

# Activated mTORC1-SIRT6 Mediates Myofibroblast Differentiation and Idiopathic Pulmonary Fibrosis

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## Research Article

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# Abstract

## Background

Idiopathic pulmonary fibrosis (IPF) is characterised by accumulation of myofibroblasts and deposition of extracellular matrix proteins. Fibroblast-to-myofibroblast transdifferentiation and myofibroblast hyperproliferation plays a major role in pulmonary fibrosis. Moreover, mTOR signaling pathway and SIRT6 play a critical role in pulmonary fibrosis. However, the mechanisms whether SIRT6 affect the myofibroblasts differentiation during IPF remain unclear.

## Method

We investigated myofibroblast differentiation using a bleomycin-induced mouse pulmonary fibrosis model and TGF- $\beta$ 1 induced human fetal lung fibroblasts (MRC5) in vitro. We used both SIRT6 siRNA and rapamycin to study the role of SIRT6 and mTOR signaling pathway in the normal human lung fibroblasts and the myofibroblasts from human IPF lungs.

## Results

Our data show that high level of SIRT6 was detected in IPF samples, and SIRT6 was significantly upregulated by TGF- $\beta$ 1 in a time and concentration-dependent manner. SIRT6 expression and activation of mTORC1 signalling pathway were upregulated in fibrotic lung tissues and primary lung fibroblasts isolated from patients with IPF and bleomycin-challenged mice. Furthermore, rapamycin treatment inhibited mTORC1 pathway activity and SIRT6 protein expression. SIRT6 SiRNA failed to mediate the activity of mTORC1 pathway and autophagy induction. However, SIRT6 knockdown could promote TGF- $\beta$ 1 induced pro-fibrotic cytokines.

## Conclusion

Activated mTORC1 signalling pathway regulated SIRT6 overexpression. Deficiency of SIRT6 mediated myofibroblasts differentiation through induced pro-fibrotic cytokines production in the present of TGF- $\beta$ 1. The study indicated that manipulations of SIRT6 expression may provide a new therapeutic strategy to prevent and reverse the progression of pulmonary fibrosis.

## Background

Idiopathic pulmonary fibrosis (IPF) is a chronic, fibrosing interstitial lung pneumonia, characterized by abnormal interstitial collagen fibers deposition by lung fibroblasts, leading to respiratory failure and affecting adults over 50 years old [1-3]. The signs and symptoms are of insidious onset and are characterized by progressive effort dyspnea and persistent dry cough [4, 5]. Increasing evidence has implicated that myofibroblasts are primary effector cells for matrix deposition and tissue remodeling in IPF patients [6, 7]. Lung fibroblasts differentiate into contractile myofibroblasts that secrete excessive extracellular matrix (ECM) proteins such as collagen [8, 9]. Although Nintedanib and Pirfenidone

significantly reduced Forced vital capacity (FVC) decline and prolonged time to first acute exacerbation, failed to improve respiratory failure [10-12]. However, the pathogenesis of IPF still have not been fully explained.

The PI3K/Akt/mTOR signaling pathway is a central signaling regulator of cell metabolism, proliferation, differentiation, and survival [13, 14]. Mammalian target of rapamycin (mTOR) is a serine/threonine kinase in the PI3K family that is an important regulator of protein and lipid biosynthesis, cell cycle progression, proliferation, survival, and senescence [15, 16]. While mTORC1 controls cell growth and metabolism and is highly sensitive to rapamycin, mTORC2 regulates cell proliferation and survival and is relatively insensitive to rapamycin [14, 17]. PI3K/Akt/mTOR inhibition attenuates transforming growth factor (TGF)- $\beta$ 1-induced collagen production in human lung fibroblasts (LFs), and collagen formation markers in IPF lung tissue [18]. Previous study showed that the aberrant PTEN/AKT/mTOR axis desensitizes IPF fibroblasts from collagen matrix-driven stress by suppressing autophagy, which produces a viable IPF fibroblast phenotype on collagen [19]. However, the mechanism of mTOR signaling regulated myofibroblasts differentiation is still unclear.

Sirtuins are a family of nicotinamide adenine dinucleotide-dependent deacetylases that are involved in regulating stress resistance, metabolism, and organismal life span [20]. Seven sirtuins are found in mammals (SIRT1 to 7) and SIRT6 plays essential roles in DNA repair, gene expression, telomere maintenance, metabolism and aging [21]. Recent studies showed that SIRT6 directly recognizes DNA damage through a tunnel-like structure that has high affinity for DNA double-strand breaks (DSB) [22]. SIRT6-deficient mice are small and develop abnormalities, including profound lymphopenia, loss of subcutaneous fat, and severe metabolic defects [23]. Currently, SIRT6 inhibits epithelial to mesenchymal transition during idiopathic pulmonary fibrosis [24]. SIRT6 prevents TGF- $\beta$ 1-induced lung myofibroblast differentiation through inhibiting TGF- $\beta$ 1/Smad2 and NF- $\kappa$ B signaling pathways. However, the role of SIRT6 in pulmonary fibrosis is controversy. SIRT6 and mTOR pathway were involved in pulmonary fibrosis, very little is known about SIRT6 regulate myofibroblast proliferation via mTOR signaling pathway in the pathogenesis of pulmonary fibrosis. It was reported that SIRT6 overexpression induced autophagy via attenuation of IGF-Akt-mTOR signaling [25]. The aim of this study was to elucidate the role of SIRT6 in the differentiation of fibroblast transmission to myofibroblast using primary human idiopathic pulmonary fibrosis myofibroblasts.

In this work, our findings indicated that mTORC1 signaling and SIRT6 activated in both TGF- $\beta$ 1-induced fibroblast and bleomycin-induced experimental model. Aberrant mTORC1 modulates myofibroblast differentiation and induces SIRT6 overexpression. Furthermore, the inhibition of SIRT6 promotes fibrotic transcription factors.

## Materials And Methods

### Ethics statement

This study involves the analysis of human IPF patient specimens. Primary fibroblast lines were obtained from unused, existing pathological human tissue samples, and therefore is exempt (exemption 4). Tissue samples were stripped of all identifiers and designated as waste. All patients underwent procedures for diagnostic or therapeutic procedures. Written informed consent was obtained on all patients prior to the procedure being performed. Use of human tissues was approved by the Xiamen University and Wuxi Lung Transplant Center.

## **Human subjects**

Cell lines were derived from lungs removed at the time of transplantation. The diagnosis of IPF was supported by history, physical examination, pulmonary function tests, and typical high resolution chest computed tomography findings of IPF. In all cases, the diagnosis of IPF was confirmed by microscopic analysis of lung tissue and demonstrated the characteristic morphological findings of usual interstitial pneumonia. All patients fulfilled the criteria for the diagnosis of IPF as established by the American Thoracic Society (ATS) and the European Respiratory Society (ERS). For this study, new primary IPF fibroblast lines were generated as tissue became available. To address concerns of biological variability, we studied 2 control cell lines and 2 IPF cells lines. Primary control and IPF lung fibroblast lines were generated by explant culture and cultured in high glucose DMEM containing 10% FCS. Fibroblasts were used between passages three and five (Supplementary Fig1). Cells were characterized as fibroblasts as previously described [26]. The levels of SIRT6 in IPF were derived from Meltzer in R2 Genomics Analysis and Visualization Platform

## **Mouse Lung Fibrosis Model**

The animal procedures were approved by the Institutional Animal Care and Use Committee at Xiamen University. SPF C57BL/6 male mice (6-8 weeks old) were adopted and divided into two groups: normal saline group and bleomycin injection group (2.5 U/kg). Under anesthesia, the neck skin was incised, and the trachea was exposed. Saline or 2.5 U/kg bleomycin (Sigma, USA) was injected into the mouse trachea from the interval of the trachea cartilaginous rings with a 25-guageneedle. The tracheal puncture points were sutured by silk thread. Mouse lungs were collected at 21 days for experiments.

## **Cell Culture and Transfection**

Human fetal lung fibroblasts (MRC5) was gained from the American Type Culture Collection (ATCC, Manassas, VA), cultured in minimal essential medium (MEM) (Gibco, USA) with 10% (v/v) FBS and 1% penicillin/streptomycin. For cell transfection, SIRT6 siRNA was synthesized by GenePharma (Shanghai, China). Before the transfection, cells were cultured to 60%–80% confluence and transfected using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions.

## **Western Blotting**

The cell lysates were centrifuged at 12000 rpm at 4°C. Cells were placed for 30 min on ice in RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) in the presence of a cocktail proteinase inhibitor (Sigma-Sigma-

Aldrich, St. Louis, MO, USA). Sample proteins were quantitated using pierce BCA protein assay (Thermo Fisher Scientific, Rockford, USA). The protein lysates were separated using SDS-PAGE and transferred to a PVDF (Millipore, Billerica, MA, USA) membrane. The membrane was blotted with specific primary and horseradish peroxidase (HRP) secondary antibodies. The immunoreactive bands were visualized using SuperSignal West Pico Chemiluminescent Substrates (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Antibody binding was detected by enhanced chemiluminescence detection kit (ECL) (Amersham International Plc., Buckinghamshire, UK). The following antibodies were used: anti-Akt (#2920) and phospho-Akt at Ser473 (#9271), 4EBP-1(#9459) and phospho-4EBP-1(#2855),  $\beta$ -actin (#3700), anti-S6 (#2317) and phospho-S6 at Ser240/244 (#5364) (diluted with 5% BSA to 1: 1000) were obtained from Cell Signaling Technology (Beverly, MA, USA). p62/SQSTM1 (#H00008878-M01) from Abnova (Taipei City, Taiwan), and LC3 (L8918) and  $\beta$ -actin (#A5316) from Sigma-Aldrich (St. Louis, MO, USA).

### **Immunohistochemistry**

Immunostaining was done on formalin-fixed, paraffin-embedded mice lung tissue specimens, and 4 mm thick paraffin sections were cut. These sections were incubated overnight with anti-SIRT6 and anti-p-Akt, anti-p-S6 antibody (Abcam) at a 1: 200 dilution in 5% FBS overnight and then incubated with the horseradish peroxidase-conjugated secondary antibody for 30 min at room temperature. The results were visualized by reaction with diaminobenzidine (DAB; 3,3'-diaminobenzidine tetrahydrochloride) and counterstaining with hematoxylin.

### **Cell Proliferation Assay**

MRC5 was plated at a density of  $1.0 \times 10^3$  cells/well in 96-well plates. The cells were cultured in the corresponding serum-free medium for 24 h. Cell viability was examined using 10  $\mu$ L/well CCK-8 solution (Dojindo, Kumamoto, Japan). After incubating for 2 h, the absorbance was measured at 450 nm.

### **Quantitative Real-Time PCR (qRT-PCR)**

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Then, reverse transcription was performed to get the first strand cDNA by using the PrimeScript<sup>®</sup> RT reagent kit (TaKaRa, Dalian, China). The expression level of  $\alpha$ -SMA, CTGF were determined by qPCR reactions and were performed by using the ABI 7500 Fast system (Applied Biosystems, CA) with SYBR green (TaKaRa). The  $2^{-\Delta\Delta C_t}$  method was used for quantification. All reactions were triplicated. The relative expression of Collagen1, 3, Fibronectin, MMP3 were respectively normalized to GAPDH.

### **Statistical Analysis**

Results are presented as means  $\pm$  SEM. Significance of the differences between means was assessed using one-way analysis of variance or a two-tailed Student's t-test. Values of P less than 0.05 were considered significant.

# Results

## 1. The expression of SIRT6 is increased in IPF lung myofibroblasts

To explore the role of SIRT6 in the pathogenesis of pulmonary fibrosis, we first assessed SIRT6 protein expression in IPF patient using immunoblotting staining. Lung tissues from IPF patient and normal controls were stained for SIRT6. Immunohistochemical analysis showed that SIRT6 localized to areas of active fibrosis, the fibroblastic foci, and subepithelial and subendothelial regions within IPF lung (Fig. 1a). We also examined SIRT6 expression in normal lung fibroblasts and those derived from patients with IPF. The expression of SIRT6 was also confirmed in primary pulmonary fibroblasts by Western blotting. Compared with control, the presence of higher levels of SIRT6 and  $\alpha$ -SMA were observed in IPF patient with the dose-dependent of TGF- $\beta$ 1 (Fig. 1b). Moreover, we found that the expression of SIRT6 in the advanced IPF was more higher than in health control ( $P=0.032$ ) by R2 platform ((Fig. 1c).

To understand the role of SIRT6 in bleomycin-induced pulmonary fibrosis, we established pulmonary fibrosis mice treated with 5U/kg BLM-induced. Lung tissues were collected at 21 days, and lung sections were stained with H&E staining and Masson's Trichrome. It was demonstrated that the alveolar structure in bleomycin-treated mice was destroyed with hyperplasia of collagen deposition from on days 21 (Fig. 2a). Immunohistochemical analysis demonstrated diffuse staining for SIRT6 protein expression within the lungs of bleomycin-induced mice (Fig. 2b). Furthermore, human fetal lung fibroblasts (MRC5) were cultured with 10ng/mL TGF- $\beta$ 1 for 72 hours to induce myofibroblast differentiation. It was showed that the expression of SIRT6 and  $\alpha$ -SMA were also upregulated by TGF- $\beta$ 1 induced in dose-dependent manner (Fig. 2c).

## 2. Activity of mTOR signaling pathway is up-regulated in myofibroblast differentiation

To investigate the activity of mTOR signaling pathway in lung myofibroblast differentiation, we measured mTOR kinase activity with Western blotting. In MRC5 line, the phosphorylated mTORC1 protein level was significantly high with increasing dose of TGF- $\beta$ 1 using phosphorylated S6 level, a direct substrate of mTORC1. Additionally, the phosphorylated-Akt ser473, Thr308 level were decreased in MRC5 due to feedback loop of up-regulated level of phosphorylated S6 (Fig. 3a). We next examined mTOR kinase activity in IPF fibroblasts. There were also a significant increasing in the activity of phosphorylated S6 kinase, when TGF- $\beta$ 1 induced pulmonary myofibroblasts differentiation in a dose dependent pattern. And TGF- $\beta$ 1 treatment decreased the level of phosphorylated-Akt ser473, Thr308 in a dose-dependent manner (Fig. 3b).

## 3. Rapamycin attenuates SIRT6 expression by inhibiting the activation of mTOR signaling pathway

To determine the regulation relationship between SIRT6 and mTOR signaling pathway in lung myofibroblast differentiation, we assessed the SIRT6 expression with rapamycin treatment. Using TGF- $\beta$ 1 (20ng/mL) induced MRC5 line differentiation, the expression of SIRT6 was inhibited by rapamycin treatment in the dose dependent manner (Fig. 4a). Furthermore, it was indicated that rapamycin markedly

suppressed SIRT6 protein with the dose dependent manner in IPF myofibroblasts (Fig. 4b). We next also investigated the activity of mTOR pathway with rapamycin treatment in pulmonary myofibroblasts differentiation. The results showed that the phosphorylated S6 protein was markedly suppressed with increasing the dose of rapamycin in MRC5. There was a different increasing in the expression of phosphorylated-Akt ser473 and Thr308 with different concentration of rapamycin treatment (Fig. 4c). After exposure to TGF- $\beta$ 1 (20ng/mL) in present of rapamycin for 48h, the level of phosphorylated S6 protein and Akt were measured in primary IPF patient. We found rapamycin significantly suppressed the expression of phosphorylation of S6, and phosphorylated Akt (Ser473 and Thr 308) were up-regulated with various concentrations of rapamycin, shown by Western blotting (Fig 4d).

#### **4. mTORC1 regulates myofibroblast differentiation through the activation of SIRT6**

To further evaluate whether mTORC1 modulated the expression of SIRT6, fibroblasts were treated with SIRT6 siRNA. Treatment with SIRT6 siRNA dramatically down-regulated the SIRT6 protein in MRC5 line (Fig. 5a). In human lung fibroblasts treated with SIRT6 siRNA, the SIRT6 expression was also decreased compared with control by Western blotting (Fig. 5b). Further, the level of SIRT6 mRNA was markedly decreased in MRC5 treated with SIRT6 siRNA (Fig. 5c).

We also evaluated the activity of mTOR signaling and SIRT6 by TGF- $\beta$ 1 induction in MRC5. The result indicated that SIRT6 siRNA failed to regulate the level of phosphorylated S6 protein. The phosphorylated-Akt (Ser473, Thr308) by TGF- $\beta$ 1 induction was increased in MRC5 cells when treated with SIRT6 siRNA (Fig. 5d). Moreover, the knockdown of the expression of SIRT6 by siRNA did not significantly affect the activity of mTORC1 signaling whether induced by TGF- $\beta$ 1 or not, as indicated by the levels of phosphorylated S6 protein in IPF patient. The level of phosphorylated serine 473 and thr 308 of Akt were increased in human lung fibroblasts treated by SIRT6 siRNA (Fig. 5e). It was indicated that activated mTORC1 pathway mediated myofibroblast differentiation through the activation of SIRT6. Meanwhile, SIRT6 also regulated mTORC2 signaling.

#### **5. TGF- $\beta$ inhibits autophagy during myofibroblast differentiation through activated mTORC1 signaling not SIRT6**

Considering that insufficient autophagy has a role in pulmonary fibrosis and mTOR signaling moderate the autophagy, whether SIRT6 regulates myofibroblast differentiation by inducing autophagy. Western blotting confirmed that TGF- $\beta$ 1 reduced autophagy in IPF fibroblasts, using the ratio of LC3 II/I, a widely recognized autophagy biomarker (Fig. 6a). We further measured the effect of TGF- $\beta$  upon autophagy in human lung fibroblasts and MRC5 by treating with TGF- $\beta$ 1 (10, 20 ng/mL) for 72h. The results indicated that LC3II levels was decreased upon addition of TGF- $\beta$ 1 in a dose-dependent manner after 48h whether in human lung fibroblasts (Fig. 6b). And TGF- $\beta$ 1-mediated decline in LC3II was also observed in MRC5 (Fig. 6c).

To demonstrate the mTORC1 signaling was involved in the regulation of autophagy, human lung fibroblasts was treated by rapamycin. It was showed that rapamycin significantly increased the protein of

LC3II with dose-dependent manner in IPF (Fig. 6d). Moreover, in order to determine whether SIRT6 also regulated the autophagy via mTORC1 pathway. We subsequently silenced SIRT6 with siRNA transfection in IPF fibroblasts and MRC5. The data indicated that the expression of LC3II was inhibited by TGF- $\beta$ 1 in human lung fibroblasts, but no significant difference was noticed by SIRT6 siRNA treatment whether TGF- $\beta$ 1-inducing or not after 48h (Fig. 6e). In MRC5, blocking SIRT6 with siRNA could not decreased the level of LC3II in the present of TGF- $\beta$ 1 or not after 48h (Fig. 6f). Taken together, TGF- $\beta$ 1 repressed autophagy through activated mTORC1 signaling or not SIRT6.

## 6. SIRT6 Knockdown regulates extracellular matrix production

In order to confirm the fibrotic role of SIRT6 in TGF- $\beta$ 1-induced myofibroblast differentiation and extracellular matrix (ECM) accumulation, we next examined whether SIRT6 modulated the expression of fibrotic gene. MRC5 was incubated with SIRT6 siRNA for 12h before TGF- $\beta$ 1 treatment. As shown in Fig 7a-d, we observed that SIRT6 siRNA transfection significantly increased TGF- $\beta$ 1-induced Collagen1, 3, MMP3, and FN mRNA expression levels in MRC5 cells.

## Discussion

In the study, we have established myofibroblast differentiation model in vitro in order to investigate the role of SIRT6 in pulmonary fibrogenesis. The mTORC1 pathway and protein level of SIRT6 was significantly upregulated in the MRC5 and primary IPF fibroblasts treated with TGF- $\beta$ 1. The results indicated that mTORC1 signaling regulated lung fibroblast-to-myofibroblast differentiation through SIRT6 under the treatment of TGF- $\beta$ 1.

Previous studies have demonstrated that SIRT6 has increased by TGF- $\beta$ 1 in human fetal lung fibroblasts (HFL1) and overexpression of SIRT6 significantly suppresses TGF- $\beta$ 1-induced myofibroblast differentiation in HFL1 cells [27]. Tian et al also found that SIRT6 was upregulated in TGF- $\beta$ 1-treated A549 cells and bleomycin-injured mice, in which have reversed TGF- $\beta$ 1-induced epithelial to mesenchymal transition (EMT) in A549 cells [24]. Studies have explored that bile duct ligation (BDL)-induced liver injury was aggravated by SIRT6 deficiency. SIRT6 activation alleviated cholestatic liver damage and fibrosis through regulating Orphan nuclear receptor estrogen-related receptor  $\gamma$  (ERR $\gamma$ ) [28]. Other findings suggested that SIRT6-deficient fibroblasts transform spontaneously to myofibroblasts through hyperactivation of transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) signaling in a cell-autonomous manner [29]. SIRT6 deficiency resulted in progressive renal inflammation and fibrosis [30]. It was also reported that SIRT6 deficiency increased expression of fibrosis-associated markers in myofibroblasts. Moreover, SIRT6 deficiency induced aging-dependent multiorgan fibrosis in SIRT6<sup>-/-</sup> mice. Cardiac microvascular endothelial cells (CMECs)-specific SIRT6 knockout appeared to worsen perivascular fibrosis and diabetic cardiomyopathy (DCM) in Type 2 diabetes mellitus (T2DM) [31]. Our results found that SIRT6 expression was up-regulated in IPF myofibroblasts and MRC5 in the time and dose-dependent of TGF- $\beta$ 1 in vitro (Supplementary Fig2-3). In addition, we used the lung sections from IPF patient and

bleomycin induced pulmonary fibrosis mice to measure the SIRT6 protein. There was a significant increasing in expression of SIRT6 in lung tissues of IPF and pulmonary fibrosis mice.

Although emerging evidence have indicated the importance of mTOR signaling pathway on the regulation of pulmonary fibrosis, current understanding of mechanism of mTOR pathway in fibrosis remain to be elucidated. Previous study demonstrated increased mTOR expression was found in lung tissue from IPF patients [32]. It has been reported that a novel dual mTOR/PI3K inhibitor (GSK2126458) inhibited PI3K signaling and functional response in IPF fibroblasts derived from patients with fibrotic foci in phase II clinical trial [18]. Furthermore, another study have showed that a dual mTORC1 and mTORC2 inhibitor (MLN0128) exhibited potent anti-fibrotic activity in both in vitro and in vivo models [33]. Although mounting evidence have indicated that mTOR inhibition is effective to suppress the fibrotic process, serious adverse event could be occurred in clinical trials [15, 34-37]. Other studies showed that the effect of rapamycin has been limited due to the inhibition of mTORC1 may remove the negative feedback loop on mTORC2/Akt [38]. mTOR is present in cells as a member of 2 complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which have different subunits and substrate specificity. The PI3K-mTORC1 complex is regulated by negative feedback loops, including the reduction of Akt activity and mTORC1 signaling in response to S6K phosphorylation of insulin receptor substrate 1 (IRS-1), which leads to IRS-1 degradation [39]. The present study showed that TGF- $\beta$ 1-induced phosphorylation of the mTORC1 substrates pS6 in MRC5 and IPF fibroblasts with a concentration-dependent manner, but the phosphorylation of mTORC2 substrates Akt (Ser473) and Akt (Thr308) were attenuated due to negative feedback loop of mTORC1. Treatment with rapamycin markedly attenuated mTORC1-dependent phosphorylation pS6 in MRC5 and IPF fibroblasts. In contrast, rapamycin increased Akt phosphorylation at both Thr308 and Ser473 sites in addition to downstream Akt signaling in TGF- $\beta$ 1-induced MRC5. Taken together, TGF- $\beta$ 1 mediated potent fibrogenic effects through activated the mTORC1 signaling.

We next identified a relationship between mTOR pathway and SIRT6 in regulating myofibroblasts differentiation. Recent article has shown that SIRT6 controls protein synthesis through the mTOR signaling [40]. Interesting, knockdown of SIRT6 failed to activate mTORC1 regardless of presence or absence of TGF- $\beta$ 1 in MRC5 or IPF fibroblasts, suggesting that activated mTORC1 regulate myofibroblasts differentiation through SIRT6 overexpression. Additionally, phosphorylation of Akt on ser473 was increased in SIRT6 knockdown lysates indicating upregulated mTORC2 activity. Our data show that activated mTORC1 modulated SIRT6 expression whereas SIRT6 knockdown enhanced activity of mTORC2.

On the other hand, mTOR pathway plays a critical role in autophagy regulation. mTOR activity may be deregulated in IPF fibroblasts, leading to the proliferative and apoptosis-resistant fibroblast phenotype through altered autophagic activity [41]. It was found that LC3B- $\beta$  expression was relatively reduced in IPF fibroblasts, a marker of autophagy when compared to control fibroblasts [19, 42]. Consistent with our studies, TGF- $\beta$ 1 significantly inhibited autophagy in MRC5 and IPF fibroblasts, which was shown by decreased conversion to LC3-II from LC3-I. Rapamycin clearly increased autophagy induction in dose

dependent manner. Intriguingly, no significant changes in autophagy induction were observed after SIRT6 siRNA treatment. Finally, we investigated the effects of SIRT6 on the expression of fibrosis-associated transcription factors. SIRT6 knockdown significantly increased the transcription levels of collagen  $\alpha_1(\text{I})$ , fibronectin, MMP3 mRNA. Thus, deficiency of SIRT6 have aggravated the expression of fibrosis-related factors.

In summary, we identified that mTORC1 was activated in fibrosis models whether vivo or vitro. Abnormal mTORC1 mediated myofibroblasts differentiation and caused SIRT6 overexpression. Effect of mTORC1 inhibition was limited in anti-fibrosis due to negative feedback loop on mTORC2 and suppressed SIRT6 induced the expression of fibrosis-related transcription factors. Future study aims to develop effective and safe agonist or target gene inhibitor for mTORC1 to treat IPF.

## Abbreviations

IPF: Idiopathic pulmonary fibrosis; FVC: [Forced Vital Capacity](#); ECM: extracellular matrix; PI3K: phosphatidylinositol 3-kinase; mTOR: Mammalian target of rapamycin; TGF- $\beta$ 1: transforming growth factor (TGF)- $\beta$ 1; EMT: Epithelial to mesenchymal transition; LC3: phosphatidylethanolamine-modified microtubule-associated protein light-chain 3; HFL1: human fetal lung fibroblasts; CMECs: Cardiac microvascular endothelial cells; IRS-1: Insulin receptor substrate 1; Estrogen-related receptor  $\gamma$  (ERR $\gamma$ ); BDL: bile duct ligation.

## Declarations

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### Authors' contributions

Yi Hu and Weixi Guo performed the experiments. Huiping Huang contributed to statistical analyses of the data. Qun Liu collected associated clinical data. Yang Du and Guangdong Wang carried out the animal and cell lines experiments. Ji Zhang provided the surgical specimens from lung transplant patients. Zhanxiang Wang and Aiping Ma wrote the manuscript and conceptualized the framework for this research. All authors read and approved the final manuscript.

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### **Availability of data and materials**

All data is available if required.

### **Declarations**

### **Ethics approval and consent to participate**

This study was conducted at The First Affiliated Hospital, School of Medicine, Xiamen University, China. This research on animals was approved by the Xiamen University Institutional Animal Care and Use Committee. The research on tumor tissues was approved by the Ethical Committee of The First Affiliated Hospital, School of Medicine, Xiamen

University.

### **Consent for publication**

All authors have read and approved the manuscript

### **Competing interests**

The authors declare that they have no competing interests

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## Figures

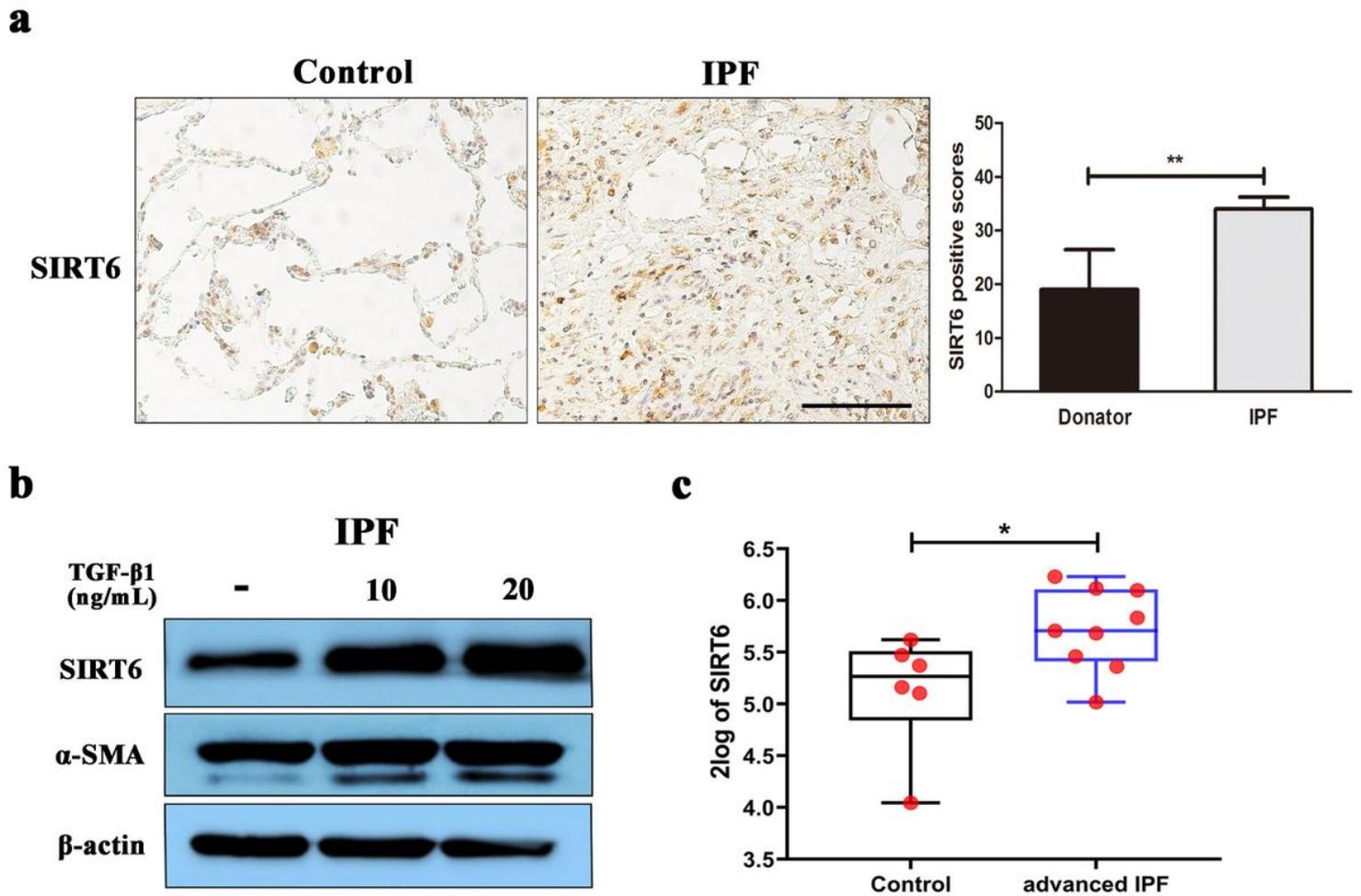
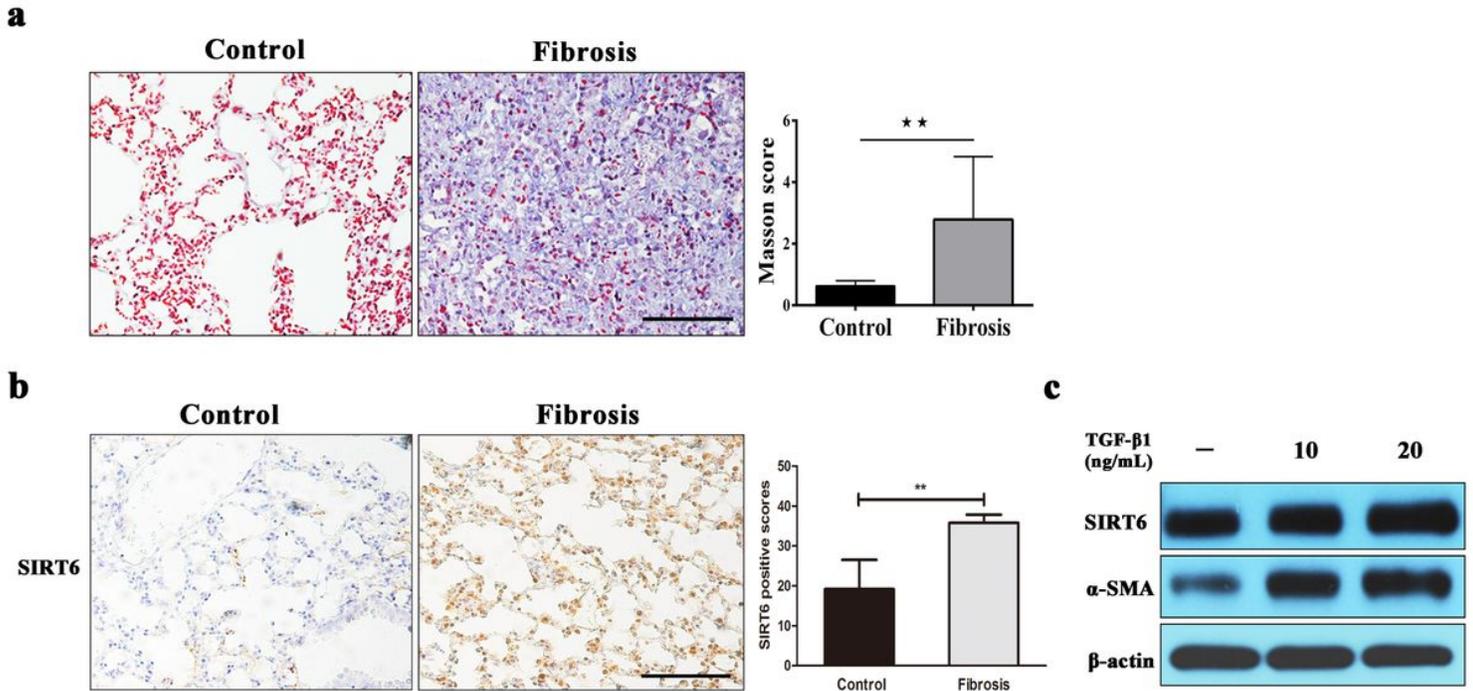


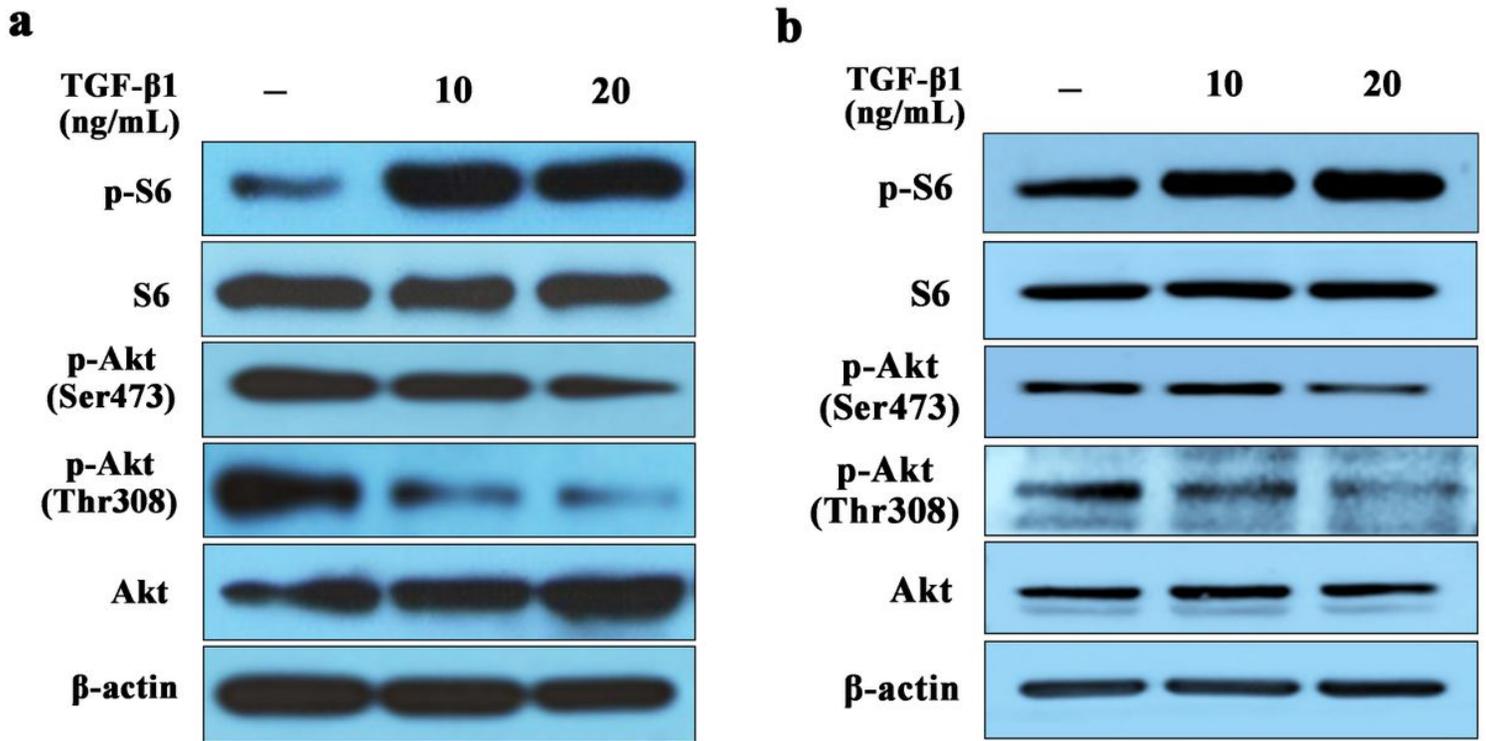
Figure 1

SIRT6 expression was up-regulated in IPF myfibroblasts. a. Immunohistochemistry staining (IHC) was performed with lung tissues from idiopathic pulmonary fibrosis (IPF) patient or from histologically normal lungs (both n=1) using SIRT6 antibody. Shown was SIRT6 expression in cells within the fibroblastic foci of IPF patients (right panel) is higher than normal lung alveoli tissue specimens (left panel), \*\*, P < 0.01. b. Primary human lung fibroblasts isolated from control subject (N=1) or IPF patient (N=1) were treated with 10, 20ng/mL of TGF- $\beta$ 1. SIRT6 and  $\alpha$ -SMA level were measured by Western blotting. Shown was SIRT6 and  $\alpha$ -SMA expression in lung myfibroblasts from IPF increased in the dose-dependent of TGF- $\beta$ 1. Bar: 100 $\mu$ m. c. The expression of SIRT6 (reporter: 219613\_s\_at) in health control (n=6) versus the advanced IPF (n=9) in R2 Genomics Analysis and Visualization Platform. P=0.032, significance was determined by unpaired Student's test.



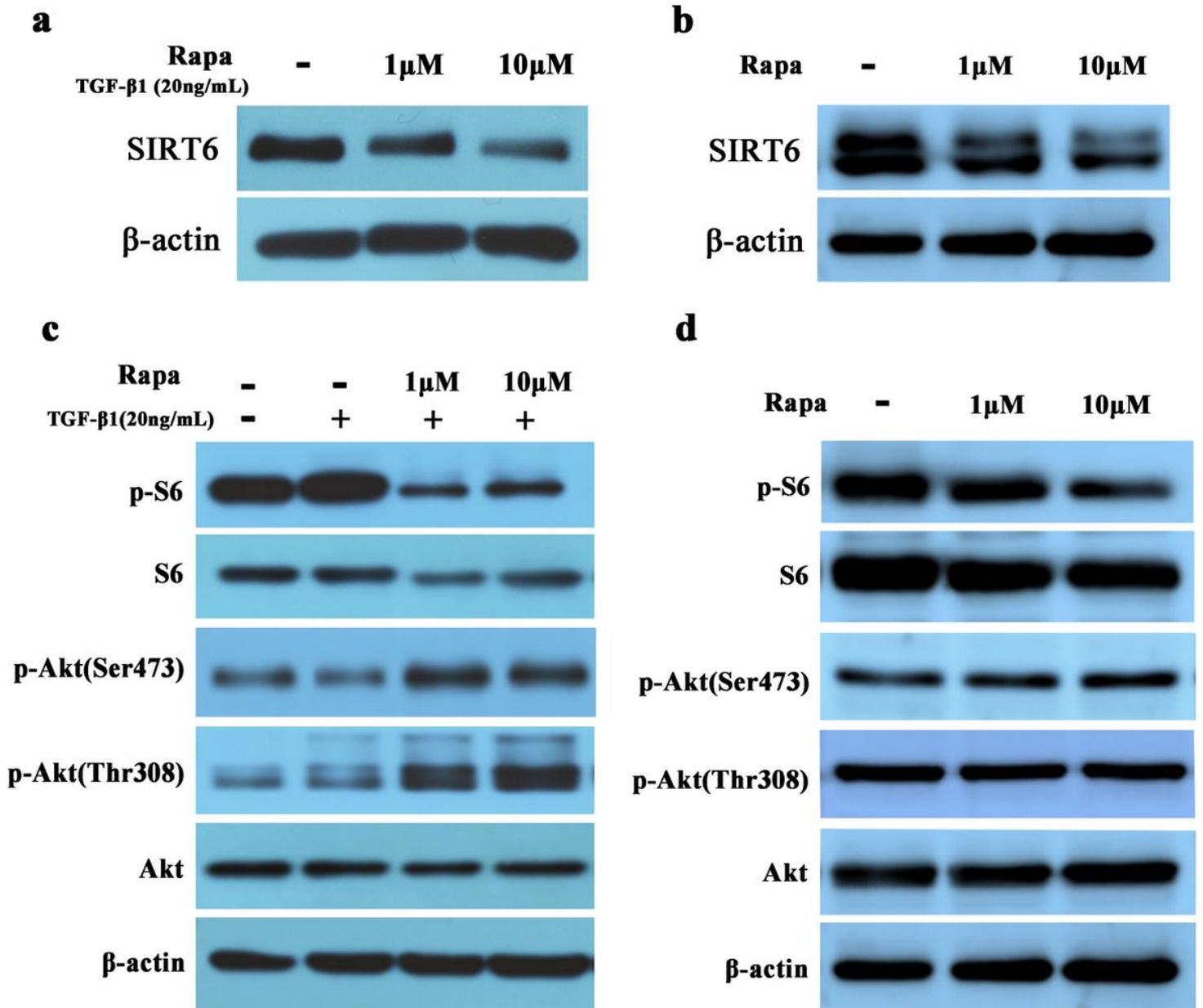
**Figure 2**

Overexpression of SIRT6 was observed in bleomycin induced pulmonary fibrosis mice. a. Lung sections of C57BL/6J mice was collected at 21 days after intratracheal bleomycin or saline injection. Mason's trichrome staining demonstrates increased lung matrix deposition in bleomycin-administered mice (Fibrosis) compared to saline-treated (Control) mice. b. IHC analysis demonstrated a significant increase in levels of SIRT6 production in the lungs of bleomycin-induced mice as compared to saline induced controls on 21 days. c. MRC5 line was cultured for 72 hours with TGF-β1-induced myofibroblast differentiation in a concentration-dependent manner (0, 10, 20ng/mL). Expression of SIRT6 and α-SMA protein induced by TGF-β1 were increased in a concentration-dependent manner. \*\*, P< 0.01.



**Figure 3**

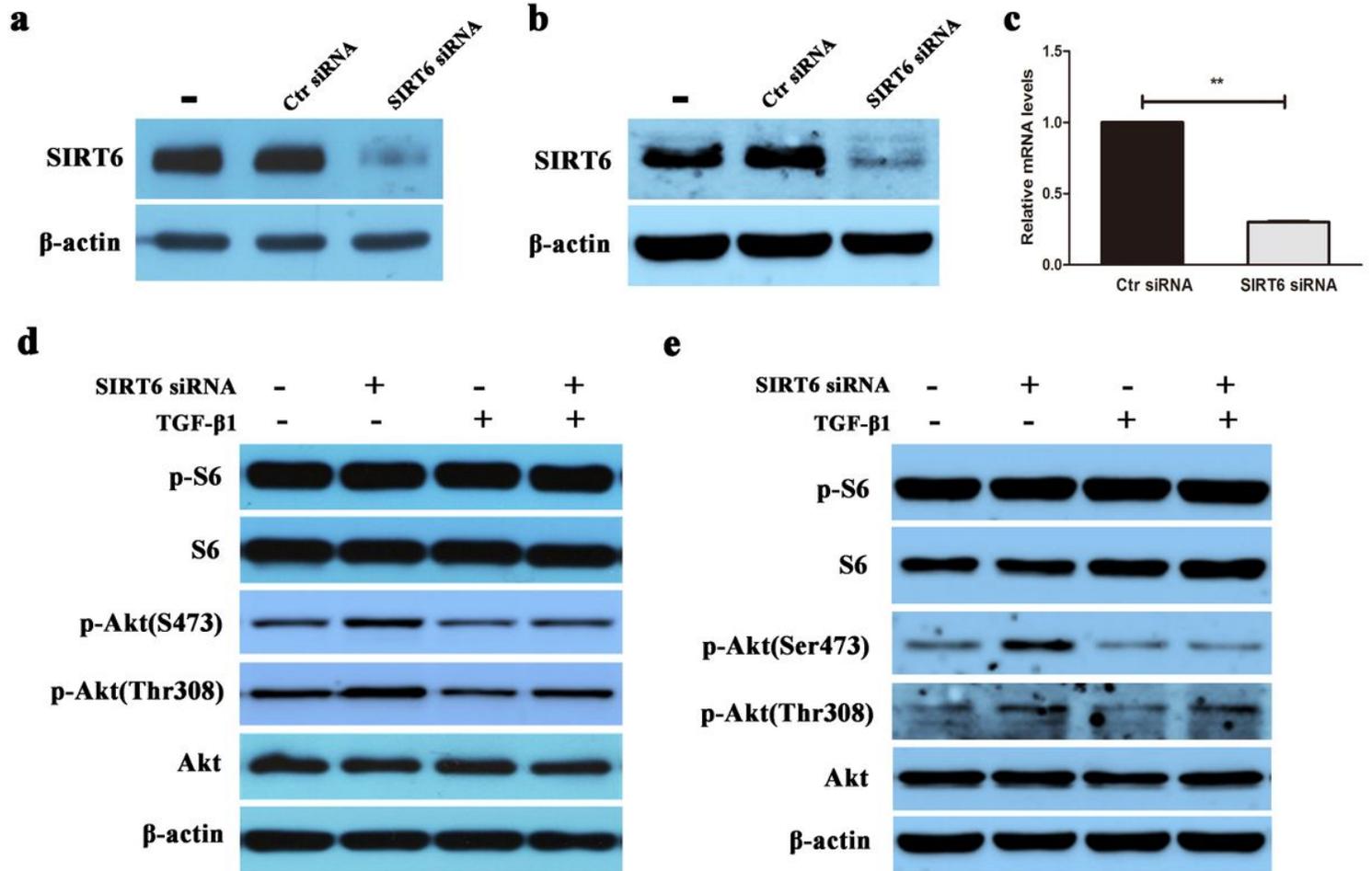
mTOR signaling pathway is highly activated in TGF-β1 induced lung myofibroblast differentiation. a. Immunoblots of MRC5 cell lysates, treated as indicated, showed that TGF-β1-induced phosphorylation of S6 was also upregulated and phosphorylated p-Akt (Ser473 and Thr308) were reduced. b. Western blotting showed the mTOR activity in primary human lung fibroblasts from patient with IPF after incubation with TGF-β1 (0, 10, 20ng/mL) for 24 hours. The expression of phosphorylated S6 level was increased induced by TGF-β1 in a concentration-dependent manner. Phosphorylated p-Akt (Ser473 and Thr308) were down-regulated by TGF-β1-induced in a concentration-dependent manner.



**Figure 4**

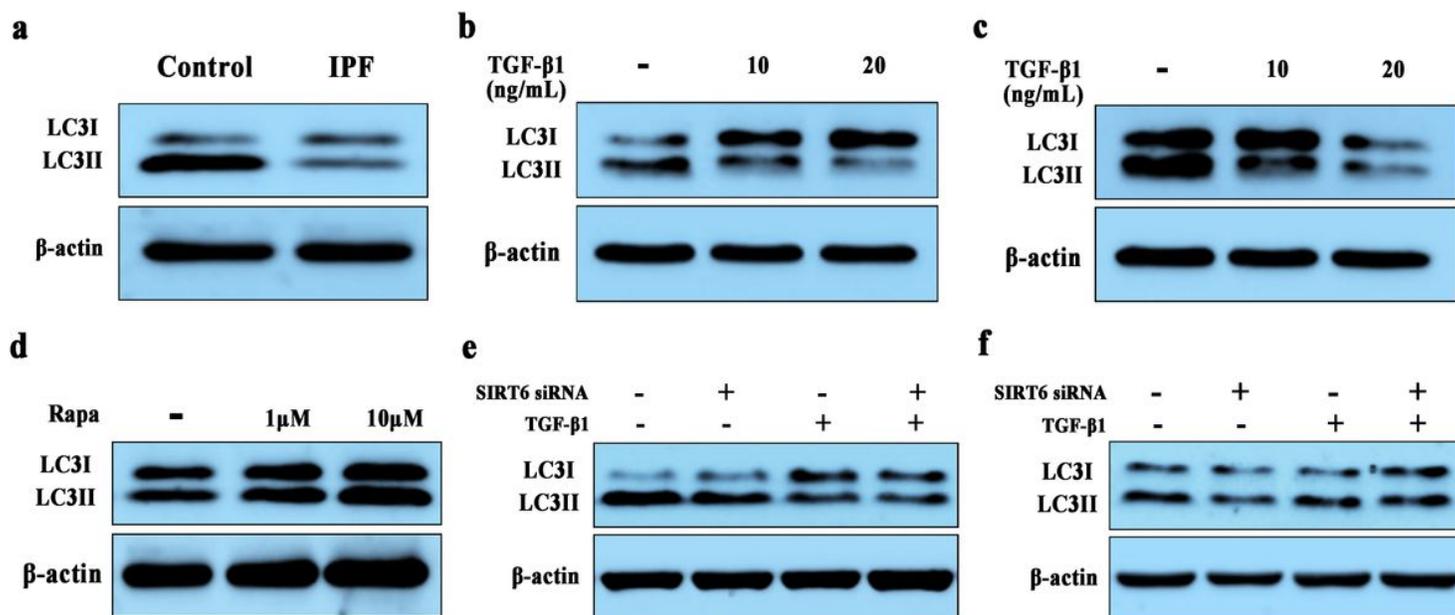
Rapamycin suppresses the expression of SIRT6 in myofibroblast differentiation. a. Western blotting showed that SIRT6 protein expression was reduced in MRC5 after TGF- $\beta$ 1 (20ng/mL) incubation for 48 hours in the presence of rapamycin (0, 1, 10 $\mu$ m). b. In primary myofibroblast from IPF patient, the level of SIRT6 was dramatically reduced by rapamycin (0, 1, 10 $\mu$ m) for 48 hours in a dose-dependent manner. c. In MRC5 line, the activity of mTOR pathway was also assessed after 48 hours incubation with 0, 1, 10 $\mu$ m rapamycin and 20 ng/mL TGF- $\beta$ 1. Rapamycin (1, 10 $\mu$ m) significantly inhibited TGF- $\beta$ 1-induced phosphorylation of S6 level in the MRC5. While rapamycin increased TGF- $\beta$ 1-induced the level of phosphorylation of Akt (Ser473 and Thr308) in a dose dependent manner. d. Primary pulmonary myofibroblast was stimulated with rapamycin (0, 1, 10 $\mu$ m) for 48 h and mTOR pathway was assessed by Western blotting. Phosphorylation of S6 was significantly inhibited with a concentration-dependent

manner of rapamycin. An increased trend was found in phosphorylation of Akt (Ser473 and Thr308) between different concentration groups of rapamycin



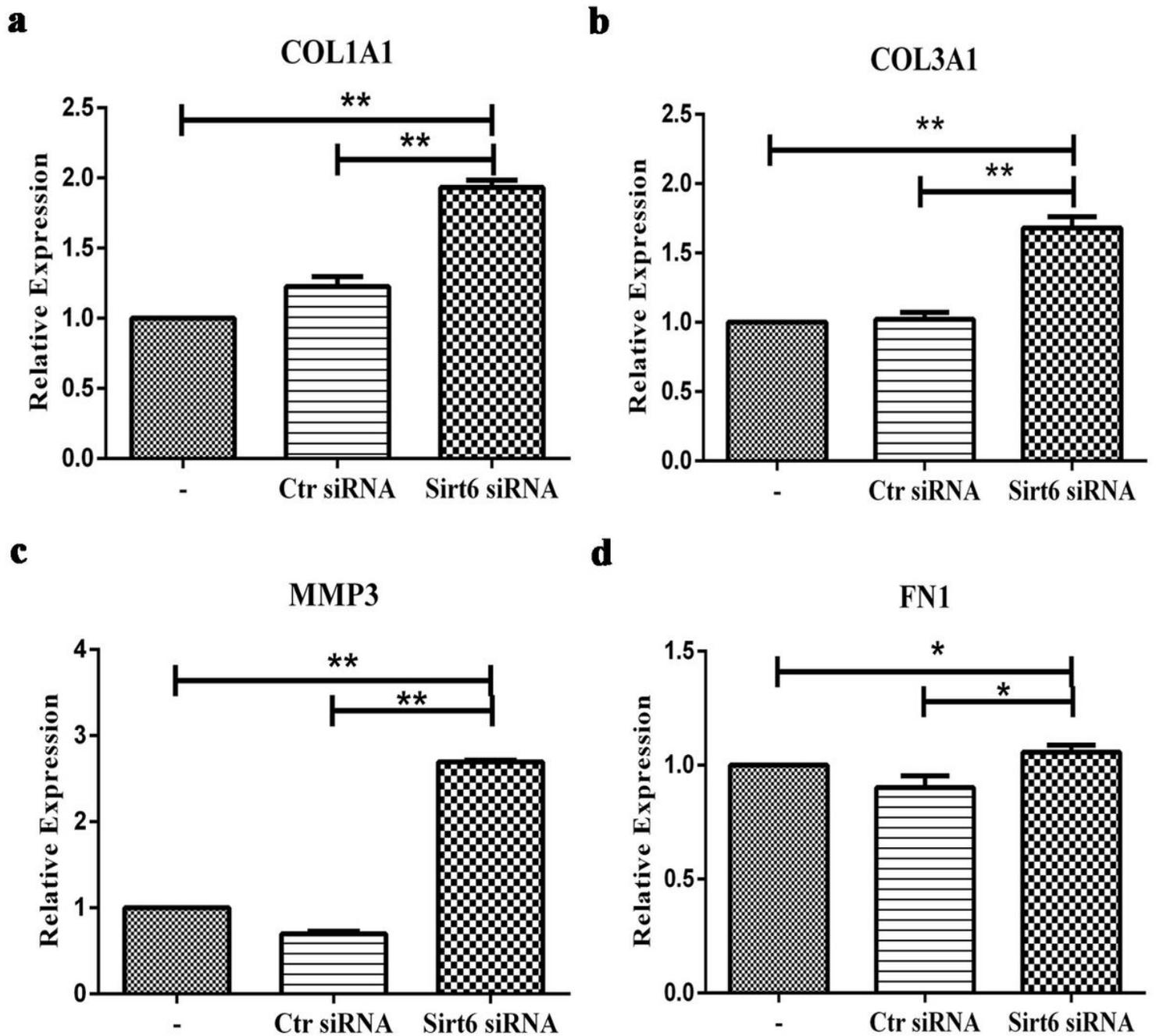
**Figure 5**

mTORC1 signaling pathway modulated the expression of SIRT6. a. SIRT6 protein was also significantly decreased in MRC5 transduced with SIRT6 siRNA. b. Primary pulmonary myofibroblast was transduced with SIRT6 siRNA showed marked decrease in the expression of SIRT6. c. Real-time qPCR analysis showed that SIRT6 siRNA treatment significantly decreased the SIRT6 mRNA level in MRC5, \*\*,  $P < 0.01$ . d. Western blotting showed that knocking down SIRT6 by siRNA in MRC5 cell did not affect phosphorylation levels of S6. The levels of the phosphorylated-Akt of Ser473 and Thr308 were reduce by TGF-β1 stimulated, while SIRT6 siRNA was able to increase phosphorylation (Ser473, Thr308) without or with TGF-β1. e. Primary pulmonary myofibroblast was transduced with SIRT6 siRNA or control siRNA followed by treatment with TGF-β1. SIRT6 deficiency could not inhibit phosphorylation of pS6 but increase the level of the Akt phosphorylation (Ser473, Thr308).



**Figure 6**

SIRT6 failed to mediate the autophagy activity in pulmonary fibrosis. a. The conversion of LC3 from LC3-I (free form) to LC3-II (phosphatidylethanolamine-conjugated form) was confirmed by western blotting in primary lung fibroblasts. Autophagy inhibition was observed in IPF. b. In primary lung fibroblast, TGF- $\beta$ 1 stimulation reduced autophagy induction as determined by decreased conversion of LC3 from LC3-I to LC3-II in a concentration-dependent manner. c. TGF- $\beta$ 1 stimulation further inhibited MRC5 autophagy by means of decreased LC3-II conversion. d. Increasing concentrations of rapamycin markedly enhanced autophagy induction as determined by increased conversion of LC3 from LC3-I to LC3-II in IPF. e. primary lung fibroblasts were transfected with siRNA SIRT6 and followed by treatment with TGF- $\beta$ 1. Western blotting showed that siRNA SIRT6 had no effect on decreasing the level of LC3-II/ I protein whether stimulation with TGF- $\beta$ 1 or not. F. Knockdown of SIRT6 was confirmed by Western blotting, and no significance difference was observed in LC3-II conversion following TGF- $\beta$ 1 treatment or not in MRC5.



**Figure 7**

SIRT6 modulates TGF- $\beta$ 1 induced myfibroblasts differentiation through pro-fibrotic cytokines. a. It was found a significant increase of Collagen1 in MRC5 cells incubated with SIRT6 siRNA in present of TGF- $\beta$ 1. b. MRC5 cells were transfected with siRNA SIRT6 for 12h before TGF- $\beta$ 1 (20ng/mL) treatment. SIRT6 siRNA significantly enhanced Collagen3 mRNA levels by TGF- $\beta$ 1-induced for 24h. c. The difference of MMP3 showed statistical significance in the presence of TGF- $\beta$ 1. d. Fibronectin levels also showed a significant increase in the present of TGF- $\beta$ 1 in MRC5 treated with siRNA SIRT6 when compared to control. \*,  $P < 0.05$ , \*\*,  $P < 0.01$ .

## Supplementary Files

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