromosome ten
PTKs	protein tyrosine kinases
PYK2	Proline-rich tyrosine Kinase 2
RGD	Arg-Gly-Asp cell-binding sequence
ROCK	Rho-associated kinase
RTK	tyrosine kinase receptor
Smurf	Smad –ubiquitination regulatory factor
TGFβ	Transforming growth factor β
VEGF	vascular endothelial growth factor

INTRODUCTION

The fundamental importance of the extracellular matrix (ECM) protein fibronectin (FN) can be inferred by its ubiquitous presence in tissues and by the non-viability of FN-deficient embryos. Under normal conditions, the ECM, specifically FN, is an important determinant of biological functions as illustrated by its high efficiency in promoting anchorage dependent cell cycle progression [1] and cell survival and proliferation [2]. Of the ECM proteins, adhesion to FN specifically stimulates cell cycle progression by the activation of RhoA in addition to growth factor mediated activation of Ras/MAPK signaling [1]. More interestingly, adhesion to FN decreases growth factor requirement for DNA synthesis by up to 1000-fold [3, 4] and selectively desensitizes specific growth factor stimulation in fibroblasts [5].

A principal component of the ECM, FN is especially abundant in tissues undergoing development or wound healing [6]. FN is critical for embryogenesis and is instrumental in various physiologic and pathologic processes. FN-rich matrices serve as regulators of cellular processes including proliferation, survival and differentiation, and provide structural support for cell adhesion and migration in ECM remodeling and tissue organization [6, 7]. Given the complexity and variability of its roles in basic cellular processes and binding partners, FN is involved in a large number of critical events including embryogenesis [8] where it has been shown to be pivotal for development as demonstrated by early embryonic lethality in mice with null mutation in FN gene.

Furthermore, FN is critical in wound healing processes, such as tissue morphogenesis [8, 9] thrombosis [10] and inflammation [11], and maintenance of normal tissue architecture [12, 13]. Abnormalities in FN, such as increased matrix deposition and assembly, have also been associated with several pathologies, including tumorigenesis and metastasis [14], fibrosis [15], and atherosclerosis [11]. Chronically elevated TGFβ1 levels are associated with excessive fibrosis which is characterized by increased FN deposition [16-19]. The hallmark of fibrotic disease is increased ECM protein synthesis by fibroblasts persistently stimulated by TGFβ1.

FN is a multi-domain adhesive glycoprotein that is multifunctional and is expressed and secreted by different cell types, particularly fibroblasts. FN regulates cell-ECM contact and cell-cell interactions by binding to different components of the ECM and to the membrane bound cell surface receptors, particularly integrins. FN that is secreted from a variety of cells is assembled into matrix by integrins, mainly α5β1, which is the primary integrin for FN

matrix assembly [20]. Furthermore, cross talk between integrins and TGFβ is an important modulator of FN transcription and assembly through a complex network of signaling pathways such as MAP Kinases and PI3K-Akt pathways [21].

Dynamic alterations in the structure and composition of the ECM, termed ECM remodeling, occur at different stages of development, during tissue repair and remodeling, and as a result of many pathologies and malignancies [22, 23]. Cells are continually integrating a plethora of signals to determine cell fate, survival and proliferation. It is in this light that the PI3K-Akt pathway can be considered as a central integrator of a tangled web of signaling networks with direct and indirect effects on each other. Thus, better understanding of FN regulatory mechanisms is crucial in order to develop novel therapeutics.

Although extensive research has established the role of MAP kinases in regulating FN mediated cell processes, we are only just beginning to understand the pivotal role of Akt [24-26]. Numerous studies have implicated the essential role of Akt, however the mechanisms by which Akt mediates and modulates FN induced signaling are just beginning to be explored. . Thus, the purpose of this review is to explore the molecular mechanisms mediating FN synthesis and matrix assembly, focusing on Akt, a major intracellular signaling protein.

STRUCTURE OF FN

FN is considered a novel, highly conserved, multi-domain glycoprotein. While encoded by a single FN gene, *FN1* on chromosome 2q35, alternative splicing can generate as many as 20 isoforms in humans [17, 27]. FN is found in two different forms: "plasma FN or pFN", a soluble dimer in the circulation which is secreted by hepatocytes directly, and "cellular FN or cFN" which is synthesized locally in tissues by many cell types including fibroblasts and endothelial cells [19, 28]. A key distinctive feature of cFN is the inclusion of variable amounts of either or both extra-domain (ED)-A and ED-B, Type III modules subject to alternative splicing by exon skipping, also termed exon shuffling. Additionally, almost all cFN subunits contain the variable, V region (also termed Type III connecting segment (IIICS) element. In contrast, pFN lacks ED-A and ED-B and only one of its subunits contains the V region [17]. Embryos that lack both ED-A and ED-B show cardiovascular defects due to altered FN functions [29].

ED-A is suggested to play a central role in the differentiation of fibroblasts to myofibroblasts [30, 31]. Indeed, the importance of the EDA domain in wound healing and fibrosis were shown in EDA null mice, which demonstrated abnormal wound healing [32] and an absence of pulmonary fibrosis despite bleomycin treatment [15]. Thus, EDA cFN plays a critical role in fibroblasts activation and mediating fibrotic disease.

FN is secreted as a soluble dimeric glycoprotein linked by inter-chain disulfide bonds near the C-terminus. Each FN monomer, 220-250 kDa, is folded into five or six domains connected by a flexible polypeptide chain. Each domain is composed of a mosaic of secondary structure modular repeats: 12 type I modules (FN-I), 2 type II modules (FN-II) and 15 to 17 type III modules (FN-III), and a variable sequence (v) [16].

Within each fragment, there are several discrete domains responsible for the binding versatility of FN. C-terminal FN- I_{10-12} is a FN binding domain, also known as an assembly domain, necessary for FN – FN interaction and initiation of FN assembly.

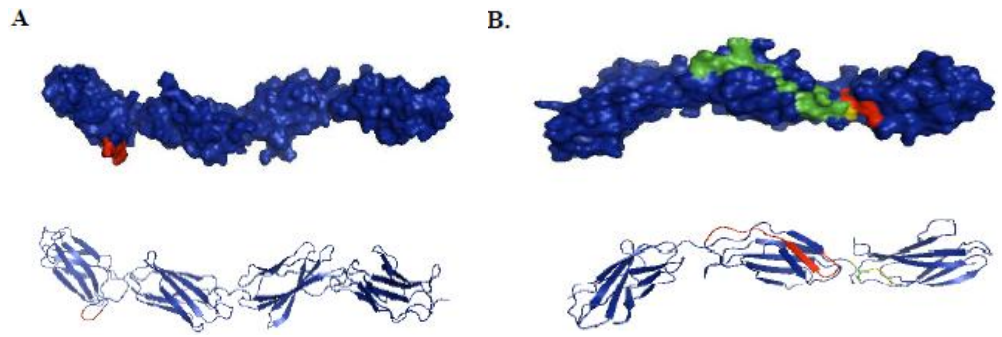

Figure 1. RGD site and integrin binding sites in human FN: A. RGD sequence (residues 1493–1496) (red). B. Integrin $\alpha_{IIb}\beta3$ (green) and $\alpha4\beta1$ (red) binding motif, and binding motif containing Asp184 residue (yellow).

The cell binding domain of FN is composed of FN- $III_{1\text{-}11}$, including the alternatively spliced ED-A and ED-B modules, specifically FN- III_{10} and FN- III_9. Several sites within this domain play a role in FN matrix assembly and/or interact with integrin. FN- III_{10} contains the most critical and primary cell binding domain, Arg-Gly-Asp (RGD). This sequence is necessary for mediating the interaction between FN and $\alpha5\beta1$ integrin; FN is the only adhesion molecule recognized by $\alpha5\beta1$ integrin, which play an integral role in FN polymerization and matrix assembly [33-35]. FN also binds to other integrins including $\alpha v\beta1$, $\alpha v\beta3$ and $\alpha IIb\beta3$ integrins, but with a lower affinity [36, 37].

In addition, FN- III_9 is also important for interaction with certain integrins through the amino acid sequence 'Pro-His-Ser-Arg-Asn (PHSRN)' [37]. This sequence is known as a "synergy site", and is unique to FN [38] as it further enhances the binding affinity of $\alpha5\beta1$ integrin for FN [39] (Figure 1). FN Type III_1 has self-association activities and a cryptic heparin binding sites [40]. The exposure of that matricryptic site is necessary for matrix FN to localize to lipid rafts, caveolae, where it regulates cytoskeletal organization and cell growth [41]. The variable composition of FN molecules facilitates a diversity of interactions with cell surface receptors that suggest a role beyond the structural considerations of the extracellular matrix.

SECRETION, POLYMERIZATION AND ASSEMBLY OF FN

FN is synthesized and secreted by different cell types including fibroblasts, one of the most ubiquitous cell types in the body, along with endothelial cells and macrophages. Following FN protein translation, FN is folded, dimerized and glycosylated in the endoplasmic reticulum [42, 43]. FN then is secreted as a soluble compact globular dimer, a process mediated by electrostatic interaction, a regulatory mechanism likely to prevent intermolecular interactions and cell aggregation prior to assembly [44]. Emerging evidence indicates that the polymerized insoluble FN is functionally distinct from soluble protomeric FN. FN matrix assembly regulates cell growth, contractility, cell migration. FN-null mice embryonic fibroblasts (MEFs) fail to spread or proliferate on type I collagen gel, while addition of FN to these cells results in increased cell proliferation and micro-tissue formation

[45]. FN matrix assembly plays an important role in preserving the structural integrity of blood vessels. FN regulates the composition and organization of the ECM. FN deposition regulates tenascin C fibrils, and the deposition of fibulin and fibrinogen in the ECM. Notably, FN secretion and FN polymerization regulate both the deposition and maintenance of collagen types I and III [46].

Cells exert mechanical force on their surroundings, and the mechanism varies according to stimuli, environment, morphology and differentiated state of cells. FN undergoes cell mediated assembly to form insoluble, elastic matrix fibrils. Cells can apply cytoskeleton-generated tension to unfold FN leading to changes in cell morphology, cell signaling and proliferation [38]. This mechanical force permit cells to change biochemical signals received from the environment, in a force- and time-dependent manner, by altering molecular recognition through unfolding and extending FN. This is achieved by exposing cryptic sites for FN-FN binding; changing the spatial positions of the RGD and synergy sites altering α5β1 integrin binding selectivity and signaling pathways; a tensile molecular recognition switch achieved by unraveling of modules such as FN-III_{10}, which was shown to straighten the RGD loop, thus, reducing accessibility and affinity to cell surface integrins. FN-III_{10} is the first module to unfold in FN-III module repeats due to its low mechanical stability [38]. Cell mediated assembly of FN into fibrils is essential to fully exploit the mechano-chemical characteristics of FN to regulate cell signaling.

FN also exhibits mechanical properties that allow the coupling of cytoskeletal generated tension to modulate fiber directionality and extension during polymerization. The mechanical coupling to the cytoskeleton exposes cryptic sites on FN that affect its affinity and selectivity to different members of the integrin family. Unmasking of previously unrecognizable receptor-binding sites may result from conformational changes due to proteolytic cleavage within a domain of an ECM molecule. Different molecular effects resulting from proteolytic cleavage of an ECM protein by matrix metalloproteinase 9 (MMP9) may lead to the exposure of a previously hidden, or 'cryptic', binding site for integrin receptors, such as an RGD-containing binding site for a β1 integrin receptor [47].

FN matrix assembly regulates cell morphology and functions by regulating the interaction of integrin with the actin cytoskeleton. The translocation and formation of α5β1 integrin-containing fibrillar adhesion requires FN matrix assembly [46] and occurs via a tensin-dependent mechanism [48]. Early FN fibrillogenesis requires α5β1 integrin translocation from focal contacts to the ECM contacts a process dependent on tensin, a primary cytoskeletal component of ECM contacts and which is also an actin binding protein.

It has been suggested that loss of cell-substrate adhesion does not result in loss of FN matrix. Several studies have observed that despite disrupting FN matrix, cells remained attached and spread, and expressed well-developed stress fiber and focal contacts [46]. Studies have shown FN matrix turnover process to involve the release of proteases into the ECM and may also include FN endocytosis [49]. Changes in cytoskeletal dynamics are suggested to be critical for FN matrix turnover. Indeed, a dynamic, reciprocal relationship exists, where disruption of actin cytoskeleton disrupts FN matrix organization, and FN polymerization regulates actin cytoskeletal assembly.

FN mechanically couples the ECM of cells to the cytoskeleton via integrin; mechanical forces are among the cues that determine cell shape and ultimately gene expression and eventual fate [38]. Integrin mediated anchorage to the ECM is required for cell proliferation

by initiating signaling pathways that are co-regulated by growth factors. Integrins mediate a "dynamic reciprocity" between cell-cell and cell-ECM [77].

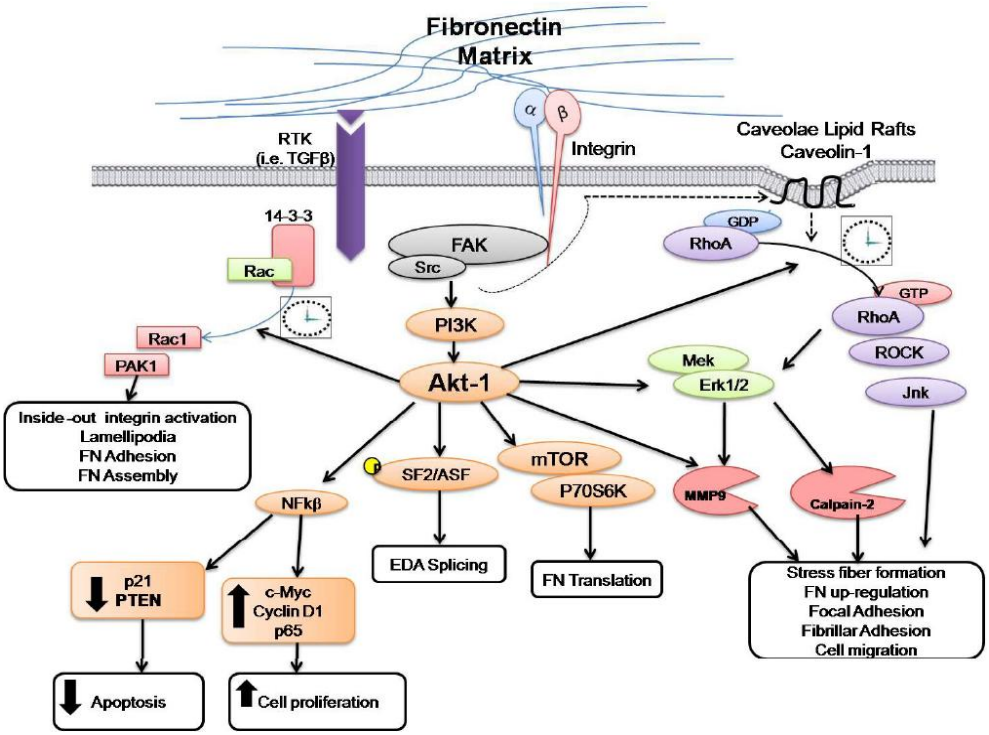

Figure 2. Schematic representation of the role of Akt1 in mediating the dynamic reciprocity between FN and integrins: Upon integrin- and/or RTK-mediated cell-FN attachment, FN induces integrin activation and clustering into focal adhesions, a process that requires the presence of FAK-Src complex. FAK/Src complexes serve as mechanosensors that converge extracellular signaling into the cytoskeleton by cross-linking signaling from integrins and RTKs, via proximal interactions. In certain cells (e.g. embryonic stem cells), activation of caveolin-1 by FN/integrin/FAK-Src is necessary for cell survival and proliferation. PI3K/Akt dynamically regulates FN-integrin interaction. FN ligation can induce the activation of Akt1, outside-in signaling, through β1 integrin cytoplasmic domain and FAK-Src complex. Conversely, Akt1 mediates FN synthesis and matrix assembly via inside-out signaling pathways. *FN stimulates its own synthesis and matrix assembly*. Akt1 enhances FN translation and ED-A alternative splicing via the activation of mTOR/P70S6k pathway and direct phosphorylation of SF2/ASF, respectively. FN is exported to ECM where it binds integrins, and Akt1 enhances inside-out activation and accumulation of integrins in focal adhesion through its cross talk with RhoA (contraction in lagging edge) and Rac (protrusion in leading edge) at different stages during cell motility and integrin clustering, simultaneously. Thus, enhanced integrin affinity for FN generates mechanical forces that reveal cryptic sites on FN necessary for FN matrix assembly. Furthermore, FN regulates cell survival and proliferation through Akt1 mediated down-regulation of PTEN and up-regulation of c-Myc and cyclin D1. *FN regulates matrix turnover.* FN binding to integrin activates Akt1 and Erk pathways that up-regulate calpain-2 and MMP9 that are necessary for de-adhesion during cell motility and matrix degradation.

MOLECULAR MECHANISMS REGULATING SECRETION AND ASSEMBLY OF FN MATRIX

Although the molecular mechanisms governing FN secretion and assembly are not fully understood, a hierarchy is evident in the assembly process. FN interaction with integrins and polymerization into the ECM is influenced by several growth factors and signaling proteins [50]. Transforming growth factor β (TGFβ), an extracellular growth factor, enhances secretion of ECM proteins including FN, increases the number of matrix assembly sites and enhances assembly of fibronectin matrix [51]. Conversely, an increase in intracellular cAMP levels decreases the number of assembly sites and inhibits FN polymerization [52]. Other signaling molecules that have been implicated in the regulation of FN matrix assembly include Src kinase, phosphatidylinositol 3-kinase [50], other growth factors [51], and protein kinase C [53].

Growth factors such as TGFβ [54-56] and cytokines [57] can regulate FN synthesis at the transcriptional level and translational level, through modulation of mRNA stability or translational efficiency. Regulation of FN synthesis also occurs at the level of protein degradation. Turnover of the FN matrix *in vivo* occurs relatively quickly, with as much as half of labeled FN that was incorporated into the ECM being lost within 24 hours [58]. ECM degradation and turnover can be mediated by many proteases, including plasmin, plasminogen activators, and MMPs [59], and by caveolin-dependent endocytosis [60] and intracellular proteolysis [61].

Integrin-FN Interactions in Cellular Adhesion and Assembly of FN Matrix

Integrins, α-β heterodimeric transmembrane molecules, are the principal receptors for ECM [34]. The α5β1 integrins, "classic" FN receptor, is the major integrin receptor responsible for initiating FN matrix assembly. Other integrins can bind FN but do not induce FN assembly [48]. In the absence of RGD-α5β1 integrin interaction, some FN matrices can still be assembled, however less efficiently, by interacting with α_v integrins, such as αvβ3, or αIIbβ3 [18]. Binding of FN induces integrin clustering, further increasing their affinity to FN [18, 48]. More generally, an individual integrin will recognize several distinct proteins [34]; for example, the αvβ3 integrin has been reported to bind collagen VI, laminin, FN, vitronectin, thrombospondin, Von Willebrand factor, and fibrinogen [62]. The β cytoplasmic domain is essential for integrins recruitment to focal contact as evident by impaired recruitment due to its truncation/mutation. The β cytoplasmic domain alone, expressed as a fusion chimera with another membrane protein, has been shown to be sufficient for focal contact localization [63, 64]. The α cytoplasmic domain serves as an auto-inhibitor of certain functions of the β cytoplasmic domain such as focal contact recruitment. Ligand binding induces conformation changes that relieve this auto-inhibition [18, 48]. Furthermore, the β cytoplasmic domain is important in signal transduction, particularly integrin activation of FAK whereas truncation or mutation of the α cytoplasmic domain does not impact this process [65].

Integrin α5β1 is critically important in vertebrate development, as demonstrated by the early embryonic lethality of null mice [66]. Notably, this phenotype is even more severe and occurs at an earlier stage than that observed in FN-null mice. Interestingly, α5 knock-out mice are embryonic lethal despite their ability to develop normal FN matrix [67, 68]. This suggests that α5β1-FN interaction conveys critical signals in development that are not compensated by other integrins including αv, thus further illustrating the importance of this integrin and FN in

embryogenesis . Furthermore, different cell types depend on different integrins for survival. For example, α5β1 integrin promotes survival in fibroblasts, endothelial cells (ECs) and epithelial cells [69, 70], and αvβ3 integrin is important for angiogenic ECs survival [71]. It has also become apparent that integrin mediated signaling pathways can be integrin specific. Whereas α5β1 integrin mediated adhesion to FN supports cell survival by up-regulating Bcl-2 and inhibiting Bad, caspase-9, and p53 through activation of FAK, PI3K/Akt, and CAMK IV signaling [69, 72-74], αvβ1 is inefficient in supporting FN matrix assembly and cell migration [75]. Cell adhesion is essential for embryonic development and tissue function and repair following injury [76] and is essential for maintenance of tissue integrity and polarity. Integrin mediated adhesions are known to undergo dynamic changes in structure and molecular composition from focal complexes to early focal adhesions- stress fiber associated focal contacts.

Integrins drive early FN fibrillogenesis, thus providing direct "dynamic reciprocity" [77] between the ECM and the cytoskeleton [48]. Inactive integrins, which are diffusely distributed in the plasma membrane, are recruited into focal contacts where they can be re-activated [18]. Cytoskeletal generated tension can occur through two types of integrin-containing structures, focal contact and focal/fibrillar adhesions [48, 78]. Focal contact mediates transmembrane linkage between the ECM and the cytoskeleton, and is composed of structural, cytoskeletal, and signaling proteins, where talin and α-catenin bind the β-integrin cytoplasmic tail domain. Soluble, compact FN dimer selectively binds to integrin via the RGD and synergy sites simultaneously, thus, initiating FN polymerization and fibril matrix assembly [46].

Integrins modulate the activity and localization of Rho-GTPases and their effector proteins that in turn regulate actin cytoskeletal dynamics that govern the morphological changes during cell adhesion and spreading on ECM [79]. Rac1 and Cdc42 stimulate the formation of "focal complexes", small cell matrix adhesions, associated with lamellipodia and filopodia [80]. RhoA stimulates actin stress fibers formation and the maturation of focal complexes to focal contacts and fibrillar adhesions [81, 82].

Fibrillar adhesions, also termed cell-matrix adhesions, contain tensin and α5β1 integrins, and co-localize with FN fibrils [46, 83]. Tensin is one of the major cytoskeletal proteins in fibrillar adhesions [78, 84]. Tensin has multiple actin-binding sites [85] and associates directly with the β1 integrin cytoplasmic tail via its phosphotyrosine-binding domain [86, 87]. There is evidence that suggests tensin is required for the formation of fibrillar adhesions [48]. In addition, when FN matrix assembly is blocked, fibrillar adhesions are not generated and tensin resides in focal adhesion plaques [46], suggesting that fibronectin regulates the distribution of tensin.

In addition to mediating adhesion to ECM, integrins are important in ECM assembly. Integrin connection to the actin cytoskeleton is essential for FN fibrillogenesis. FN fibrillogenesis requires integrin-mediated binding to soluble FN dimers and conformation changes in FN mediated by Rho-dependent cytoskeletal contractility to stretch integrin-bound FN molecules exposing cryptic FN binding sites [88-90]. The specific translocation of α5β1 integrin together with tensin from focal contacts to another cell-matrix adhesion "fibrillar adhesions" is of critical importance in FN matrix assembly [48, 78]. A fragment of tensin inhibits both the translocation of α5β1 integrin and FN fibrillogenesis without affecting focal

contacts [48], thus demonstrating that the high efficiency with which α5β1 modulates FN is partly due to its association with tensin.

Cell-cell adhesion is mediated by an analogous process but with different players which include tight junctions and adherens junctions, which include cadherin mediated adhesion [91]. The adhesive functions of both integrins and cadherins are well established and are known to require cytoskeletal interactions [92]. However the dynamic interplay between them is not well understood. Lefort and colleagues have recently identified α5β1 integrin and tensin as novel components of N-cadherin adhesion complexes and demonstrated the regulatory role of matrix FN on cell-cell and cell-ECM dynamics. In the absence of FN, α5β1 integrin and tensin localize to cell-cell adhesion complexes Ligation of FN to α5β1 integrin triggers the dissociation of tensin from N-cadherin and promotes tensin association with α5β1 integrin. The α5β1 integrin and tensin complex then translocates to the ECM fibrillar adhesions [93]. Thus, FN matrix assembly coordinates the interaction between cell-ECM and cell–cell adhesion.

Integrin-mediated adhesion to matrix proteins such as FN and collagen results in activation of complex pro-survival signal transduction pathways. Cross linking of different integrin heterodimers initiates different signaling pathways [94]. Mutual and cooperative crosstalk of integrins with growth factor receptors such as TGFβR regulates intracellular signaling pathways such as FAK/Src, MAPK, PI3K signaling and the activity of Rho family of small GTPases [95].

Integrin-Growth Factor Cross-Talk in the Assembly of FN Matrix and Fibrogenesis

Integrins can also regulate cell-ECM interaction through their association with growth factor receptors [96]. There is extensive cross-talk between integrins and growth factors that modulate mitogenic signaling; For example, whereas an acute and robust activation of Erk-MAPK signaling occurs in response to growth factor stimulation, FN adhesion leads to milder Erk activation with slower kinetics [79, 97].

Several integrins can regulate TGFβ signaling directly by TGFβ activation, or indirectly through regulating SMAD dependent and independent signaling. The importance of the synergy between growth factors and integrin has been demonstrated best in cell migration through FAK/Src signaling. FAK (focal adhesion kinase) a non-receptor tyrosine kinase, has been shown to act as a receptor proximal regulatory protein that directly integrates growth factor and integrin signals.

FAK binds though its C-terminus, to the ECM-ligated integrins induces autophosphorylation at tyrosine 397 (Y397) which leads to binding to another tyrosine kinase, Src, which in turn phosphorylates FAK at tyrosine 861 (Y861). In addition to the localization through the C-terminus into the integrin adhesion complex, FAK proximally regulates and directly interacts with the cytoplasmic domain of cognate growth factor receptors, thus conveying both integrin and growth factors mediated signaling [98].

TGFβ Signaling in FN Secretion and Matrix Assembly

TGFβ is crucial due to its important role as a transcriptional regulator where it stimulates the production of ECM proteins including FN and tissue inhibitors of metalloproteinases, and decreases the synthesis of FN degrading MMPs.

TGFβ superfamily ligands are secreted morphogenes, some of which are crucial regulatory cytokines that are essential in activating myriad of signaling cascades that modulate diverse cellular processes in embryonic development and adult tissue homeostasis [99].The source of the activity was identified as two distinct factors, TGF-α and –β Roberts [100-102]of which TGF- α belongs to the epidermal growth factor (EGF) family of growth factors. In mammals, TGFβ has three different isoforms TGFβ -1, -2, and -3 that are known to function as modulators of ECM proteins by induction of both gene activation and protein formation. Studies showed that mechanical strain/loading/stretch induces TGFβ expression and FN interaction.

TGFβ 1, the prototypical cytokine, acts primarily through the SMAD protein to stimulate fibroblast activation and fibronectin deposition. Mature TGFβ remains associated with latency associated peptide (LAP), thus inhibiting TGFβ activation [103]. Upon release from the latent complex, mature dimeric TGFβ ligand binds to a serine-threonine transmembrane kinase, TGFβ Receptor type II (TGFβRII), which is intrinsically active. This is followed by the recruitment and phosphorylation of TGFβR type I (TGFβRI) kinase resulting in the formation of a heterodimeric serine/threonine kinase receptor complex and propagation of signal cascade via downstream Smad proteins. Activated TGFβRI initiates the canonical signaling pathway where it directly phosphorylates and activates effector proteins Smad 2 and Smad 3, also known as receptor-regulated Smad (R-Smad) which are regulators of transcription. Smad-2 and -3 are anchored by the cytoskeletal protein filamin A. Phosphorylated Smad 2/3 undergo conformational changes through unfolding and subsequently forming a heterotrimeric complex with Co-Smad4 and translocating into the nucleus. Smad4 is not crucial for TGFβ activity [104], and nuclear import of Smads does not require Smad4. The TGFβR complex remains active for at least 3 to 4 hours after ligand binding and continuous receptor stimulation maintains Smad complex accumulation in the nucleus where they regulate gene transcription. Smads interacts with transcriptional co-activators such as CBP, p300 or ARC105 to regulate the expression of target genes. Smad proteins can mediate the activation or repression of target genes, including those that encode integrins; a process regulated by cell type and Smad protein associated partners [99, 105, 106]. Initially, studies have identified Smad target specific genes through direct binding to DNA. However, it has become apparent that Smads act as transcriptional co-modulators and require the association with other transcription factors such as ATF/CREB, AP-1, FAST and SP1 to mediate transcriptional activation or repression of different genes [107-110].

TGFβ signaling is negatively regulated by inhibitory Smads (I-Smad), Smad 6/7, which compete with R-Smads for TGFβRI or Co-Smad complex formation. I-Smads regulate the transcription of Smurf (Smad –ubiquitination regulatory factor) proteins, which promotes the degradation of TGFβRI through proteosomal pathways [106, 111].

More recently, it has been shown that ligand-activated TGFβ receptors can stimulate signaling through non-canonical (Smad-independent) signaling predominantly via PI3K/

Akt/mTOR, RhoGTPases and MAPK pathways. This in turn controls a variety of cellular responses which modify cell growth and survival. Some of these pathways have been shown to also cross-talk and regulate Smad activation and signaling, including JNK and p38 MAPK pathways [112].

In wound healing, TGFβ suppresses the inflammatory response and promotes the formation of granulation tissue by inducing fibroblast proliferation and differentiation. TGFβ induces the expression of FN and different integrins including α1β1 and α2β1, which mediate fibroblast contraction, and αv integrins that activate latent TGFβ. Additionally, αvβ3 can suppress TGFβ signaling by inhibiting TGFβR expression. Over-expression of the α5β1 integrin leads to up-regulation of TGFβR expression and establishes a growth inhibitory autocrine loop for TGFβ [113]. Conversely, TGFβ treatment of NRK fibroblasts leads to upregulation of α5β1 expression and a loss of anchorage dependence of growth [114]. Additionally, latent forms of TGFβ are ligands of αvβ6 and αvβ1 integrins, and the association of TGFβ with αvβ6 leads to spatially restricted activation of TGFβ.

Interestingly, the transcriptional control exerted by TGFβ leads to regulation of integrin activation and function in a feedback loop. TGFβ regulates integrin expression and activation via 'inside-out' mechanisms, it also regulates the expression of integrin ligands, including FN and tenascin, and integrin associated proteins, such as paxilin and ILK. (Fransvea et al 2009, Pechkovsky et al, 2008).Cell adhesion and spreading, migration, survival and proliferation all depend on integrin signaling through FAK, PI3K activated downstream of growth factor stimulation and ECM attachment [50].

FAK/Src Pathway Regulating Assembly of FN Matrix

Mounting evidence lends its weight to a critical role of Focal adhesion kinase (FAK) in FN-integrin mediated signaling. Immunoprecipitation studies demonstrated that FAK undergoes enhanced tyrosine phosphorylation in response to FN [115] [116], and its tyrosine kinase activity in NIH 3T3 fibroblasts increases 2 to 3 folds and is further enhanced in v-Src transfected cells [115]. It is ubiquitously expressed in mammalian cells, and the targeting and localization of FAK to the focal adhesion has been demonstrated in a variety of cells including fibroblasts, ECs, keratinocytes, and osteoclasts. Following its discovery as a focal adhesion protein that was phosphorylated in response to Src transformation [117, 118], extensive research has revealed that FAK plays an important role in normal development and has been implicated in diseases including cancer. Germ line deletion of FAK results in embryonic lethality as demonstrated in FAK deficient mice, embryos showed gastrulation abnormalities mesodermal deficiency similar to those found in the FN-deficient mouse [8, 119]. Furthermore, FAK-/- cells showed a dramatic reduction in cell motility, survival and migration due to the inability to turnover adhesion complexes [119]. Further studies showed poor vascularization and defects in the fusion of cardiac plates [120].

Due to the lack of enzymatic activity of the short integrin cytoplasmic tails, recruitment of adaptor and signaling proteins are essential for effector functions [83]. Activation of FAK and Src family protein tyrosine kinases (PTKs) and subsequent protein tyrosine phosphorylation is a prominent event in integrin outside-in signaling [121]. Hence, the activation of the non- receptor protein tyrosine kinases, FAK and Src, are the best characterized key coordinators of integrin signaling.

FAK plays a central role in integrin signaling and is essential for fibroblast assembly of FN fibrils [120]. FAK is a unique member of the FA-PTK family, which also contains PYK2. Unlike other non-receptor tyrosine kinases, FAK does not contain SH2 or SH3 domains. Instead, it contains a central catalytic domain flanked by large non- catalytic domains, the N-terminal FERM homology domain that interacts with b1-containing integrins, and a C-terminal domain containing a focal adhesion targeting (FAT) sequence [117, 118]. These contain multiple binding sites for proteins including Src and paxillin and PI3K; Tyr-397 on FAK serves as a binding site for PI3K. Tyrosine phosphorylation of FAK is regarded as not only important in integrin mediated signaling, but also in integrin-independent signaling.

The activity of FAK can be influenced by diverse input signals, from integrins to growth factors. Thereby, the multiple tyrosine residues of FAK act as a platform for many different interaction proteins and because of this, FAK plays an indispensable role in multiple signal transduction pathways. It has been clearly shown, that FAK is necessary for cells to survive and resist anoikis, a term describing apoptosis as a result of loss of adherence. Moreover, FAK is indispensable in various aspects of cell motility. Due to its very upstream position in signaling, it is still unknown in which way certain signal inputs lead to specific and well-timed arrangements of FAK-protein complexes and to very distinct downstream effects.

Cell-ECM attachment induces FAK auto-phosphorylation at Tyr397, which correlates with increased catalytic activity, either directly through integrin clustering or indirectly through Src [122, 123]. Upon integrin clustering, FAK is recruited to the focal adhesion complex. In the inactive form, FAK is in a closed conformation in which the N-terminal FERM domain, auto-inhibitor, directly binds and inhibits the catalytic domain rendering tyrosine sites inaccessible. Upon competitive binding of integrin with the FERM domain, FAK unfolds and undergoes rapid auto-phosphorylation at Tyr397, which creates high affinity binding for Src through its SH2 domain. Although integrin ligation is the main activator of FAK, FAK can also be activated by growth factors, mechanical stimuli and GPCR agonists.

FAK and paxillin localize to focal complexes - a process mediated by Rho family members Rac and Cdc42, not RhoA by itself. The interaction with actin cytoskeleton and Rho modulated actino-myosin tension regulates the maturation of focal complexes into focal adhesions. Rho also mediates FA turnover. Paxilin, which is phosphorylated following integrin activation, serves as a scaffold protein to mediate downstream integrin-signaling. Germ line deletion of paxilin showed results in a similar phenotype as the deletion of FAK and FN. In ex vivo studies, paxillin null cells showed deficient focal adhesion formation, migration and localization of FAK to focal adhesion complexes. Therefore, paxillin is considered to be a critical transducer of FN signals in development [124].

Rho Pathway in FN Matrix Assembly and Fibrogenesis

RhoA was isolated by Richard Axel's group in 1985 and was the first member of Rho/Rac family identified [125, 126]. Of the six subfamilies, we will focus on Rho, Rac, and Cdc42 owing to their essential role as molecular switches in regulating cellular responses such as cytoskeletal changes, microtubule dynamics, cell polarity and cell cycle progression [127].

Renand and colleagues [128] developed as assay to detect GTP-Rho and reported initial observations that underscored the importance of these molecular switches and laid the foundation for extensive studies aimed at understanding Rho regulation. Utilizing direct measurement of Rho activity, they reported that Rho can be activated upon ECM ligation or by soluble factors such as LPA and that the protein exhibits a biphasic regulation during cell spreading on FN. The initial phase is associated with a period of low Rho activity that corresponds with rapid spreading on FN with cells showing small focal complexes at periphery with minimal stress fiber or focal adhesions. This is followed by a transient increase in Rho activity leading to integrin clustering and formation of focal adhesions and stress fibers. Furthermore, cell adhesion to FN down-regulates growth factor induced Rho activation whereas in suspended cells its activity and MLC phosphorylation remain elevated. They suggested the existence of a negative-feedback loop following Rho-dependent focal adhesion formation that serves the dual purpose of preventing excessive formation of focal adhesion and contraction, and regulating focal adhesion disassembly necessary for cell migration.

Rho activation generates contractile force through phosphorylation of myosin light chain (MLC) on Ser19 [129] and inhibition of MLC phosphatase (MLCP) through the action of Rho-associated kinase (ROCK). Furthermore, RhoA can mediate TGFβ-induced matrix accumulation. For example, TGFβ-induced RhoA activation correlates with fibronectin upregulation in mesangial cells [110].

The initial cell attachment and spreading over a substrate is accompanied by reduced activity of the GTPase RhoA and simultaneous activation of the GTPases Rac1 and cell division cycle 42 (Cdc42), which suppresses actomyosin contractility and enhances actin-mediated protrusion. During early stages of cell spreading, α5β1 integrins inhibit RhoA activity through a Src-dependent tyrosine phosphorylation of p190RhoGAP [130]. Integrins also activate Rac1 and Cdc42 by activating FAK-Src-mediated tyrosine phosphorylation of p130CAS [130, 131]. The FAK–Src complex also phosphorylates paxillin, which recruits ADP-ribosylation factor (ARF)–GTPase-activating protein (GAP), as well as recruiting Rho guanine nucleotide exchange factor 7 (ARHGEF7; also termed β-PIX), the GDP–GTP exchange factor (GEF) for Cdc42 and Rac1 activation. Through a direct interaction within focal adhesions and membrane ruffles, ARHGEF7 recruits and activates Rac1 [132]. Thus, integrin signaling through the Src family kinases and FAK regulates the localization and activity of GEFs and GAPs to coordinate actin cytoskeleton stabilization with membrane protrusion thus mediating cell matrix adhesion and migration.

Adhesion to FN stimulates "outside-in" signaling through integrins that triggers a complex network of intracellular signaling events. For instance, fibroblasts plated on FN induce the activation of MAPK [133-135]. In converging adhesive signals between the ECM with cytoskeleton, integrins stimulates certain pathways that are critical for the initiation and maintenance of matrix assembly. FAK [120] and PI3K [50] are essential for assembly of FN fibrils. Members of the Src kinase family are essential for maintaining matrix association with the cell. FN matrix assembly was delayed in SYF cells (which lack Src family kinases Src, Yes, and Fyn) but not eliminated and this delay is overcome following increased Src expression and activity [131]. This suggests that Src plays an important supportive role in matrix assembly dynamics. After matrix accumulation, inhibition of Src activity causes rapid loss of matrix [136]. Consequently, these pathways are essential for cell adhesion, spreading, migration survival and proliferation.

PI3K/Akt Cascade in FN Secretion, Assembly and Fibrogenesis

Perhaps the most pivotal mediator and modulator of FN activity is protein kinase B (Akt), a serine/threonine kinase. Akt, a major effector downstream of PI3K, is a key survival kinase and is a multi-functional protein that regulates a plethora of cellular functions including survival, proliferation, and transcription, integrin trafficking and recycling, actin organization, angiogenesis and apoptosis. Although not fully understood, mounting evidence suggests that the versatility of Akt activity is associated with the complex network formed between Akt and its substrates such as GSK-3, mTOR, TSC, FOXO as well as cross-talk with other signaling pathways such as p38 MAPK and Rac –PAK [137-143].

The Akt family consists of three highly homologous isoforms that are transcribed from independent genes and have overlapping but distinct functions [138, 144]. Of the three Akt isoforms, we identified Akt1 as the predominant isoform that accounts for approximately 75% of total Akt activity in vascular cells [145].

It has been shown that owing to its complexity in regulating a variety of cell responses, Akt signaling is very fine-tuned. Prolonged Akt signaling is associated with embryonic lethality, edema and vascular malformation and remodeling abnormalities [146]. Loss of the Akt1 gene leads to growth retardation accompanied by a decrease in size of multiple organs and increased cell apoptosis [147]. Environmental cues including growth factors, cytokines, and ECM regulate the highly orchestrated multistep process of cell migration involving Akt [148].

Research from our laboratory has established the central role of Akt in the regulation of integrin activation in ECs and fibroblasts, ECM assembly, tissue remodeling and angiogenesis [56, 145, 149-152]. Several *in vivo* studies have shown the crucial role of Akt1 in wound healing and vascular maturation [151]. We previously reported several interesting features of an Akt1 knock-out mouse model including impaired migration, impaired secretion and assembly of ECM proteins and enhanced angiogenesis resulting in vascular leakage and skin abnormalities [145]. Akt mediates VEGF induced integrin activation in ECs [153, 154].

Specifically, VEGF-165 was shown to activate integrin αvβ3 leading to enhanced adhesion and migration of ECs a process that involves PI3K and Akt. Integrin activation is important for EC and fibroblast adhesion and migration by regulating ECM assembly. ECs isolated from Akt1-/- mice exhibited impaired integrin activation which was associated with impaired migration on matrix proteins including FN, and impaired ECM assembly in skin and vascular basement membrane of Akt1-/- mice [145]. Additionally, genetic ablation of Akt1 but not Akt2, leads to impaired wound healing characterized by a reduced vascular area and impaired vascular maturation, further demonstrating the importance of Akt1[151]. These finding suggests that Akt1 is essential for inside-out integrin signaling.

The tryptophan residue at position 775 of β1 integrin cytoplasmic domain was shown to be a specific regulator of Akt activation by repressing the activity of the protein phosphatase 2A (PP2A) in ECM assembly [143]. Mutation of Trp775 (W775A) in β1 integrin specifically suppresses Akt activation and fibroblast survival without interfering with other integrin-mediated signaling pathways. This has been shown to be due to rapid Akt de-phosphorylation through increased local activation of PP2A, which was 2.5 times higher compared to β1 WT

in response to PDGF [143].A subsequent study demonstrated similar results and further illustrated the importance of two distinct amino acid residues on β1integrin cytoplasmic domain, Trp-775 and Argenine-760 (Arg-760)in modulating cell spreading and FN matrix assembly.Trp-775 and Arg-760 differentially modulate FN assembly and cell spreading through talin and Akt-1respectively. While both Trp-775 and Arg-760 exhibit overlapping roles in regulating FN assembly through recruiting talin to the β1integrin cytoplasmic complex, Trp-775 selectively regulates Akt1 signaling in cell spreading on FN. W775A mutation disrupts Akt1 and talin activity leading to impaired cell adhesion and spreading, and defects in FN matrix assembly and fibrillar adhesion. Although over-expression of Akt1 in W775A mutant cells rescues cell spreading activity, it does not reverse impaired FN fibrillogenesis. This suggests that Akt specifically regulates cell spreading not matrix assembly, although the effect of Akt knockdown on FN assembly in β1WT integrin cells was not investigated directly. Thus the role of Akt in mediating inside-out FN assembly remains unclear [155].

Originally, it was thought that Akt mediates cellular functions in response to outside-in signaling induced by ECM-integrin ligation [156]. However, recent studies have emphasized the role of Akt in regulating integrin activity and, subsequently, mediating inside-out signaling [138, 145, 154].

FN matrix assembly is impaired in Akt1-/- fibroblasts [150]. Akt1over-expression results in significant increase in integrin activation, while Akt deletion impairs integrin activation and, in turn, impairs EC and fibroblast adhesion and migration [150]. Fibroblasts are necessary for effective repair in tissue injury and are known to express α5β1 integrin, while αvβ3, which mainly binds vitronectin, is expressed to a lower extent [89]. Deletion of Akt in fibroblasts significantly impairs adhesion to FN without affecting integrin expression in the presence and absence of growth factor stimulation; implying that Akt regulates integrin functional activity and FN recognition. Although Akt -/- and WT fibroblasts adhere to vitronectin to the same extent, it was to a much lower level compared to FN for both WT and Akt-/- cells. Similarly, Akt1-/- fibroblasts show a significant decrease in migration on FN compared to vitronectin [150].

Additionally, bFGF, not VEGF, induces PI3K dependent Akt phosphorylation and activation in fibroblasts. In addition to impaired FN matrix recognition, Akt deficiency – Akt1-/-, PI3K inhibitor, or Akt inhibitor (SH-5) - results in impaired FN matrix assembly in fibroblasts. Treatment with integrin β1blocking antibody significantly blunted FN assembly in Akt1-/- and Akt1+/+ fibroblasts. Notably, exogenousβ1 integrin activation, using TS2/16, rescues the defect on FN assembly caused by SH5. Similarly, restoration of Akt activity by reconstitution of Akt in Akt-/- ECs and fibroblasts rescues the defect in FN assembly and leads to enhanced migration. Thus, demonstrating that, in the absence of Akt1, impaired FN assembly is due to impaired ability of β1 integrin to recognize FN. Collectively, we demonstrate the essential role of Akt1 in regulating inside-out activation of integrins, specifically β1 integrin, FN recognition and matrix assembly in fibroblasts and ECs [150]. Furthermore, while α5β1 and αvβ3 internalization and transport to the recycling compartment are Akt-independent, PI3K and Akt regulate the trafficking and recycling of integrins back to the plasma membrane by regulating GSK-3 [157].

Numerous studies demonstrate that FN stimulates cytoskeletal remodeling, cell growth, survival, and migration via the PI3K/Akt/mTOR pathway [7, 158-160]. However, evidence of PI3K/Akt induced FN expression is not fully understood. Our previous findings indicate that

impaired adhesion and migration of Akt -/- fibroblasts results in impaired FN assembly in vitro [150] and in vivo [145, 151]. Akt1 regulates FN assembly through the activation of α5β1 integrin. We proposed that Akt1 also regulates FN synthesis and secretion [56]. Indeed, in TGFβ stimulated fibroblasts, FN synthesis and secretion in the conditioned media increases in a dose and time dependent manner; an effect that is further enhanced with over expression of Akt and impaired in Akt deficient cells. This suggests that Akt can modulate FN expression possibly through mTOR, known to be involved in protein translation and control of the overall rate of protein synthesis. Specific inhibition of mTOR results in diminished growth factor mediated FN synthesis and secretion.

Interestingly, we observed that mTOR regulates FN at translational not transcriptional level through activation of P70S6Kinase and S6Ribosomal Protein, both of which are downstream effectors of mTOR [56]. Similar results are observed in bleomycin stimulated fibroblasts; bleomycin is known to induce fibrosis potentially through TGFβ signaling pathways. Notably, we observed that mTOR is involved in transcriptional repression of several proteins including α4 and β3 integrins, matrix metalloprotease-8, -10, -11, and laminin. All together, we conclude that PI3K/Akt/mTOR regulates FN by controlling protein levels at translational level. A recent study supported this finding and also showed that Akt enhances EDA alternative splicing by directly phosphorylating and activating SF2/ASF [161]. To date, evidence for the mechanism of transcriptional regulation by mTOR has not been clearly illustrated this area requires further investigation. Collectively these data suggests a reciprocal dynamic regulation between FN and the Akt pathway.

FN-MEDIATED REGULATION OF DIRECTIONAL CELL MIGRATION THROUGH AKT SIGNALING PATHWAY

Recent studies have demonstrated caveolin-1 (cav-1) can serve as a scaffold protein that plays an important role in sequestering and modulating signaling pathways (reviewed in [162]) as evident in TGFβR internalization and degradation [163, 164] and association with β integrins to promote cell growth [165] and proliferation [166, 167]. In cav-1 deficient cells, TGFβ-mediated RhoA-activation is repressed, and cav-1 is required for TGFβ1 mediated FN expression [110]. Absence of cav-1 correlates with loss of membrane anchorage and cell cycle arrest [168]. A novel dynamic regulatory function between FN and cav-1 was recently illustrated by two groups. First, it was shown that FN stimulates cell proliferation, in part, by initiating and maintaining the proximal association between β1 integrin/FAK-Src and cav-1,and phosphorylation of cav-1is necessary to activate RhoA-PI3K/Akt-Erk1/2 pathways [169]. This study also suggested that the localization of RhoA to the caveolae microdomain is necessary for mediating cell functions. Indeed, another study demonstrated that the cav-1-Rho-GTPase interaction is crucial for α5 integrin expression and the activation of Src, Ras and Erk [170].

The Rho family of GTPases (namely, Rho, Rac and Cdc42) acting downstream of integrins, are critical regulators of the cytoskeletal dynamics involved in cell morphology, cell polarization and directed migration, and ECM assembly in vascular cells [81, 171-174]. Additionally, activation and localization of Rac and Cdc42 activate p21-activated kinase (PAK) auto-phosphorylation, a major downstream effector [175, 176] which is responsible

for myosin light chain phosphorylation which regulates cytoskeletal organization and cell migration. The process of cell migration and cell guidance migratory mechanisms has been extensively investigated [148, 177]. PAK1 is the major isoform of the PAK family, which contains seven isoforms, and which was recently identified as a substrate of Akt [178].

Although most studies on Rho have illuminated its role in stress fiber and focal adhesion formation in fibroblasts, it has become apparent that Rho also plays an important role in physiological cell migration and invasion in tumors.

The process of tread milling (please explain this term) and directed migration is a highly regulated step wise process. Chemo-attractants activate RhoA and Rac/Cdc24 and localize them to the trailing edge and leading edge, respectively. Cdc42 induces actin spikes and promotes filopodia extension leading to directional sensing, and establishment of cell polarity during adhesion [179]. Rac is required for F-actin formation and promotes lamellipodia and membrane ruffles formation leading to protrusion and extension of the leading edge. Rho activation is required for focal adhesion assembly, actin stress fiber formation which inhibits protrusion while promoting attachment and contraction at the trailing edge [180]. Contraction at the lagging end can in turn push the membrane at the other side outwards forming polarized directed migration. There exists a localized incompatibility of the two actin cytoskeletal responses, actin-myosin and actin polymerization, due to the ability of Rho and Rac to inhibit signals that promotes the other. Thus, each actin assembly gradually segregates spatially to the lagging or leading edge of the cell and, simultaneously, promotes the other's activity at a distance [181]. PI3K is necessary for localization of Rac- Cdc42/PAK1 signaling at the leading edge. Simultaneously, active PAK1 regulates PTEN and F-actin localization to the trailing edge [182].

Much attention has been focused on identifying the downstream signaling proteins of PI3K involved in regulating cell morphology and motility. It is known that PI3K is required for growth factor, and integrin mediated cell migration and is involved in actin filament remodeling induced by growth factors, integrins [183] and Ras. For example, whereas insulin stimulated cells exhibit rapid actin filament reorganization which is correlated with PI3K recruitment and activation in that region, PI3K inhibition abolishes insulin induced actin remodeling and membrane ruffling. Similarly, inhibiting actin filament formation disrupts PI3K distribution and signal transduction [184-187]. Furthermore, inhibition of actin filament formation also results in attenuated PI3K- and Akt-mediated cell migration [158]. This suggests an important crosstalk between RhoGTPases such as Rac and PI3K/Akt signaling pathways.

Previous studies have revealed the importance of Akt in modulating Rac-PAK activity and subsequent actin cytoskeleton reorganization and cell migration [178, 188-190]. Through an SH3 binding domain, PAK is localized in focal adhesions via the adaptor proteins Nck and PIX. Akt directly phosphorylates serine 21 on PAK, releasing it from Nck. PAK then translocates to the cytosol, therefore facilitating PAK activity in directed cell migration [178]. The cross-talk between PI3K and Rho-GTPase family is complex, since PI3K can act both up- and down-stream of the GTPases.

'AKT–INTEGRIN–FN' PARADIGM

Akt1 regulates inside-out activation of integrins, cell adhesion and migration and FN matrix assembly through cross talk with 14-3-3β-Rac1-PAK [152]. Rac1 localization is Akt1 dependent; Akt1 activity correlates with the formation of lamellipodia, but not with filopodia or stress fibers. In Akt-/- fibroblasts and ECs, lamellipodia formation and directional migration on FN is impaired.

In both cell types over-expression of Akt results in Rac1 translocation to membrane ruffles and enhanced Rac1 activity and PAK phosphorylation, which is blunted by treatment with inhibitors of PI3K and Akt, but not GSK-3, or in cells over expressing inactive Akt. These findings emphasize the strong regulatory role of Akt on Rac1-Pak signaling [152].

The14-3-3 proteins are a family of regulator molecules expressed in all eukaryotic cells. These adaptor proteins bind a multitude of functionally diverse signaling proteins; hence 14-3-3 is implicated in a wide range of vital cellular processes. Akt substrates, such as Gsk-3, FOXO, and cRaf, are known to interact with 14-3-3 [191]. Also, 14-3-3 mediates Rac1 localization. This suggests that Akt modulates Rac1 activity through 14-3-3. Indeed, enhanced Rac1 localization and formation of lamellipodia, adhesion to FN, and FN assembly are observed in fibroblasts over-expressing 14-3-3 and inhibited with co-expression of inactive Akt (DN-Akt). Furthermore, lamellipodia formation and cell adhesion and spreading on FN is enhanced in DN-Akt fibroblasts co-expressing active- Rac1 or -PAK1, and inhibited in fibroblasts over-expressing Akt with inactive-Rac1 or -PAK1. This indicates that lamellipodia formation and cell adhesion, as discussed above is through Akt mediated inside-out activation of integrins and is regulated by 14-3-3-Rac-Pak signaling downstream of Akt1 [152].

Notably, the defect in Akt-/- fibroblast to adhere to FN is rescued by co-expression of active-Rac1 or PAK1, but not 14-3-3. Interestingly, constitutively active myristoylated Akt (myr-Akt) fibroblasts co-expressing inactive-Rac1 or –PAK1 show impaired FN assembly. In contrast, impaired FN matrix assembly in DN-Akt and Akt null fibroblasts is rescued with co-expression of active-Rac1 or -Pak1 [152]. Collectively, we identified a novel cross talk between 14-3-3-Rac-Pak and Akt in regulating inside-out integrin activation, cell-FN adhesion, FN matrix assembly.

FN IN DISEASE

Under normal physiological conditions, signaling pathways are highly regulated resulting in dynamic and finely orchestrated intra- and inter-molecular interactions to modulate a wide range of cellular functions [169, 192-196]. However, in tumorigenesis [197, 198] and fibrogenesis [31, 199, 200], many essential pathways are de-regulated and dynamic interaction between pathways is perturbed. FN expression is up-regulated in several cancers, especially in non-small cell lung carcinoma (NSCLC) [201]. FN stimulates NSCLC cell growth and resistance to apoptosis through the activation of α5β1 integrin and inhibition of cyclin-dependent kinase inhibitor p21 gene expression; hence FN is implicated in drug

resistance [202]. Notably, these effects are specific to FN and are not shared by other ECM proteins such as type I collagen.

Mutations in Smad proteins, specifically Smad4, were found in a wide range of human cancers. Recently it has been shown that Smad4 C324Y mutation plays an important role in metastasis of papillary thyroid carcinoma. Such mutation leads to the acquisition of transformed and invasive phenotype through significant activation of TGFβ signaling, lengthening of nuclear localization with R-Smads survival and growth in anchorage independent conditions and decreased adhesion to FN and levels of E-cadherin, which is crucial for the EMT process [203].

FN stimulates fibroblast proliferation and differentiation into myofibroblasts in many fibrotic diseases including pulmonary fibrosis. Excess myofibroblasts in fibroblast foci represent the hallmark lesions of IPF. They are responsible for increased ECM deposition which leads to impaired gas exchange and ultimately fibrosis. Glomerular sclerosis of diverse etiologies is characterized by excess matrix accumulation mediated by up-regulation of TGFβ and FN as an important pathogenic factor [110].

FN-mediated aberrant activation of the classic PI3K/Akt and MAP Kinase cascades has been implicated in signaling toward pro-oncogenic outcomes in many malignancies including prostate cancer [18, 204, 205] as well as in fibrotic disease [18, 206, 207]. Transformed cells are characterized by their ability to survive in the absence of matrix adhesion [208]. For example, PI3K/Akt is a key mediator of survival of both primary and Ras transformed cells in the presence or absence of matrix adhesion [209]. One important implication of this observation is that activated Ras can induce cell survival in the absence of integrin signaling, thus contributing to tumor cell survival.

Several studies have demonstrated that up-regulation of both PI3K/Akt and MAPK is required for metabolic transformation, thus, initiating oncogenic transformation in oncoproteins such as Ras, Src, Akt, Raf, and c-Myc [198, 210]. This creates a dysregulated continuous positive feedback loop leading to sustained growth, proliferation and metastasis. Additionally, loss of tumor suppressor genes encoding p53 and PTEN, and secretion of MMPs are involved in this process [198]. Furthermore, induction of epithelial-mesenchymal transition has been identified as a maker of aggressive phenotypes in certain cancers and fibrotic diseases. EMT has been shown to be PI3K-Rho dependent or MAPK-Smad dependent [99, 211-215].

The observation of increased plasma levels of MMP9 in NSCLC patients suggests that the metastatic and invasive capability of tumor cells correlates with enhanced MMP9 expression [201, 216].

Indeed, a study showed that, in lung cancer cells, FN induces the activation of the FAK-Src complex that subsequently activates PI3K/Akt and Erk1/2 pathways. Further, FN induces cell invasion through enhanced expression of MMP9 that degrades matrix components. FN also induces cell de-adhesion and migration via enhanced expression of calpain proteases, specifically calpain-2, and RhoA, respectively [49].

In fibrosis excessive synthesis and matrix assembly of FN induces cell differentiation towards a contractile phenotype as evident in fibroblast to myofibroblast differentiation [31, 217]. Also, sustained FN accumulation mediates sustained activation of myofibroblasts, resistance to apoptosis, and deposition of excessive collagen through Akt-mTOR-PTEN pathways in various fibrotic diseases including lung [218, 219] and liver fibrosis [220, 221].

Therapeutic targeting of PI3K and Akt has proven more difficult and the progress of a number of compounds has been limited by specificity and / or toxicity. This is disappointing as Akt is often activated in response to FN adhesion in the *in vitro* setting and inhibiting this pathway with the laboratory based agents' wortmannin and/or LY294002 has been shown to have a beneficial effect in combination with radiation in many cell systems [222]. mTOR, however, which is downstream of Akt and controls the translation of proteins for cell cycle progression and cell proliferation, is perhaps a more promising target. mTOR inhibitors are currently under evaluation, particularly in endometrial cancer, glioblastoma and pulmonary fibrosis, all of which commonly possess mutations in PTEN [223]. Identifying the principal elements of the Akt-mTOR-PTEN signaling network and determining the dynamic with FN will improve our understanding of cancer and fibrosis pathogenesis and lead to the rational development of novel therapeutics.

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