

# Akt1 Mediates $\alpha$ -Smooth Muscle Actin Expression and Myofibroblast Differentiation via Myocardin and Serum Response Factor<sup>\*S</sup>

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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  $\alpha$ -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  $\alpha$ -actin ( $\alpha$ SMA) 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  $\alpha$ SMA expression and assembly; an effect that is blunted in cells expressing inactive Akt1 despite TGF $\beta$  stimulation. Mechanistically, Akt1 mediated TGF $\beta$ -induced  $\alpha$ SMA 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 over-expressing active Akt1. Akt1 deficiency was associated with decreased myocardin, SRF, and  $\alpha$ SMA expressions *in vivo*. Furthermore, sustained Akt1-induced  $\alpha$ SMA synthesis markedly decreased upon RNA silencing of SRF and myocardin. In addition to its integral role in  $\alpha$ SMA 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 TGF $\beta$ -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)<sup>2</sup> 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  $\alpha$ -smooth muscle cell like actin ( $\alpha$ SMA) 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  $\beta$ 1 (TGF $\beta$ ) 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 TGF $\beta$ -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  $\alpha$ SMA, a principal component of MFs, is a highly regulated process that is controlled by TGF $\beta$ , splice variant ED-A fibronectin (ED-A FN), and mechanical tension (3, 5). Numerous lines of evidence indicate that the transcription of  $\alpha$ SMA 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 B $\alpha$  (Akt1) in wound healing, ECM remodeling, and vascular maturation (28). Furthermore, we have shown

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<sup>2</sup> The abbreviations used are: MF, myofibroblast; Akt, protein kinase B;  $\alpha$ SMA,  $\alpha$ -smooth muscle like actin; TGF $\beta$ , transforming growth factor  $\beta$ ; 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.

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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  $\alpha$ SMA (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  $\alpha$ SMA synthesis through myocardin and SRF leading to MF differentiation.

In the present study, our results demonstrate that Akt1 is critical for  $\alpha$ SMA synthesis in MF differentiation. Our studies constitute evidence of a novel signaling cascade that links Akt1 to  $\alpha$ SMA synthesis through enhanced expression of myocardin and SRF, a previously uncharacterized link in MFs. We also demonstrate the dual role of Akt1 in mediating  $\alpha$ SMA 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 TGF $\beta$  (a pre-determined dose (31)) 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 TGF $\beta$  (100 pM) for 48 h. This was followed by co-treatment for 24 h (total 72 h) with inhibitors of PI3 kinase (25  $\mu$ M LY294002), Akt (10 nM triciribine), mammalian target of rapamycin (mTOR) (25 nM rapamycin), or SRF/Rho (1  $\mu$ M CCG1423). Cells were subjected to Western analyses as described below.

**Antibodies**—Anti- $\alpha$ SMA (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- $\alpha$ SMA (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  $\mu$ M okadaic acid), protease inhibitor mixture (Roche Diagnostics) and 1 mM PMSF). SDS-PAGE and Western blotting were performed as described previously (31).

**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 TGF $\beta$  (100 pM) treatment for 72 h (supplemental Fig. S1). Cells were subjected to Western analyses and immunocytochemistry to detect  $\alpha$ SMA 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 (Cytoskelton, CO) by mixing rat tail tendon collagen, distilled water, 4 $\times$  DMEM, and cells. The final concentration was 1 $\times$  DMEM, 0.75 mg/ml of collagen, and fibroblasts were present at 3 $\times$  10<sup>5</sup> cells/ml. Following this, 500  $\mu$ l 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, TGF $\beta$ , 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 TGF $\alpha$  for 72 h. Next, cells were fixed with 4% paraformaldehyde in 1 $\times$  PBS followed by permeabilization with 0.1% Triton X-100 in 1 $\times$  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- $\alpha$ SMA antibody (Abcam) (dilution 1:1000) overnight at 4 $^{\circ}$ 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<sup>-/-</sup> mice were generated as previously described (28) and were maintained in the C57BL/6 background. Sex and age-matched wild-type and Akt1<sup>-/-</sup> were randomized to normobaric hypoxia (10% O<sub>2</sub>)

(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.

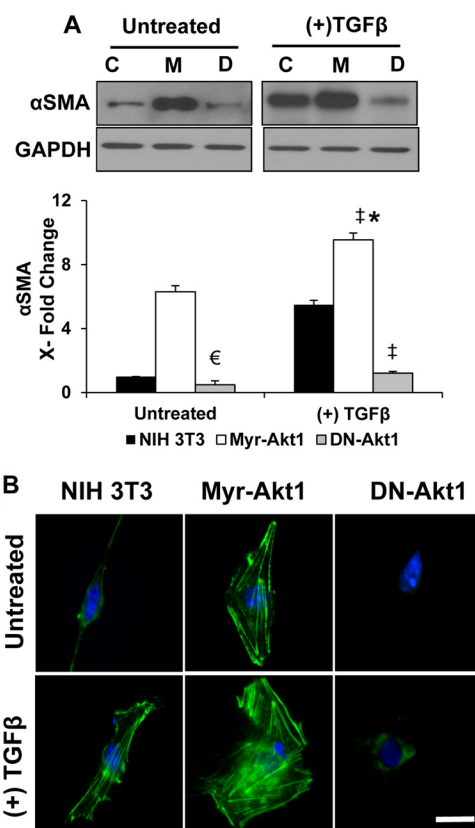
**Statistical Analysis**—All data are presented as mean  $\pm$  S.D. To determine significant differences between treatment and control values, we used the Student's two-tailed *t* test. The significance was set at 0.05 levels (marked with symbols wherever data are statistically significant).

## RESULTS

**Akt1 Inactivation Abolishes TGF $\beta$ -induced Myfibroblast Differentiation**—We first determined that 72 h is the optimal time for TGF $\beta$ -induced MF differentiation in NIH 3T3 fibroblasts, as measured by a 5-fold increase in  $\alpha$ SMA expression). To examine whether Akt1 activation is required for  $\alpha$ SMA 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 TGF $\beta$  (100 pM) for 72 h. The results showed that despite the absence of TGF $\beta$  stimulation, sustained hyperactivation of Akt1 significantly enhanced  $\alpha$ SMA expression ( $\sim$ 6-fold) and assembly compared with control fibroblasts; this effect was further amplified with TGF $\beta$  stimulation (Fig. 1, A and B). In contrast, inactivation of Akt1 blunted the stimulatory effects of TGF $\beta$  on  $\alpha$ SMA expression and assembly (Fig. 1, A and B). Thus, the absence of Akt1 impedes TGF $\beta$ -induced  $\alpha$ SMA 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  $\alpha$ SMA 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, TGF $\beta$  (100 pM), or bFGF (20 ng/ml). In contrast to the stimulatory effect of TGF $\beta$ , bFGF was associated with diminished  $\alpha$ SMA expression in control and myr-Akt1 expressing NIH 3T3 fibroblasts (Fig. 2B). This suggested a molecular see-saw of bFGF and TGF $\beta$ , 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  $\alpha$ SMA expression by bFGF and TGF $\beta$  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 a 72-h TGF $\beta$  stimulation (total 96 h). Although treatment with bFGF partially, but significantly, inhibited TGF $\beta$ -induced  $\alpha$ SMA 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 TGF $\beta$ , it decreased the

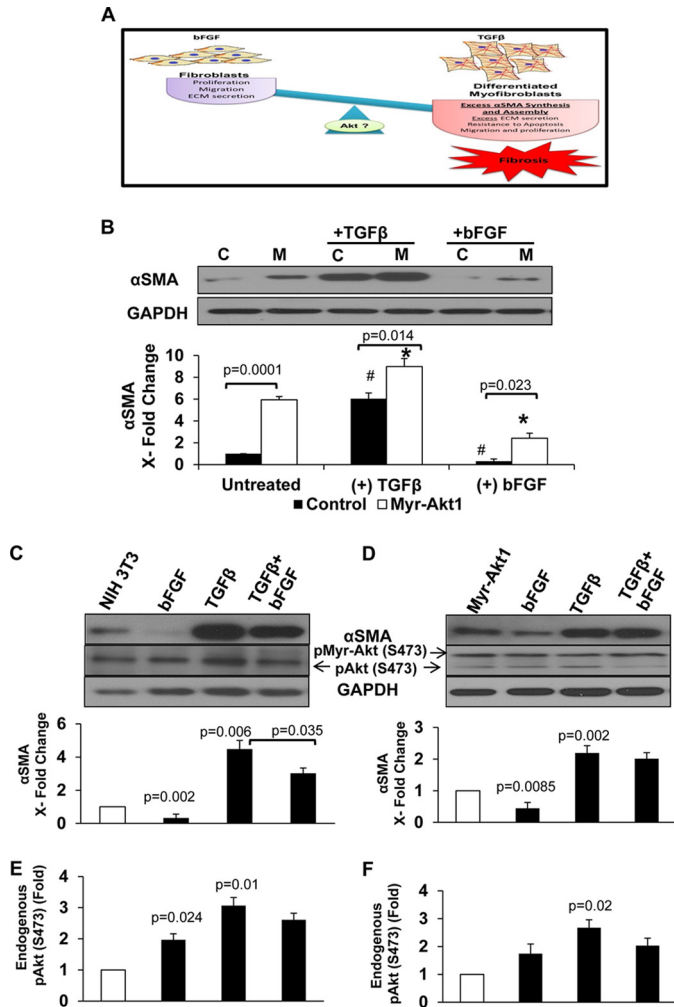


**FIGURE 1. Akt1 is critical for  $\alpha$ SMA expression in response to TGF $\beta$ .** A, control vector (pBabe-Puro), myr-Akt1, and DN-Akt1 expressing NIH 3T3 fibroblasts grown to 80% confluence were subjected to serum-free medium in the presence and absence of 100 pM TGF $\beta$  for 72 h. Lysates were prepared and subjected to Western analyses with antibodies against  $\alpha$ SMA ( $n = 3$ ) (C, control NIH 3T3; M, myr-Akt1; D, DN-Akt1) ( $\blacktriangle$ ,  $p = 0.0001$  and  $\epsilon$ ,  $p = 0.02$  compared with control untreated NIH 3T3;  $*$ , compared with myr-Akt1 control;  $\ddagger$ , compared with TGF $\beta$ -treated NIH 3T3 cells). B, cells, subjected to serum-free medium in the presence and absence of 100 pM TGF $\beta$  for 72 h, were fixed using 4% paraformaldehyde and stained using anti- $\alpha$ SMA antibodies. Fluorescent images of  $\alpha$ SMA assembly were visualized using a fluorescence microscope and photographed. Scale bar, 50  $\mu$ m.

TGF $\beta$ -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  $\alpha$ SMA 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 TGF $\beta$ -induced MF differentiation.

**Akt1 Modulates TGF $\beta$ -induced MF Differentiation through SRF**—Once we identified Akt1 as a key modulator of  $\alpha$ SMA expression, we sought to define and characterize the mechanisms by which Akt1 mediates TGF $\beta$ -induced  $\alpha$ SMA expression and MF differentiation. Because studies have shown that the transcription factor SRF is integral for  $\alpha$ SMA synthesis, we explored whether SRF is also a potential target of the PI3K/Akt/mTOR pathway during MF differentiation. To investigate

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**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 (TGFβ) 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).

this, we utilized inhibitors of PI3K (25 μM LY294002), 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 resis-

tance 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*<sup>-/-</sup>) mice to continuous hypoxia exposure for 14 days and lung tissues were subjected for Western analysis. As expected, *Akt1*<sup>-/-</sup> 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.

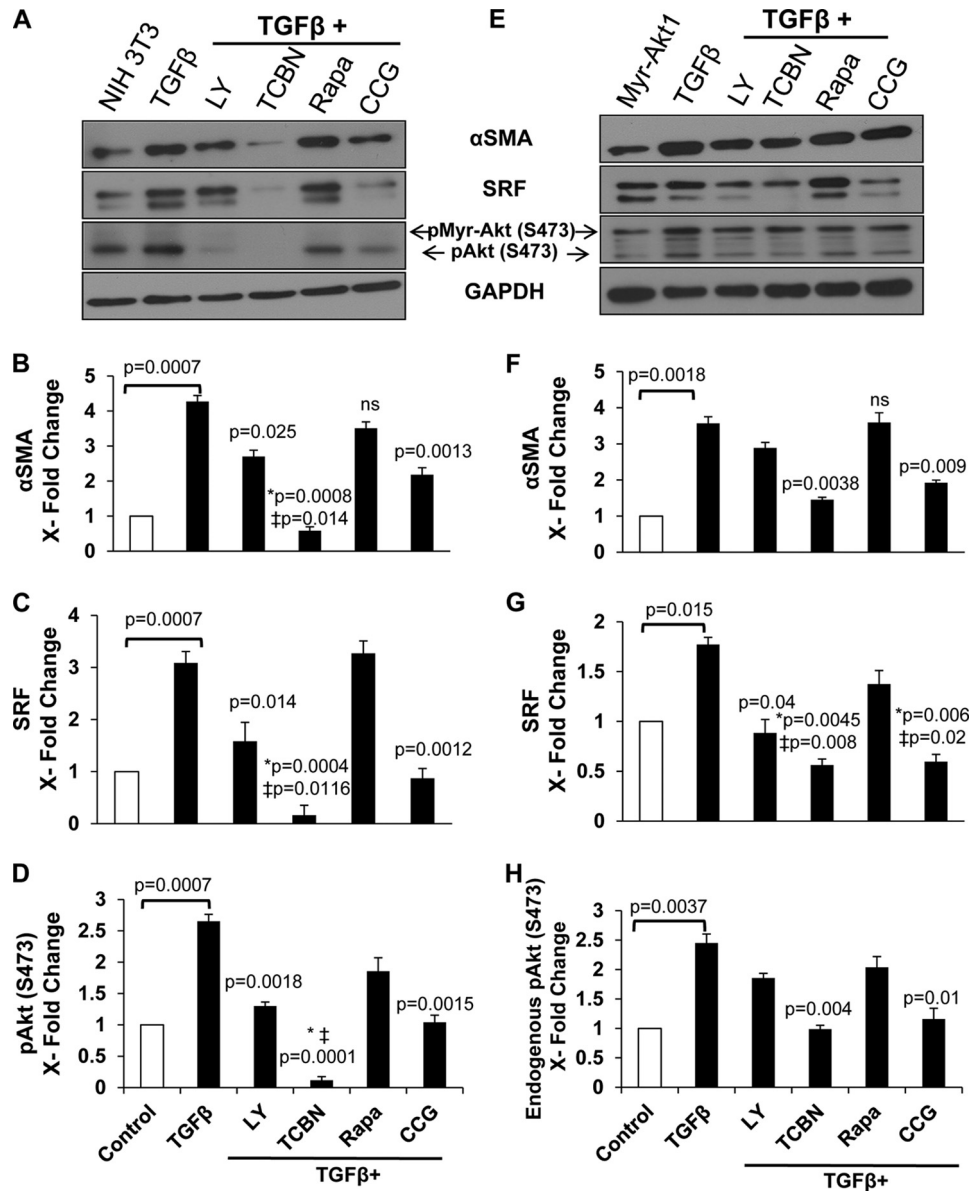
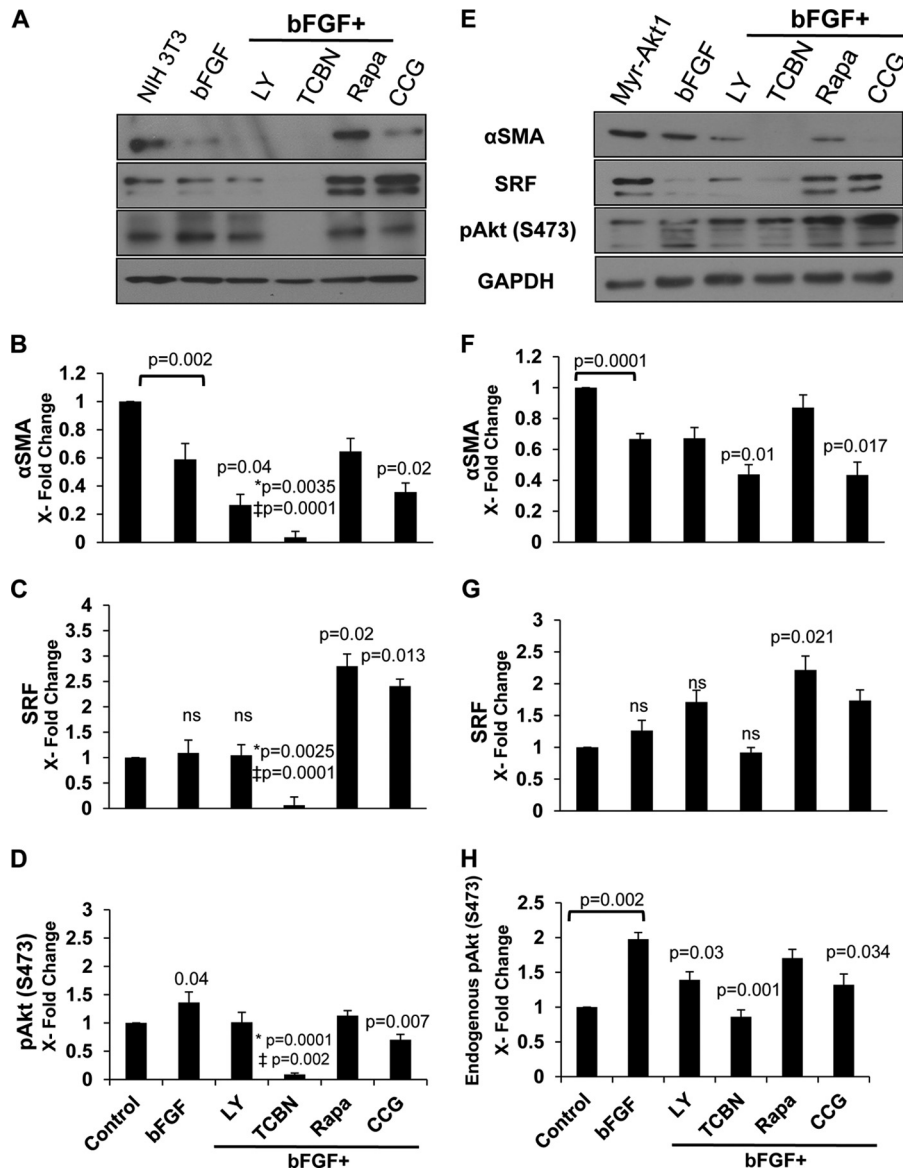


FIGURE 3. **TGF $\beta$ -induced  $\alpha$ SMA expression is Akt1-dependent through SRF, independent of mTOR.** NIH 3T3 fibroblasts (A–D) and myr-Akt1 expressing fibroblasts (E–H) grown to 70% confluence were treated with TGF $\beta$  for 72 h in the absence of FBS. To examine pathways governing MF differentiation, inhibitors were applied before significant differentiation occurred: cells were treated with TGF $\beta$  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  $\alpha$ SMA (B and F), SRF (C and G), and pAkt (Ser-473) (D and H) ( $n = 4$ ). \*, compared with TGF $\beta$ ; †, compared with control untreated cells.

*Akt1 Plays a Dual Role as a Modulator of TGF $\beta$ -induced  $\alpha$ SMA 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, 34, 40, 41) and having seen that Akt1 is crucial for  $\alpha$ SMA 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 (31). Fibronectin splice variant ED-A FN, which is not typically expressed in undifferentiated fibroblasts, is mandatory for TGF $\beta$ -induced  $\alpha$ SMA synthesis and MF differentiation (3). Furthermore, total fibronectin, which is secreted excessively by differentiated MFs, also exerts mechan-

ical 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 TGF $\beta$  for 48 h and co-treated with inhibitors of Akt1 and mTOR for an additional 24 h. Expression of  $\alpha$ SMA, ED-A FN, total fibronectin and phosphorylated Akt was determined. Inhibiting Akt significantly reduced the stimulatory effect of TGF $\beta$  on  $\alpha$ SMA, ED-A FN, and fibronectin expressions. However, treatment with the mTOR inhibitor rapamycin only inhibited total fibronectin expression with no discernible decrease in  $\alpha$ SMA 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

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**FIGURE 4. bFGF-induced  $\alpha$ SMA 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  $\alpha$ SMA (B and F), SRF (C and G), and pAkt (Ser-473) (D and H) ( $n = 4$ ). \*, compared with bFGF; †, compared with control untreated cells.

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 (31), 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 TGF $\beta$ -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 TGF $\beta$ -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 TGF $\beta$ -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 TGF $\beta$ -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, TGF $\beta$  effects on MF differentiation.

## DISCUSSION

In the present study, we provide the first conclusive evidence on the significance of sustained Akt1 activation in persistent

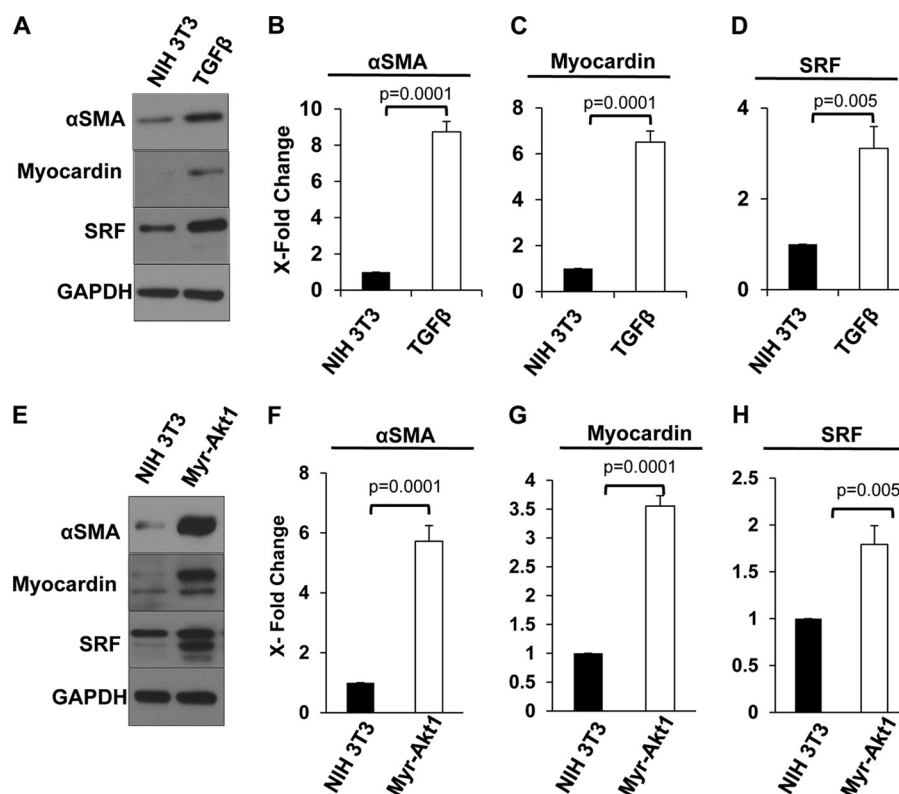


FIGURE 5. Akt1 induces MF differentiation through enhanced expression of myocardin and SRF. NIH 3T3 fibroblasts treated with TGF $\beta$  for 72 h (A–D) and myr-Akt1 fibroblasts (E–H) were subjected to Western analysis for SMA, myocardin, and SRF expression ( $n = 4$ ).

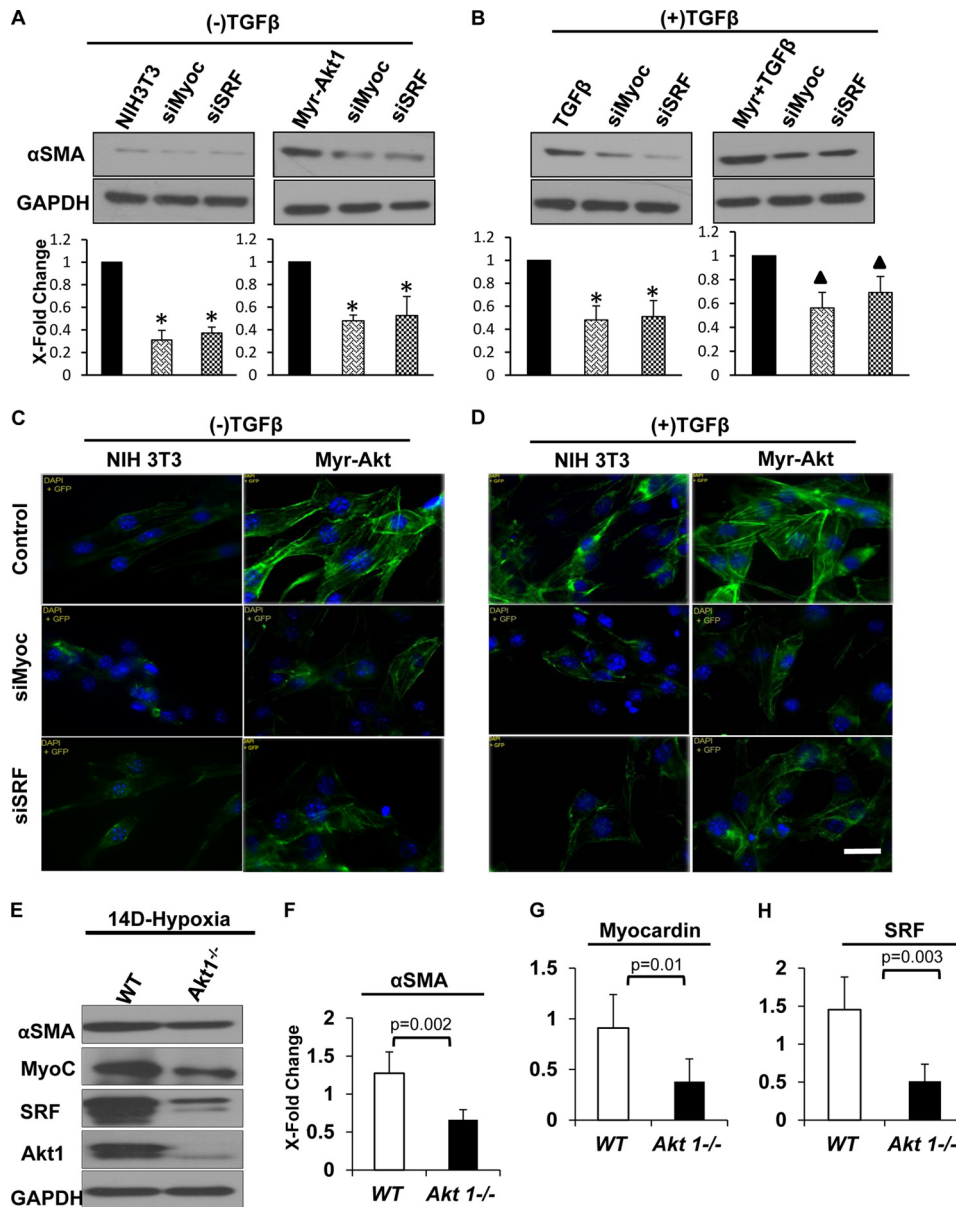
MF differentiation with potential pathophysiological implications in tissue fibrosis. First, whereas sustained activation of Akt1 mimicked TGF $\beta$  stimulation and induced an  $\sim 6$ -fold increase in  $\alpha$ SMA, inactivation of Akt1 blunted TGF $\beta$ -induced  $\alpha$ SMA expression and assembly. Second, Akt1 mediates  $\alpha$ SMA 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, TGF $\beta$ -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 TGF $\beta$ -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  $\alpha$ SMA synthesis in smooth muscle cells and MFs. Literature also suggests a correlation between Akt activation and  $\alpha$ SMA 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  $\alpha$ SMA 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 TGF $\beta$ -induced  $\alpha$ SMA expression. Noteworthy, by conducting our studies in NIH 3T3 fibroblasts treated with TGF $\beta$ , 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  $\alpha$ SMA synthesis (4, 16–24, 26, 44, 45). Notably, SRF, a ubiquitous transcription factor, is not smooth muscle-specific. Furthermore, Olson and colleagues (25) discovered that myocardin, the transcription co-activator of SRF, is a critical master activator of  $\alpha$ SMA 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  $\alpha$ SMA in fibroblasts (46). Our studies showed that the expression of both SRF and myocardin were up-regulated during TGF $\beta$ -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  $\alpha$ SMA synthesis and MF differentiation, we utilized an siRNA-mediated gene knockdown approach to target myocardin and SRF. Our studies revealed that in unstimulated myr-Akt1 fibroblasts, myocardin and SRF knockdown

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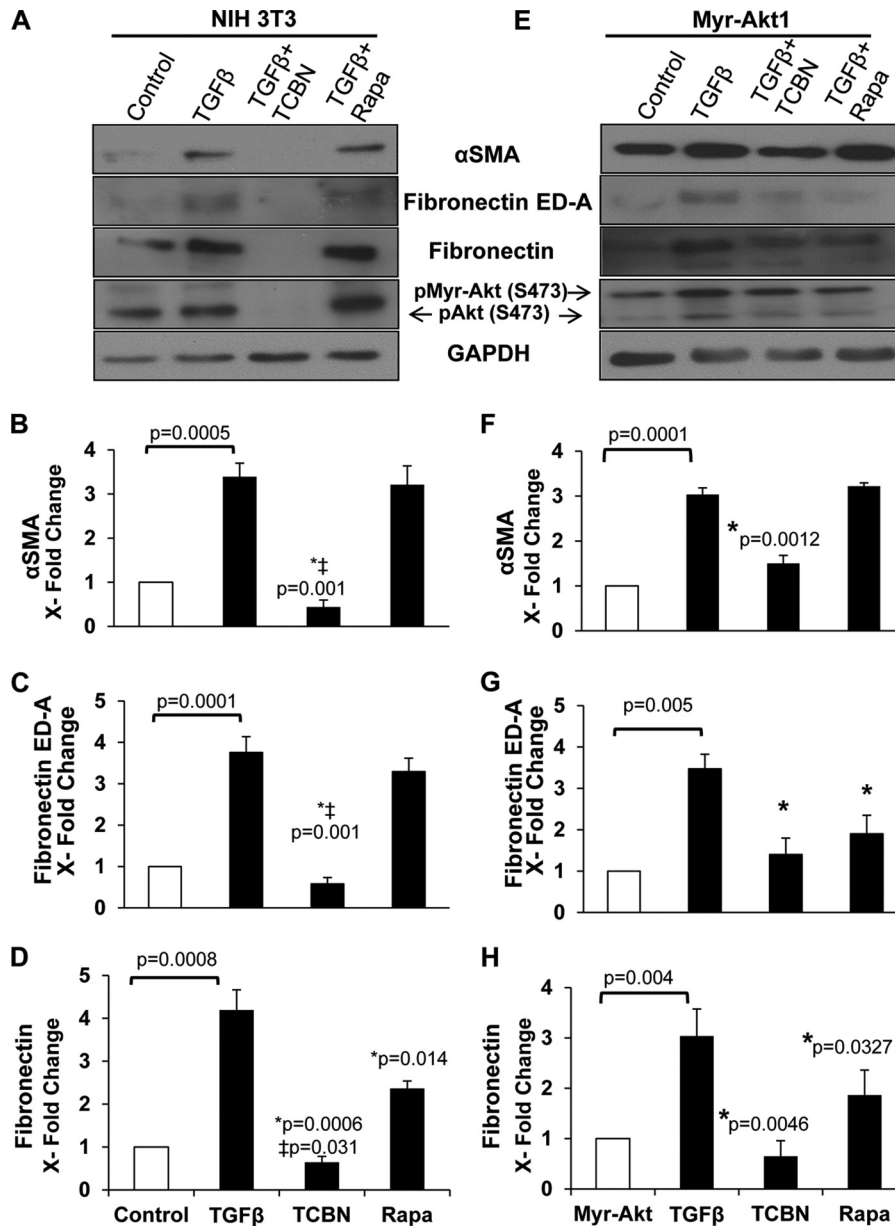
**FIGURE 6. siRNA-mediated knockdown of myocardin and SRF ablates Akt1-induced SMA expression and MF differentiation.** Control NIH 3T3 fibroblasts (A and C) and myr-Akt1 fibroblasts (B and D) were transfected with control, myocardin, or SRF siRNA (*siMyoc*, 100 nM and *siSRF*, 150 nM), and 24 to 48 h later, cells were treated with 100 pM TGFβ for 72 h. Lysates were prepared and subjected to Western analyses, quantification of αSMA expression 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<sup>-/-</sup>*) 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 ( $n = 4-5$ ).

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





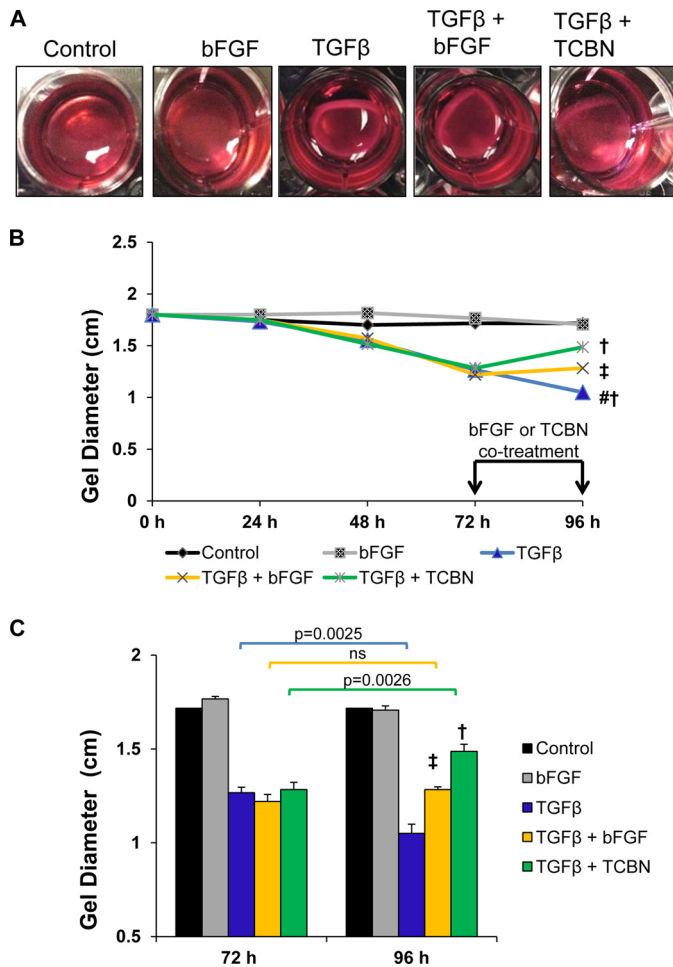
**FIGURE 7. Akt1-mediated expression  $\alpha$ SMA and ED-A FN in response to TGF $\beta$  is independent of mTOR activity.** Control NIH 3T3 (A–D) and myr-Akt1 expressing fibroblasts (E–H) grown to 70% confluence were subjected to serum-free medium and treated with TGF $\beta$  for 72 or 48 h and then co-treated with inhibitors for an additional 24 h targeting the Akt/mTOR pathway (trichiribine (TCBN) and rapamycin, respectively). Lysates were prepared and subjected to Western analyses with antibodies against  $\alpha$ SMA, ED-A FN, total fibronectin, and pAkt(Ser-473) ( $n = 3$ ). \*, compared with TGF $\beta$ ; †, compared with control untreated cells.

increase in  $\alpha$ SMA 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 TGF $\beta$ , 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, 31). 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 TGF $\beta$ -induced  $\alpha$ SMA 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  $\alpha$ SMA 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 TGF $\beta$  stimulation, rapamycin treatment did infer some effect on ED-A FN in addition to total FN in myr-

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**FIGURE 8. Akt inhibition reverses TGF $\beta$ -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 TGF $\beta$  for 96 h or TGF $\beta$  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 TGF $\beta$ ; ‡,  $p = 0.001$  compared with TGF $\beta$ .

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 (50, 51). It has been suggested that elevated basal levels of Akt can directly mediate alternative splicing of ED-A FN (50, 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  $\alpha$ SMA 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 TGF $\beta$  up-regulation, respectively. Collectively, these findings highlight the importance of Akt1 as a novel therapeutic target for fibrotic diseases.

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