TGF- β -induced α -SMA expression is mediated by C/EBP β acetylation in human alveolar epithelial cells in vitro

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Abstract

Background This study sought to determine whether binding of acetylated C/EBP β to α -SMA promoter could affect its activity and was essential for EMT and extracellular matrix deposition in IPF using in vitro model.

Methods Through western blotting, the expression of EMT and C/EBP β were detected in A549 cells with TGF- β as pulmonary fibrotic model in vitro. Moreover, the expression of C/EBP β mRNA via Real Time-PCR and Collagen-I expression using ELISA were performed. The luciferase activity assay was used to examine the activity of C/EBP β . The knockdown expression of C/EBP β gene was prepared in A549 cells with C/EBP β siRNA. We also investigated the effect of deacetylation of C/EBP β on EMT using SIRT1. The binding ability of C/EBP β with the α -SMA promoter was affirmed via ChIP and EMSA. Furthermore, the relationship between α -SMA and acetylated C/EBP β was investigated using the co-immunoprecipitation.

Results SiRNA-mediated knockdown of C/EBP β in A549 cells attenuated TGF- β 1-induced myofibroblast differentiation and ECM deposition. The extent of association between acetylated C/EBP β and the α -SMA promoter was dynamically monitored. Furthermore, it was confirmed that deacetylation of C/EBP β in A549 cells successfully ameliorated TGF- β 1-induced EMT, as shown by reduction in α -SMA expression and excessive collagen-I accumulation.

Conclusions Collectively, our data suggested that the EMT and fibrotic effect of TGF- β 1 could be dependent on acetylated C/EBP β -mediated regulation of α -SMA gene activity. This thus suggests that C/EBP β acetylation may play a central role in pulmonary fibrosis.

Introduction

Idiopathic pulmonary fibrosis (IPF) is diagnosed as an irreversible progressive fibrotic disease with a median survival rate of up to 2.5–3.5 years after diagnosis [1]. The morbidity and mortality rates associated with IPF are high and the abnormal pulmonary function of patients with IPF adversely affects their quality of life. Unfortunately, powerful and precise therapies coupled with the exception of lung transplantation, for improving the prognosis for patients with IPF are lacking. Therefore, the investigation of the mechanisms of IPF pathogenesis is critical for the development of efficient IPF therapeutics.

The characteristic pathological feature of IPF is the usual interstitial pneumonia (UIP), marked by increased deposition of extracellular matrix (ECM) components such as fibronectin and collagen-I (Col-I) [2], which causes abnormality of lung tissue, resulting in pulmonary function insufficiency. Although the precise mechanism underlying the pathogenesis of lung fibrosis is unclear, accelerated proliferation of myofibroblasts has been shown to be possibly responsible for the excessive accumulation of ECM in the alveolar and interstitial compartments of the lung coupled with the development and prognosis of IPF [3, 4]. Previous studies have reported that epithelial-to-mesenchymal transition (EMT) due to injuries to alveolar epithelial cells (AECs) could contribute considerably to the initiation and maintenance of fibrosis [5, 6]. During the EMT process, the epithelial cells lose cellular interaction and gain mesenchymal

phenotype as well as the ability to generate ECM. Furthermore, reduced expression of E-cadherin, an epithelial cell marker and increased expression of α -smooth muscle actin (α -SMA), a marker for myofibroblast differentiation has been observed in fibrotic lung tissue [4]. Hence, regulation of α -SMA expression is critical for induction of myofibroblasts and genesis of fibrosis. Indeed, inner and outer injuries to the epithelium could lead to the accumulation of cytokines and molecular sensors, which normally support the active EMT process, resulting in ECM deposition. Pertinently, transforming growth factor- β (TGF- β) is known to be a major inducer of the initiation and maintenance of lung fibrosis [7]. Willis et al reported that AECs that respond to TGF- β induced conversion to the mesenchymal phenotype, is associated with α -SMA-positive expression [8]. Hence, TGF- β 1-induced EMT has been implicated to play a dominant role in the pathology of pulmonary fibrosis.

Molecular signalling controlling TGF- β 1-induced EMT are complex as TGF- β 1 activation of the various pathways, including Smads [9], mitogen-activated protein kinase (MAPK) [10], and phosphatidylinositol 3-kinase (PI3K) [11]. In rodents, CCAAT/enhancer binding protein β (C/EBP β), a leucine zipper transcription factor, has been shown to be localised in the AECs and bronchiolar epithelium using immunohistochemistry [12]. Indeed, other studies have shown that C/EBP β is required for maintaining the stability of epithelial cells. Hu et al. reported that C/EBP β activation was essential for cytokine secretion and differentiation of myofibroblasts in vivo [13]. Furthermore, another study demonstrated that mice lacking C/EBP β antagonised the development of bleomycin-induced lung fibrosis [14]. Nonetheless, the post-translational modification of C/EBP β responsible for activating α -SMA expression is still unclear.

Schwartz et al. confirmed that acetylation of C/EBP β was necessary for cytokine activation and downstream transcription [15]. Based on this assertion, we hypothesised that acetylation of C/EBP β could induce the binding to the α -SMA promoter, which subsequently may activate gene expression that is critical for ECM deposition and lung fibrosis. To affirm this hypothesis, we treated the human alveolar epithelial cell line A549 with TGF- β 1 to establish an in vitro model of lung fibrosis. The level of C/EBP β acetylation associated with the α -SMA promoter to indicate the EMT progression was dynamically monitored. Furthermore, siRNA-mediated knockdown of C/EBP β in A549 cells attenuated TGF- β 1-activated myofibroblast differentiation and ECM deposition. We therefore postulated that deacetylation of C/EBP β in A549 cells may successfully ameliorate TGF- β 1-induced EMT via decrease in α -SMA expression, followed by reduction in excess collagen-I accumulation, a characteristic of lung fibrosis.

Methods

Cell culture and stimulation

Human alveolar epithelial A549 cells were purchased from ScienCell® and propagated as described previously [16]. The A549 cells were plated in six-well plates and grown overnight under serum deprivation till 70% confluence was reached. For knockdown experiments, the A549 cells were pre-treated with C/EBP β small interfering RNAs (siRNA) (10 nM) according to the manufacturer's protocol (Santa Cruz, USA) for 24 h. SIRT1 (R&D Systems, USA), a class III histone deacetylase, was added to the cells at

the final concentration of 2 μ M 24 h prior to the addition of 10 ng/ml TGF- β (R&D Systems) for C/EBP β deacetylation. All the experiments were performed in A549 cells between passages 4 and 8. Whole-cell lysates were prepared for protein and mRNA collection.

Western blot analysis

The proteins isolated from A549 cell lysates were quantified using the bicinchoninic acid (BCA) method. Equal amounts (60 μ g) of protein was electrophoresed on 12% sodium dodecyl sulphate-polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were incubated overnight with specific antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (R&D Systems), acetylated-C/EBP β (Ac-C/EBP β ; Pocono Rabbit Farm & Laboratory, USA), C/EBP β (Abcam, UK), and α -SMA (Abcam) at 4 °C and then incubated with appropriate secondary antibodies at room temperature for 4 h. The chemiluminescence signals developed using the enhanced chemiluminescence (ECL) kit (Santa Cruz) was quantified using the Quantity One software. All independent experiments were repeated thrice.

Real time-polymerase chain reaction (RT-PCR)

Total RNA was extracted from A549 cells using the RNA extraction kit (Lucigen, USA) according to the manufacturer's instruction and was quantified using a spectrophotometer. The integrity of RNA (300 ng) was determined for each assay. The sequences of the primers (Applied Biosystems Inc., Foster City, CA, USA) are as follows: C/EBP β forward 5'-GCCTCTCCACGTCCTC-CTCGT-3' and reverse 5'-CACCTTCACCGTTCCAGTTT-3'; α -SMA forward 5'-GTGACTACTGCCGAGCGTG-3' and reverse 5'-ATAGGTGGTTTCGTGGATGC-3'; forward 5'-CACCTTCACCGTTCCAGTTT-3' and reverse 5'-CTCTTCC-AGCCTTCCTTCCT-3'. RT-PCR was performed for 30 cycles of 51°C for 30 min, 95°C for 30 s, 94°C for 45 s, 60°C for 45 s, and 72 °C for 10 min in a GeneAmp 7500 sequence detection system (Applied Biosystems Inc.). The Taqman One Step RT-PCR master mix was used. The PCR products were electrophoresed on a 2% agarose gel and data were quantified with GAPDH as the internal control using the UVP bioimaging system GDS-8000. The mean Ct value of triplicate experiments was used for data calculation.

ChIP assays

The A549 cells exposed to TGF- β for 24 h were prepared for CHIP assays using a high sensitivity ChIP assay kit (Abcam) according to the manufacturer's instructions. A PCR was performed using the following primers: 5'-GCT GTC GTV TTA TCT CCA CCA-3' and 5'-GCA GGA GTC TAG CAG AAG TTC-3'. All the data were collected from three independent studies.

Co-immunoprecipitation

Protein samples were immunoprecipitated with either polyclonal antibody against C/EBP β (Abcam) or control IgG (Santa Cruz) at 4 °C overnight. Then, the samples were constantly agitated with A/G-agarose beads (Santa Cruz) at 4 °C for 4 h. After five washes with the buffer, the beads were used for protein

extraction. Proteins from deposits with lysate were used for western blotting as mentioned above. The antibodies used for western blotting were anti-acetylated-lysine antibody (Santa Cruz) and anti- α -SMA (Abcam).

Enzyme-linked immunosorbent assay (ELISA)

The levels of collagen-I were measured in cell-free supernatants from A549 cells after stimulation with factors using ELISA kits (R&D Systems) according to the manufacturer's protocol. The data were obtained from three separate experiments.

Luciferase reporter assay

The luciferase activity assay was performed as described previously [17]. Briefly, luciferase reporter gene constructs under the control of the 5'-flanking promoter sequence of the human α -SMA gene with the conserved putative C/EBP β -binding motif TTGGGCAA were constructed by inserting DNA fragment generated by PCR into the pGL4.10 vector (Promega, Madison, WI). The A549 cells in 12-well plates were treated with C/EBP β -Luc (0.8 μ g) or 0.5 μ g α -SMA-Luc from Stratagene (USA) for 24 h and subsequently used for luciferase reporter assays. The cells were treated with exogenous SIRT1 (2 μ M) for 24 h as described in earlier work with some modifications [18]. Then, the cells were activated with or without TGF- β (10 ng/ml) for 16 h. The luciferase activity of the stimulated cells was measured using the luciferase assay system (Promega, USA).

Statistical analysis

All data were presented as mean $\pm$ standard error of mean (SEM). For the significance between two group samples, a two-tailed parametric or non-parametric t-test was used. Two-way analysis of variance (ANOVA), followed by Boferroni's test was performed for multiple comparison between more than two groups. The control samples from separate experiments were normalised to a value of one. A P-value < 0.05 was considered statistically significant.

Results

TGF- β induces EMT of A549 cells

The A549 cells were first treated with various amounts of TGF- β at different time periods to determine the safe and effective concentration of TGF- β and incubation time required for investigating EMT in vitro. When A549 cells were treated with 1.0, 2.5, 5.0, 10 and 20 ng/ml TGF- β for 24 h, we observed a dose-dependent increase in the expression of the mesenchymal marker α -SMA and decrease in the expression of the mesenchymal marker E-cadherin using western blotting (Fig. 1a). Although α -SMA level increased in A549 cells after treatment with 20 ng/ml TGF- β , cell viability was poor. In addition, A549 cells were treated with 10 ng/ml TGF- β at different time periods (0, 6, 12, 24, and 48 h) to determine the appropriate

time frame of treatment. We observed an increase in the expression of α -SMA in a time-dependent manner, which peaked at 48 h by 8.72 ± 0.45 -fold compared with that of the control. Nevertheless, cell death was also evident. E-cadherin expression decreased significantly in a time-dependent manner after 24 h of exposure to TGF- β (Fig. 1b). Therefore, the optimal TGF- β concentration and incubation time were 10 ng/ml and 24 h, respectively. Indeed, the increase in the level of collagen-I (determined using ELISA) correlated with the degree of fibrosis in A549 cells exposed to TGF- β (Fig. 1c). Thus, we successfully established an in vitro model of EMT-induced fibrosis using A549 cells treated with optimal concentration of TGF- β for specific exposure duration.

Up-regulation of C/EBP β is involved in TGF- β -induced EMT

Previous study has shown that phosphorylation of C/EBP β is involved in pulmonary fibrosis in mice [19]. To investigate the precise roles of C/EBP β in fibrosis, the relationship between C/EBP β activation and TGF- β -induced EMT was investigated. In this study, we observed that TGF- β was up-regulated C/EBP β mRNA level in dose-and time-dependent manner (Fig. 2a, b). Furthermore, western blot analysis showed that the elevated levels of C/EBP β in A549 cells correlated closely with dose-and time of TGF- β treatment, as it peaked when 10 ng/ml TGF- β was used for 48 h (Fig. 2c, d). Obviously, the TGF- β -mediated increase in C/EBP β expression correlated well with occurrence of EMT in A549 cells. Furthermore, we used luciferase reporter assay to better understand C/EBP β activation after TGF- β -induced EMT. Indeed, exposure of A549 cells to TGF- β generated time-and dose-dependent increase in C/EBP β -luciferase activity and exhibited a 2.83 ± 0.42 -fold increase in expression compared with that of the control (Fig. 2e, f). Collectively, these observations suggested that TGF- β increased C/EBP β expression and activation during the EMT.

Loss of C/EBP β shifts TGF- β -induced collagen deposition following EMT

Previous studies have suggested that C/EBP β activation is involved in the pulmonary fibrotic process [13, 14]. Other studies have reported that mice with C/EBP β deficiency antagonise BLM-induced pulmonary fibrosis in vivo [13]. Hence, we investigated whether C/EBP β is required for TGF- β -activated EMT and collagen-I deposition. In this regard, C/EBP β siRNA (10 nM) was used to establish the reducing gene model in A549 cells. As shown in Fig. 3a, the C/EBP β siRNA successfully decreased gene expression. A549 cells transfected with the C/EBP β siRNA showed attenuation of TGF- β -induced α -SMA and collagen-I expression (Fig. 3a, b). Furthermore, TGF- β could not increase C/EBP β -luciferase activity in A549 cells treated with the C/EBP β siRNA (Fig. 3c). Taken together, our results suggest that C/EBP β is a crucial factor for regulating TGF- β -induced EMT and collagen-I deposition in pulmonary fibrosis.

TGF- β induced C/EBP β binding to α -SMA promoter in A549 cells

As mentioned above, our data confirmed that C/EBP β played a pivotal role in EMT and pulmonary fibrosis in vitro. As an important transcription factor, C/EBP β triggered the expression of downstream

genes by binding to its cognate sites in the gene promoters. However, the C/EBP β binding site on the α -SMA promoter region in A549 cells is not known. Therefore, to elucidate the molecular mechanism through which TGF- β regulates α -SMA expression, we checked the putative C/EBP β -binding sites in the 5' promoter region of human α -SMA gene. Also, the 5' promoter region of the human, mouse and rat α -SMA gene was examined by using Multiple Sequence Alignment. We found a conserved putative C/EBP β -binding motif TTGGGCAA in the 5' promoter region within 200 bp from the transcription start site was identified (Figure 4A). Therefore, we hypothesized that the putative C/EBP β -binding motif is a C/EBP β -responsive *cis*-element that mediates the upregulation of the α -SMA gene, and that the activation of this *cis*-element is critical to the development of EMT. To test our hypothesis, we evaluated C/EBP β binding to the putative binding motif present in the α -SMA promoter in A549 cells by using the chromatin immunoprecipitation (ChIP) assay. We observed that TGF- β -treated A549 cells showed increased C/EBP β binding to the α -SMA promoter region (Fig. 4B). These results suggested that activated C/EBP β accelerates TGF- β -induced EMT by binding to the α -SMA promoter region in A549 cells.

Role of acetylation of C/EBP β in binding to the α -SMA promoter in A549 cells

Other reports have shown that phosphorylation of C/EBP β plays a critical role in alveolar EMT and acts an essential step in pulmonary fibrosis. As a transcription factor, C/EBP β activates its downstream signals via post-translational modification, such as phosphorylation, acetylation and methylation. However, reports that regard to the role of C/EBP β acetylation in pulmonary fibrosis are lacking. As shown above, activated C/EBP β binds to the α -SMA promoter region in TGF- β -treated A549 cells. Nonetheless, the mechanism connecting C/EBP β binding to α -SMA expression is not clearly understood. Hence, we investigated the effect of TGF- β on C/EBP β modification and observed that acetylation of C/EBP β increased significantly by 6.42 ± 0.72 -fold in A549 cells treated with TGF- β (Fig. 5). Interestingly, enhanced α -SMA expression was observed in samples treated with the C/EBP β antibody but not in TGF- β -treated cells with IgG. Collectively, our results showed that α -SMA expression is triggered by activated C/EBP β , which is acetylated in an in vitro model of pulmonary fibrosis.

Acetylation and deacetylation of C/EBP β in TGF- β -induced EMT and collagen deposition

In the present study, we used TGF- β -treated A549 cells to mimic the pulmonary fibrotic model in vitro. The binding of C/EBP β to α -SMA and the increased expression and acetylation of C/EBP β indicated that acetylated C/EBP β was involved in EMT and pulmonary fibrosis. As shown in Fig. 6a, TGF- β treatment led to acetylation of C/EBP β and accelerated EMT-induced collagen-I deposition. Acetylation of C/EBP β is important for its activation. To clarify whether acetylation of C/EBP β is necessary for pulmonary fibrosis, we subsequently investigated the effect of deacetylation of C/EBP β on EMT using sirtuin1 (SIRT1). As reported, SIRT1, a class III histone deacetylase (HDAC), specifically deacetylates histone or non-histone proteins. C/EBP β is one of the deacetylation targets of SIRT1 [20]. We observed that SIRT1 reversed TGF- β -induced C/EBP β acetylation (Fig. 6a). Interestingly, C/EBP β deacetylation significantly reversed the elevated expression of α -SMA and collagen-I in TGF- β -treated A549 cells (Fig. 6a, b). As shown above, SIRT1 stimulation also suppressed the increased C/EBP β -luciferase activity in TGF- β -treated A549 cells

(Fig. 6c). These observations suggested that acetylation and deacetylation are useful steps in regulating C/EBP β functions. All these results confirm that C/EBP β acetylation is a key player in alveolar EMT and that pulmonary fibrosis is blocked by its deacetylation.

Discussion

Induction of C/EBP β phosphorylation corresponds to lung fibrosis in mice [18]. In this study, we demonstrated that TGF- β leads to acetylation of C/EBP β , which in turn enhances its activation and up-regulates collagen-I deposition. Furthermore, acetylation of C/EBP β accelerates EMT in A549 cells by activating α -SMA after binding to the α -SMA promoter. In addition, C/EBP β deficiency attenuates α -SMA and collagen-I production. Deacetylation of C/EBP β is a major hurdle for TGF- β -induced EMT and collagen-I synthesis. Our observations revealed that acetylation/deacetylation of C/EBP β play a central role in EMT and pulmonary fibrosis via α -SMA activation.

A growing body of evidence shows that myofibroblasts are the main players involved in extracellular matrix (collagen-I) production during IPF [21]. Multiple factors stimulate the EMT of lung epithelial cells, which contribute significantly to myofibroblast formation during pulmonary fibrosis. During EMT, lung epithelial cells lose cellular polarity and gain migratory properties; this is accompanied by the deficiency of the epithelial marker E-cadherin and acquisition of the mesenchymal marker α -SMA [22,23]. Hence, EMT is studied by determining changes in the expression of these markers. We used TGF- β -treated A549 cells to mimic pulmonary fibrosis in vitro. Along with the reduction in E-cadherin expression, TGF- β improved α -SMA expression in a dose- and time-dependent manner. With the reduction in E-cadherin expression and increase α -SMA expression, A549 cells gradually transformed into myofibroblasts, which secrete excess collagen-I, resulting in pulmonary fibrosis. Therefore, blockage of α -SMA expression is a promising way of inhibiting EMT in pulmonary fibrosis. The molecular mechanism through which α -SMA regulates fibrosis has been the focus of investigation. In this study, the TGF- β -treated A549 cells provided a credible in vitro model for studying these mechanisms.

As a critical transcription factor, C/EBP β expression in the alveolar and bronchiolar epithelium of rodents significantly affects cellular proliferation and differentiation [24]. In vivo data confirmed that mice with C/EBP β deficiency showed significant attenuation of bleomycin-induced myofibroblast accumulation and pulmonary fibrosis [14]. Available evidence suggests that hypoxia-induced expression of connective tissue growth factor (CTGF), resulting in pulmonary fibrosis, was dependent on C/EBP β activation [17]. Besides, a previous study confirmed that C/EBP β was a key mediator of TGF- β -dependent fibroblast remodelling in asthma [25]. Similarly, C/EBP β may play a pivotal role in the development of pulmonary fibrosis. Indeed, Yuka et al. observed that TGF- β regulated the target genes of C/EBP β , which indicated that C/EBP β was a potential downstream effector of TGF- β signalling [6]. Furthermore, augmented expression of the TGF- β receptor 2 in embryonic stem cells with over-expression of C/EBP β indicated the reciprocal relationship between TGF- β and C/EBP β signalling [26]. A previous study has shown that TGF-

β regulated cellular growth, invasion and metastasis depending on C/EBP β activity in the mammary epithelial cells [27]. However, the involvement of C/EBP β in EMT of lung was unclear. Thus, we reasoned that C/EBP β acts as a key player in TGF- β -induced EMT in human alveolar epithelial cells. In this study, we observed that TGF- β increased C/EBP β mRNA and protein levels. Furthermore, the enhanced activity of C/EBP β -luciferase in TGF- β -treated A549 cells strengthened the evidence supporting the existence of a TGF- β -C/EBP β signalling pathway. In addition, we observed that the C/EBP β siRNA suppressed TGF- β -induced EMT, as observed by reduction in α -SMA expression and collagen-I [synthesis](#). Our study reveals that C/EBP β is required for profibrotic processes in TGF- β -treated A549 cells. Altogether, C/EBP β -dependent α -SMA activation is involved in TGF- β -induced pulmonary fibrosis.

Evidence suggests that C/EBP β regulates signalling by binding to its cognate sites in target genes. Chen et al. reported that hypoxia-induced CTGF-luciferase activity depended on the binding of C/EBP β to the ADAM 17 promoter site in human lung fibroblasts [17]. A related study showed that the binding of C/EBP β to the α -SMA promoter was involved in IL-1-regulated inflammation in rat lung myofibroblasts [28]. However, C/EBP β binding to the α -SMA promoter in pulmonary fibrosis had not been previously investigated. Herein, we demonstrated binding of C/EBP β to the α -SMA promoter in A549 cells. Furthermore, TGF- β promoted the expression of α -SMA in human alveolar epithelium, which was dependent on C/EBP β . The above studies clearly indicated that TGF- β -induced EMT was dependent on the binding of C/EBP β to α -SMA promoter sites.

The C/EBP β binding to this region of α -SMA promoter indicates that C/EBP β may block TGF- β -activated α -SMA expression; however, the specific pathway remains unclear. As a transcription factor, C/EBP β activation is regulated by multiple mechanisms of post-translational modification, including phosphorylation and acetylation. Indeed, human C/EBP β has several known phosphorylation sites, including Thr235, Thr266 and Thr273, which are important for intracellular localisation and transcriptional activity. An in vivo study demonstrated that C/EBP β phosphorylation on Thr217 contributed to bleomycin-induced lung fibrosis in mice [19]. Chen et al. observed that C/EBP β and C/EBP β -luciferase activity, which depended on its phosphorylation, was involved in hypoxia-activated lung fibrosis [17]. A recent study revealed that regulation of artificial C/EBP β phosphorylation may ease the membrane damage in ALI and improve membrane repair [29]. Chromatin remodelling is an essential mechanism that regulates gene transcription and acetylation/deacetylation play pivotal roles in the functioning of several transcription factors [30,31]. The mechanism via which acetylation/deacetylation regulates C/EBP β activity has been described previously. Huang et al. observed that C/EBP β acetylation by p300, a nuclear co-activator with intrinsic acetyltransferase activity, plays a key role in inflammatory responses in human lung epithelial cells [32]. However, the roles of C/EBP β acetylation in lung fibrosis are unclear. Hu et al. identified the binding of C/EBP β to the α -SMA promoter in rat lung fibroblasts [28]. We proposed that acetylation of C/EBP β determines the outcome of collagen-I expression and α -SMA activation in TGF- β -treated A549 cells. Our results showed that acetylation of C/EBP β were involved in TGF- β -induced EMT and lung fibrosis in vitro. Results of the luciferase reporter assay showed that C/EBP β activity was moderated by the extent of acetylation. Furthermore, using co-immunoprecipitation, the enhanced expression of α -SMA in TGF- β -treated A549 cells was shown to depend on C/EBP β

activation. To understand the importance of C/EBP β acetylation in α -SMA gene expression and lung fibrosis, we used SIRT1, a class III histone deacetylase, to construct a cellular model of C/EBP β deacetylation in vitro. We observed that enhanced C/EBP β acetylation and C/EBP β -luciferase activity caused by TGF- β treatment was reduced in A549 cells after SIRT1 administration. Probably, Sirt1 could deacetylate C/EBP β intracellularly through its ability to dynamically shuttle between cytoplasm and nucleus as reported in bronchial epithelial cells [33]. Nevertheless, our future study will further investigate the mechanism underlying cellular uptake of exogenous Sirt1. We further observed that the deacetylation of C/EBP β successfully limited α -SMA expression and collagen-I deposition. This inhibition of C/EBP β binding to the α -SMA promoter correlates with the suppressive effect of SIRT1 in A549 cells. Thus, this is the first study to suggest that the EMT and fibrosis-promoting roles of C/EBP β acetylation in A549 cells were attenuated by its deacetylation.

Conclusions

The present study showed that TGF- β could induce EMT, as evident from α -SMA expression and fibrosis in A549 cells, via C/EBP β activation. Furthermore, acetylation of C/EBP β could be a necessary step for binding to the α -SMA gene promoter and collagen-I deposition. Deacetylation of C/EBP β could serve as an effective approach for restraining EMT and lung fibrosis.

Abbreviations

C/EBP β - CCAAT/enhancer binding protein beta

IPF- Idiopathic Pulmonary Fibrosis

α -SMA- α -smooth muscle actin

UIP- Usual interstitial pneumonia

EMT- Epithelial-mesenchymal transition

ECM- Extracellular matrix

TGF- β - Transformed growth factor-beta

Col-I- Collagen-I

AECs- Alveolar epithelial cells

MAPK- Mitogen-activated protein kinase

PI3K- Phosphatidylinositol 3-kinase

siRNA- Interfering RNAs

PVDF- Polyvinylidene fluoride

ECL- Enhanced chemiluminescence

ChIP- Chromatin immunoprecipitation

SIRT1- Sirtuin1

HDAC- Histone deacetylase

Declarations

Ethics approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the Helsinki Declaration and its later amendments or comparable ethical standards.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declaration of Competing interest

The authors declare no conflict of interest.

Fundings

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Author Contribution statement

RH C and YZ L designed the experiments and contributed to the writing of the manuscript. HD carried out the study and partly wrote the manuscript. JZ C performed the figures. QJ P statistically analyzed the data. All the authors reviewed the manuscript.

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Figures

Figure 1

TGF- β induced EMT and deposition of collagen-I in A549 cells. (a) A549 cells were exposed to TGF- β (0–20 ng/ml) for 24 h. Protein levels of α -SMA and E-cadherin were determined using western blotting. In agreement with the elevated expression of α -SMA, E-cadherin expression decreased in a dose-dependent manner, compared to non-stimulated cells. (b) Expression of α -SMA and E-cadherin in A549 cells treated with TGF- β (10 ng/ml) for 0, 6, 12, 24, and 48 h was assessed using western blotting. (c) ELISA analysis of collagen-I levels in the supernatant of A549 treated with 10 ng/ml TGF- β for 24 h or controls. All data were from three separate experiments. Asterisk (*) denotes significant difference ($P < 0.05$).

Figure 2

Up-regulation of C/EBPβ is involved in TGF-β-induced EMT. (a) Real-time PCR analysis of C/EBPβ mRNA levels in A549 cells treated with 0–20 ng/ml TGF-β for 24 h. (b) C/EBPβ mRNA levels increased in a time-dependent manner of TGF-β (10 ng/ml) treatment (0–48 h) in A549 cells. (c) TGF-β increased C/EBPβ protein levels in A549 cells in a concentration- (0, 1, 2.5, 5, 10, and 20 ng/ml) dependent manner. (d) C/EBPβ expression increased gradually by 7.05 ± 0.81 folds by 24 h when TGF-β treatment was

prolonged. (e) Luciferase activity assay was performed to assess C/EBPβ promoter activity. Generation of reporter gene constructs controlled by the human α-SMA gene promoter. Reporter constructs were generated by inserting fragments of the α-SMA gene promoter into a luciferase reporter vector pGL4.10. The location of the C/EBPβ-binding motif is as indicated. Relative to the control, the activity of C/EBPβ-luciferase increased with TGF-β concentration and peaked at 10 ng/ml by 2.74 ± 0.42 -fold. (f) TGF-β up-regulated C/EBPβ-luciferase activity. Traces represent three experiments with similar results. Means $\pm$ SEM of data are shown. Asterisk (*) denotes significant difference ($P < 0.05$).

Figure 3

TGF-β up-regulated the expression of α-SMA via C/EBPβ activation in A549 cells. (a) TGF-β increased α-SMA protein levels via C/EBPβ activation. A549 cells were treated with TGF-β (10 ng/ml) for 24 h with or without the presence of C/EBPβ siRNA (10 nM) for 8 h. Levels of C/EBPβ and α-SMA in cell lysate were analysed using western blotting. (b) The C/EBPβ inhibitor blocked TGF-β-induced increase in collagen-I synthesis. The effect of C/EBPβ siRNA on collagen-I deposition in TGF-β-treated A549 cells was analysed using ELISA. (c) TGF-β-induced C/EBPβ-luciferase activity is regulated by the C/EBPβ siRNA. A549 cells transfected with C/EBPβ siRNA successfully attenuated TGF-β-activated C/EBPβ-luciferase activity. Asterisk (*) denotes significant difference ($P < 0.05$).

A

Human	-130	TCCAATGAGAGGCGATGGGGGAAGT	TTGGGCAA	CTCGGCCTGGCCAATGGAGAGCCTCG	-70
Mouse	-128	TCCAATGAGAGGCGCTGGGGGAAGT	TTGGGCAA	CTCGGCCTGGCCAATGGAGAACCTCG	-68
Rat	-128	TCCAATGAGAGGCGCTGGGGGAAGT	TTGGGCAA	CTCGGCCTGGCCAATAGAGAACCTCG	-68

*****. *****. ****. *****

B

Figure 4

TGF- β induced C/EBP β binding to α -SMA promoter in A549 cells. (A) The 5' promoter sequence of the human, mouse and rat α -SMA gene contains a putative C/EBP β -binding motif (TTGGGCAA) within 200 bp from the transcription start site. *Indicates conserved sequences. (B) A549 cells were exposed to TGF- β (10 ng/ml) for 3 h, followed by ChIP assay. ChIP primer pairs, yielding 237-bp PCR products, were designed to amplify DNA corresponding to the C/EBP β -binding site. A rabbit IgG clone was used as a negative control. Typical traces are representative of two experiments with similar results. Asterisk (*) denotes significant difference ($P < 0.05$) compared to the control.

Figure 5

Involvement of C/EBP β acetylation in TGF- β -induced EMT in A549 cells. Cell lysates were prepared from A549 cells treated with 10 ng/ml TGF- β for 24 h. After treatment with the C/EBP β antibody (described in "Methods"), C/EBP β acetylation was shown to be involved in TGF- β -induced α -SMA expression. However, elevated expression of acetylated C/EBP β and α -SMA proteins was not observed in A549 cells treated

with IgG during immunoprecipitation. Data show mean $\pm$ SEM. Asterisk (*) denotes significant difference ($P < 0.05$) compared to the control.

Figure 6

Roles of C/EBP β deacetylation in TGF- β -induced EMT and collagen deposition. (a) A549 cells were treated with TGF- β (10 ng/ml) for 24 h. Western blotting showing TGF- β treatment led to acetylation of C/EBP β and increased the levels of α -SMA proteins. (b) Deacetylation of C/EBP β via SIRT1 administered in A549 cells rectified TGF- β -induced elevation in collagen-I deposition. (c) SIRT1 suppressed the increased C/EBP β -luciferase activity in TGF- β -treated A549 cells. Data show mean $\pm$ SEM. Asterisk (*) denotes significant difference ($P < 0.05$) compared to the control.