

Loss of Family with Sequence Similarity 13, Member A Exacerbates Pulmonary Fibrosis Potentially by Promoting Epithelial to Mesenchymal Transition

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Idiopathic pulmonary fibrosis (IPF) is a devastating disease with poor prognosis due to limited clinical treatment options. IPF is characterized by the augmented deposition of extracellular matrix driven by myofibroblasts, and the epithelial-mesenchymal transition (EMT) has been known to play an essential role in the mechanism of pulmonary fibrosis. Previous genome-wide association study identified Fam13a as one of genes that showed genetic link with IPF and chronic obstructive pulmonary disease. Here, we analyzed the role of Fam13a in the pathogenesis of pulmonary fibrosis using Fam13a-deficient mice. We found that Fam13a was down-regulated in mouse lungs of bleomycin-induced pulmonary fibrosis model. Of note, genetic deletion of Fam13a exacerbated the lung fibrosis induced by bleomycin in association with enhanced EMT in mice. Moreover, silencing of Fam13a accelerated EMT induced by TGF- β and TNF- α in alveolar epithelial cells, accompanied by increased active β -catenin and its nuclear accumulation. Our data revealed a crucial role of Fam13a in the development of pulmonary fibrosis potentially through inhibiting EMT, and thus Fam13a has a therapeutic potential in the treatment of IPF.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most common type of interstitial lung disease with unknown etiology that occurs in elderly or middle-aged adults, which predominantly affect males than females (1-3). Chronic inflammation has been considered as the primary driver of IPF; however, many recent studies proved that repetitive injury to alveolar epithelial cells (AEC) was the main culprit that induces the generation of fibroblast and myofibroblast foci as the hallmark of IPF (2,4). The revelation that TGF- β plays a vital role in the mechanism of lung fibrosis by activating fibroblast and inducing epithelial-mesenchymal transition (EMT) has followed by numerous studies (5,6). EMT is a process by which the epithelial cells characters shift into mesenchymal cells phenotypes (7). Numerous studies reveal AEC as the origin of myofibroblast through EMT after TGF- β exposure (4).

Previous genome-wide association studies identified family with sequence similarity 13, member A (Fam13a) as one out of 7 newly associated loci that vulnerable to chronic obstructive pulmonary disease (COPD) and pulmonary fibrosis (8,9). A recent study also discovered the polymorphism of Fam13a in IPF patients, which affect the prognosis and susceptibility (10). It has been also reported that Fam13a is a modifier gene in cystic fibrosis by affecting RhoA function and EMT (11), and that genetic deletion of Fam13a protects mice from cigarette smokes-induced emphysema through modulating the β -catenin stability (12).

According to these findings, we hypothesized that Fam13a might be involved in the pathogenesis of IPF potentially through regulating the EMT. Here, we studied a role of Fam13a in pulmonary fibrosis using Fam13a-deficient mice.

MATERIAL AND METHODS

Materials

Recombinant human TGF- β and TNF- α were obtained from R&D systems. Small interfering RNA (siRNA) for FAM13a was purchased from Dharmacon (M-020516-0005). The antibodies used were; β -catenin (Cell Phone: +81-78-441-7537 Fax: +81-75-441-7538 E-mail: ikedak-circ@umin.ac.jp

LOSS OF FAM13A EXACERBATES PF BY PROMOTING EMT

Signaling Technology, D10A8), active β -catenin (Cell Signaling Technology, D13A1), Fam13a (SIGMA, HPA038108), phospho-smad 2/3 (Cell Signaling Technology, D27F4), total-smad 2/3 (Cell Signaling Technology, #3102S), E-cadherin (Cell Signaling Technology, 24E10), α -smooth muscle actin (Sigma, F3777), and β -actin (Cell Signaling Technology, #4970S).

Animal study

All experimental animal procedures were approved by the Ethics Review Committee for Animal Experimentation of Kobe Pharmaceutical University, Kobe, Japan.

Fam13a^{-/-} mice [Fam13a tm1e(KOMP)Wtsi; C57BL6N background] were obtained from Knockout Mouse Project (KOMP) at UC Davis. Mice were maintained under standard conditions with free access to food and water. To induce pulmonary fibrosis, 12 weeks old mice were anesthetized with isoflurane inhalation, and then 22-gauge needle was inserted into the trachea to instill bleomycin (10 μ g/g body weight; Jena Bioscience) in 60 μ L of 0.9% saline vehicle as previously reported (13). Sham control mice were received 60 μ L of the vehicle. Mice were later sacrificed 28 days after bleomycin instillation. Whole lung samples were collected for lung tissue section, western blot analysis, and quantitative PCR.

Quantitative PCR

RNAs were extracted from whole lung tissue and A549 cells using RNAiso plus (TAKARA), followed by purification using Nucleospin RNA clean-up kit (Macherey-Nagel). cDNA was made from \sim 1 μ g of total RNA using PrimeScript RT reagent Kit with gDNA Eraser (TAKARA). FastStart SYBR Green Master (Roche Applied Science) was used and followed by the real-time PCR analysis using LightCycler96 (Roche Applied Science). mRNA levels of target genes relative to 18S were analyzed. Nucleotide sequences of the primers used are shown in Table I.

Cell Culture

A549 cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% FBS and 1% antibiotic-antimycotic (Gibco). Epithelial-mesenchymal transition was induced by the treatment with TGF- β 1 (10 ng/mL) and TNF- α (20 ng/mL) for 24 hours as described previously (14,15).

A549 cells were transfected with 10 μ M siRNA when cells were at \sim 70% confluence using RNAiMax lipofectamine (Invitrogen).

Histopathology

Lung tissues were fixed in 4% paraformaldehyde and embedded with paraffin, followed by cutting into 5 μ m sections. Sections were stained with hematoxylin-eosin (H-E) for structure observation, or with Masson's trichrome for detection of collagen deposits. Ashcroft score was analyzed following previous study (3). For immunohistochemistry, lung sections were deparaffinized and treated with antigen unmasking solution (Vector Laboratories). Subsequently, sections were incubated with first antibodies at 4°C for overnight, followed by incubation with fluorescence-labeled secondary antibodies.

Immunocytochemistry

A549 cells were cultured on coverslips and fixed with 4% PFA. Subsequently, cells were incubated with 5% skim-milk in PBS containing 0.2% Triton-X for 1 h, followed by incubation with first antibody at 4°C for overnight. Secondary antibody labeled with Alexa Fluor 594 was used, and cells were co-stained with Wheat Germ Agglutinin conjugated with Alexa Fluor 488 to stain plasma membrane, followed by mounting with Vectashield mounting medium with DAPI (Vector Laboratories). The immunofluorescent images were captured under Zeiss LSM700 confocal laser scanning microscope.

Statistical analysis

All data are expressed as mean \pm SEM. The differences between groups were calculated for significance ($P < 0.05$) by two-tailed student's t-test or Mann-Whitney U-test. Comparison in three groups or more were analyzed with one-way ANOVA or Fisher's exact test using GraphPad Prism 7.

RESULTS

Genetic deletion of Fam13a deteriorates lung fibrosis induced by bleomycin in mice

We first explored the expressional regulation of Fam13a during pulmonary fibrosis (PF), and found that Fam13a was substantially reduced in the lungs of bleomycin-induced PF model comparing to that of saline-instilled control mice (Fig. 1A). We then analyzed lung histology in mice with targeted deletion of

Fam13a (Fam13a^{-/-}) that we previously generated (16). No apparent difference was detected in the lung histology assessed by H-E and Masson's trichrome staining between wild-type (WT) and Fam13a^{-/-} mice (Fig. 1B). To investigate a role of Fam13a in pathological PF, WT and Fam13a^{-/-} mice were subjected to intratracheal injection of bleomycin. Histopathology of the lungs was assessed through H-E and Masson's trichrome staining (Fig. 1C). Pulmonary fibrosis assessed by Ashcroft score analysis revealed that loss of Fam13a increased the lung fibrosis severity (Fig. 1D). Also, the fibrosis area in the lungs was significantly augmented in Fam13a^{-/-} mice comparing to that in WT mice (Fig. 1E). These data indicate that loss of Fam13a exacerbates PF, and thus Fam13a should play a protective role in the progression of PF.

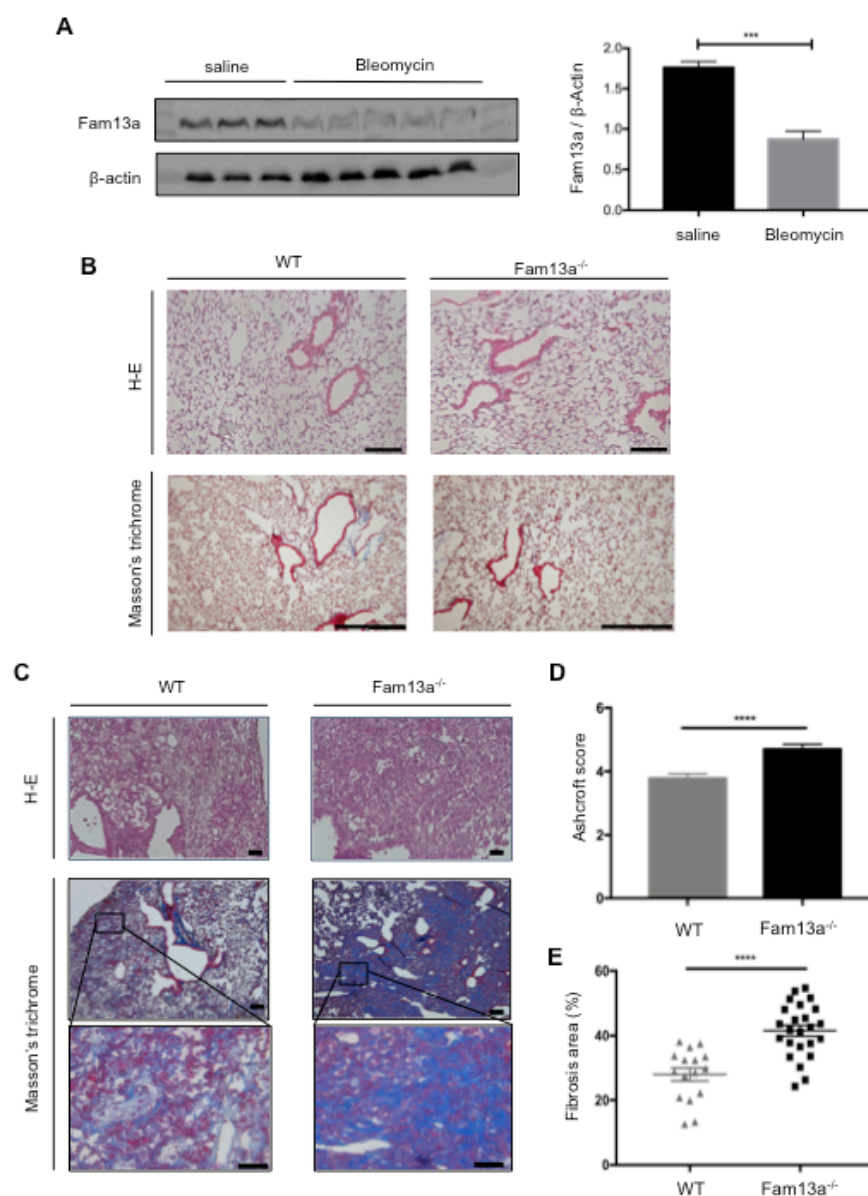


Figure 1. Genetic deletion of Fam13a deteriorates pulmonary fibrosis. (A) WT mice were intubated and injected with single-dose bleomycin intratracheally to induce pulmonary fibrosis. Control mice were injected with saline. Fam13a expression in whole lung samples was analyzed by immunoblotting ($n = 3$ for saline-group; $n = 5$ for bleomycin-group). (B) The lungs were isolated from saline-treated WT and Fam13a^{-/-} mice. Representative images of lung sections stained with H-E and Masson's trichrome were shown. Bars: 100 μm . (C) The lungs were isolated from WT and Fam13a^{-/-} mice after 4 weeks of bleomycin instillation. Representative images of lung sections stained with H-E and Masson's trichrome were shown. Bars: 100 μm . (D) Lung fibrosis severity was assessed by using the Ashcroft score ($n = 7-9$). (E) The collagen fiber area was quantified in lung sections stained with Masson's trichrome ($n = 7-8$). The values were expressed as mean \pm SEM. *** $P < 0.001$ and **** $P < 0.0001$.

Loss of Fam13a accelerates EMT in association with increased active β -catenin

We then analyzed the appearance of myofibroblasts or mesenchymal-like cells in the lung fibrotic lesion (17). Fam13a^{-/-} mice showed an enormous amount of α -SMA-positive myofibroblast in the lungs after bleomycin-treatment comparing to that in WT mice (Fig. 2A). Since Fam13a has been reported to modulate EMT in cystic fibrosis (11), we assessed the EMT in the lungs by immunohistochemistry for epithelial and mesenchymal markers. Cells positive for both markers were considered to be cells undergoing EMT. EMT was significantly enhanced in the lungs of Fam13a^{-/-} mice comparing to that in WT mice (Fig. 2B and 2C). Consistently, gene expressions for mesenchymal markers were largely increased, while epithelial marker genes expressions showed tendency toward decrease in the lungs of Fam13a^{-/-} mice (Fig. 2D). Fam13a has been reported to promote the GSK-3 β -mediated β -catenin degradation (12), while β -catenin has been involved in the EMT process (18-21). We found that non-phosphorylated active β -catenin was increased, whereas total

LOSS OF FAM13A EXACERBATES PF BY PROMOTING EMT

β -catenin levels did not change in the lungs of *Fam13a*^{-/-} mice comparing to those in WT mice (Fig. 2E). These data strongly suggest that *Fam13a* protects the lung from fibrosis potentially by inhibiting β -catenin-mediated EMT in the lungs. In contrast, active β -catenin was not increased in the lungs of *Fam13a*^{-/-} mice treated with saline (Fig. 2F). Therefore, *Fam13a* is unlikely an indispensable regulator for β -catenin signaling at least under physiological condition.

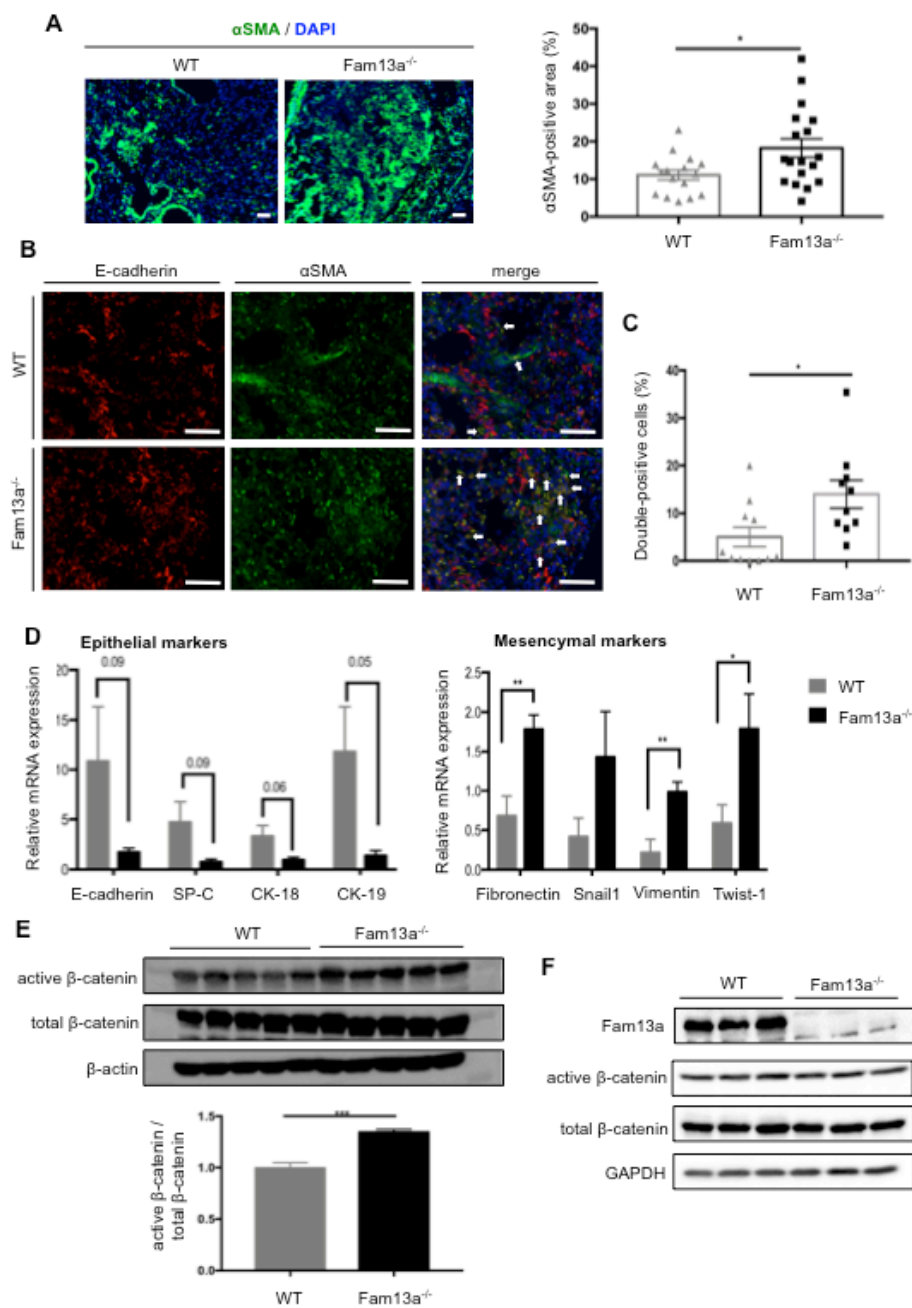


Figure 2. Loss of *Fam13a* enhances EMT and accentuates myfibroblast appearance in the lungs after bleomycin instillation. (A) Representative images of lung immunohistochemistry for α -SMA were shown. Bars: 100 μ m. α -SMA staining-positive areas were quantified (n = 7–8). (B) Immunohistochemistry for E-cadherin (red) and α -SMA (green) in the lungs. Bars: 100 μ m. Arrows indicates the cells double-positive cells for epithelial and mesenchymal markers. (C) Cells undergoing EMT were quantitatively analyzed (n = 3–5). (D) mRNA expression for epithelial markers such as E-cadherin, surfactant protein-C (SP-C), cytokeratin 18 and 19 (CK18 and CK19), and mesenchymal markers such as fibronectin, snail1, vimentin and twist-1 was quantitatively analyzed in whole lung samples isolated from bleomycin-treated WT and *Fam13a*^{-/-} mice (n = 5–8). (E) Immunoblotting for active β -catenin, β -catenin, and β -actin in whole lung samples isolated from bleomycin-treated WT and *Fam13a*^{-/-} mice (n = 5 each). (F) Immunoblotting for *Fam13a*, active β -catenin, β -catenin, and GAPDH in whole lung samples isolated from saline-treated WT and

Fam13a^{-/-} mice. The value was expressed as mean \pm SEM. *P < 0.05, **P < 0.01, and ***P < 0.001.

Silencing of *Fam13a* accelerates EMT in alveolar epithelial A549 cells

We further explored the role of *Fam13a* in EMT process using alveolar epithelial A549 cells. When EMT was induced by the treatment with TGF- β and TNF- α , *Fam13a* was significantly reduced in A549 cells (Fig. 3A). EMT induction was confirmed by the reduction of E-cadherin and induction of fibronectin in these cells (Fig. 3B). We then analyzed effects of gene silencing of *Fam13a* on the EMT process. Successful gene silencing was confirmed by immunoblotting (Fig. 3C). A549 cells appeared to be cuboidal-shaped, and *Fam13a*-silencing did not affect the cell morphology under basal condition (Fig. 3D). When treated with TGF- β and TNF- α , A549 cells showed a spindle-shaped morphology, and this morphological change was more apparent when *Fam13a* was knocked down (Fig. 3D). We then quantified the expression levels of epithelial and mesenchymal marker

genes at different time points after the EMT induction. Reduction of E-cadherin and induction of fibronectin was significantly enhanced in A549 cells by Fam13a-silencing, although the difference was relatively small (Fig. 3E). These data collectively indicate that loss of Fam13a accelerates the EMT in alveolar epithelial cells.

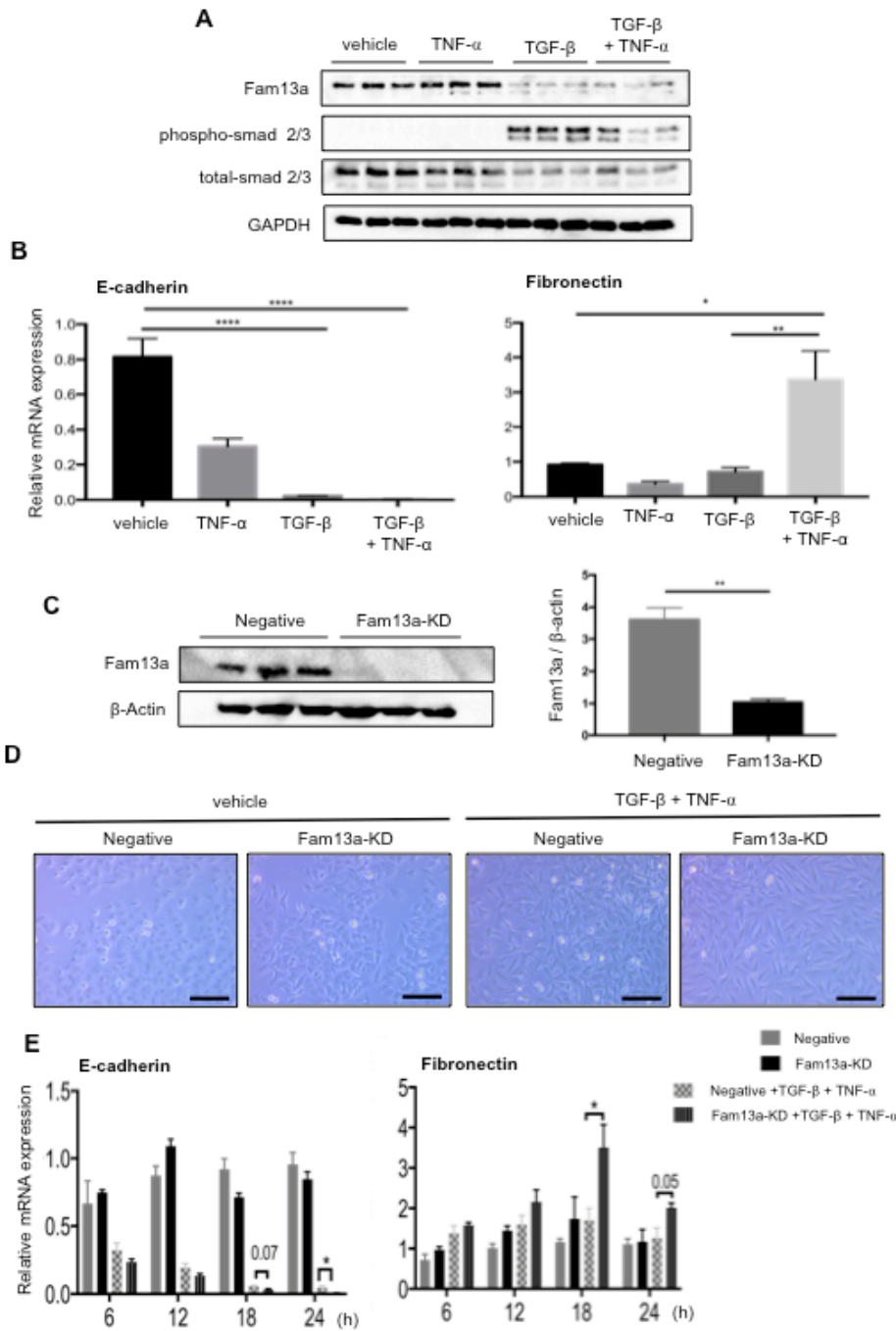


Figure 3. Gene silencing for Fam13a accelerates EMT in alveolar A549 cells. (A) A549 cells were treated with 4 different treatment; vehicle, TNF- α (20 ng/mL), TGF- β 1 (10 ng/mL), and combination of TNF- α (20 ng/mL) and TGF- β 1 (10 ng/mL). After 24 h, the protein was collected and analyzed for Fam13a, phospho- and total-smad 2/3 and GAPDH by immunoblotting. (B) mRNA expression for epithelial (E-cadherin) and mesenchymal (fibronectin) marker was quantitatively analyzed in A549 cells treated with TNF- α and/or TGF- β 1 (n = 3 each). (C) Immunoblotting for Fam13a in A549 cells transfected with either negative or Fam13a siRNA (Fam13a-KD). (D) EMT was induced by TNF- α and TGF- β 1 in A549 cells transfected with either negative or Fam13a siRNA (Fam13a-KD). Morphological changes associated with EMT were observed under microscopy. Bars: 100 μ m. (E) mRNA expression for epithelial (E-cadherin) and mesenchymal (fibronectin) marker was quantitatively analyzed in A549 cells transfected with either negative or Fam13a siRNA (Fam13a-KD) at indicated times after the treatment with TGF- β 1 and TNF- α (n = 3 each). The values were expressed as mean \pm SEM. *P < 0.05, **P < 0.01, and ****P < 0.0001.

Loss of Fam13a increases active β -catenin and its accumulation in the nucleus

β -catenin is a transcriptional co-activator that is crucially involved in the EMT process (22,23). β -catenin activity is mainly regulated by Wnt signaling (22,24); Wnt activation leads to increase of non-phosphorylated active β -catenin in the cytosol, which enters and accumulates in the nucleus to induce transcription of target genes. Consistent with the finding that active β -catenin increased in the lungs of Fam13a^{-/-} mice after bleomycin instillation, silencing of Fam13a increased active β -catenin without increasing total β -catenin protein levels in A549 cells (Fig. 4A). We then investigated the subcellular localization of active β -catenin in A549 cells using immunocytochemistry. Active β -catenin was mostly detected on the membrane of the control cells, whereas significant active β -catenin accumulation in the nucleus was detected in cells in which Fam13a was knocked down (Fig. 4B). When EMT was induced, nuclear accumulation of active β -catenin was detected in control

LOSS OF FAM13A EXACERBATES PF BY PROMOTING EMT

A549 cells as well (Fig. 4B and 4C), and this nuclear accumulation of active β -catenin associated with EMT was significantly augmented by Fam13a-silencing (Fig. 4B and 4C). Considering a crucial role of β -catenin in the EMT process, Fam13a might inhibit the EMT by promoting the inactivation of β -catenin in alveolar epithelial cells.

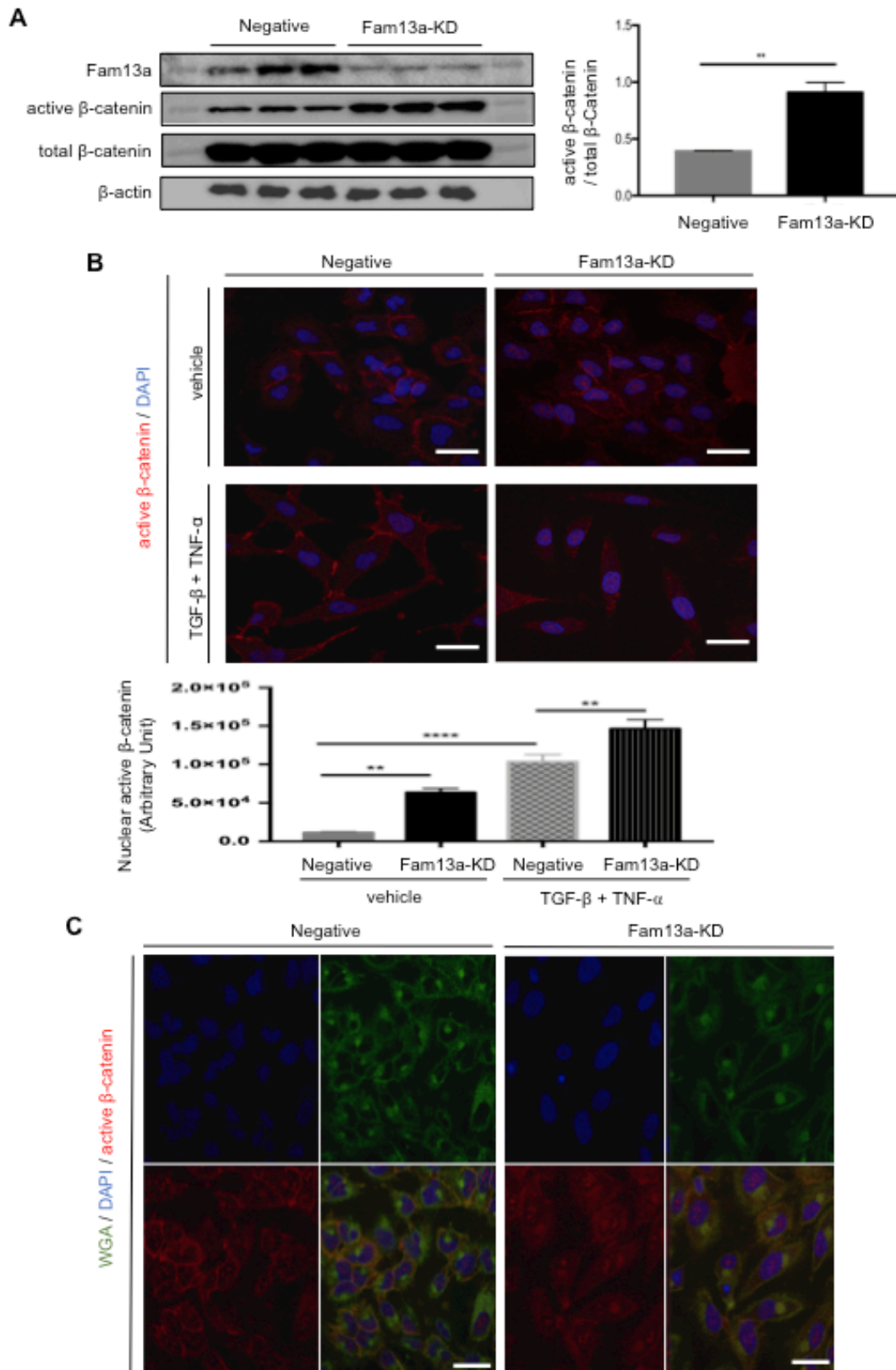


Figure 4. Loss of Fam13a increases active β -catenin, and enhances its nuclear localization. (A) Immunoblotting for Fam13a, active β -catenin, β -catenin, and β -actin in A549 cells transfected with either negative or Fam13a siRNA (Fam13a-KD). (B) A549 cells were transfected with either negative or Fam13a siRNA (Fam13a-KD). Cells were then treated with either vehicle or TGF- β 1 and TNF- α , followed by observation under fluorescence microscopy. Representative images of the cells immunostained for active β -catenin were shown. Nuclei were stained with DAPI. Bars: 50 μ m. Nuclear accumulation of active β -catenin was quantitatively analyzed. The intensity of staining obtained with anti-active β -catenin antibody was measured in each group (>20 cells each). Quantification of fluorescence was analyzed by Image J software with nuclear demarcation. (C) A549 cells were transfected with either negative or Fam13a siRNA (Fam13a-KD). Cells were then treated with TGF- β 1 and TNF- α , followed by observation under confocal laser scanning microscopy. Representative images of the cells immunostained for active β -catenin were shown. Plasma membrane and nuclei were stained with Wheat Germ Agglutinin (green) and DAPI, respectively. Bars: 50 μ m. The values were expressed as mean \pm SEM. **P < 0.01 and ****P < 0.0001.

DISCUSSION

Previous studies revealed a strong association of Fam13a in various lung diseases such as cystic fibrosis, COPD, and pulmonary fibrosis (10-12,16,25,26). It has been reported that Fam13a is expressed in the lung, especially in mucosal cells, club cells, airway epithelial cells, and alveolar type II cells (11,12). To our knowledge, there was no report investigating a role of Fam13a in the pathogenesis of PF *in vivo* using genetically modified mice. In the current study, we found that Fam13a was down-regulated in the lungs of bleomycin-induced PF model mice. We further analyzed a role of Fam13a in the pathogenesis of pulmonary fibrosis using Fam13a-deficient mice, and revealed a protective role of Fam13a against PF through inhibiting the EMT.

EMT is a reversible process of an epithelial cell that gains the mesenchymal marker and progressively lose the epithelial characteristics (27), and it contributes around 36% as the source of myofibroblasts in lung fibrosis (28). Our study consistently showed enhanced EMT in association with exacerbated PF in Fam13a-deficient mice. Also, our *in vitro* studies using A549 cells strongly support an inhibitory role of Fam13a in the EMT process. TGF- β is a master regulator for the EMT process, and Fam13a was diminished by TGF- β treatment in A549 cells. These results collectively suggest that the down-regulation of Fam13a is crucially involved in the TGF- β -mediated EMT process, while mechanisms underlying the TGF- β -mediated Fam13a down-regulation remain to be elucidated.

The previous study reported that Fam13a promotes β -catenin degradation through enhancing its phosphorylation by GSK-3 β and thus loss of Fam13a increased β -catenin protein levels (12). We found that gene silencing of Fam13a increased non-phosphorylated active β -catenin both *in vitro* and *in vivo*; however, protein levels of total β -catenin did not increase in our study. These differences could be accounted for the difference in mouse disease models *in vivo*, and for the different experimental conditions *in vitro*. In addition, we observed that loss of Fam13a enhanced nuclear accumulation of active β -catenin. We presume that increased active β -catenin protein levels in cytosol causes its enhanced nuclear accumulation; however, it remains unclear whether Fam13a could actively regulate the β -catenin nuclear translocation.

Our present study demonstrated that the loss of Fam13a exaggerated bleomycin-induced PF potentially through activating β -catenin pathway, which accelerates the EMT. Therefore, Fam13a in lung epithelial cells is an attractive pharmacotherapeutic target for the prevention and treatment of IPF.

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Table I. Nucleotide sequence of primers

Human E-cadherin	TGCACCAACCCTCATGAGTG GTCAGTATCAGCCGCTTTCAG
Human Fibronectin	TCGCCATCAGTAGAAGGTAGCA TGTTATACTGAACACCAGGTTGCA
Mouse E-cadherin	GACCACTATGCATGCTGCACACGTC TGGCACTCCAGTCTCTCTCAGAGAAC
Mouse CK-18	TACAAGGCACAGATGGAGCA ACGGGTAGTTGTCTTCTGCA
Mouse CK-19	GGGCTGTGTCTGGCAATGAG CGCAAGGCGTGTCTGTCTCA
Mouse SP-C	CCTCAAACGCCTTCTATCG CAGTGGAGCCGATGGAAAAG
Mouse α -SMA	ACAGAGGCACCACTGAACCCTAAG ACAATCTCACGCTCGGCAGTAGTC

LOSS OF FAM13A EXACERBATES PF BY PROMOTING EMT

Mouse TGF β	AGCCCGAAGCGGACTACTAT
	TCCACATGTTGCTCCACACT
Mouse Collagen 1A1	CCGTGCTTCTCAGAACATCA
	AGCATCCATCTTGCAGCCTTG
Mouse Collagen 3A1	TGCTCGGAACTGCAGAGACCTA
	AGCATCCATCTTGCAGCCTTG
Mouse Fibronectin	TGTGACAACTGCCGTAGACC
	GACCAACTGTCACCATTGAGG
Mouse Snail-1	CGAGCTGCAGGATTCTA
	GTGGGATGGCTGCCAGC
Mouse Vimentin	TAGCAGGACACTATTGGCCG
	CTGTTGCACCAAGTGTGTGC
Mouse Twist-1	ACGCAGTCGCTGAACGAGGCGTTCG
	GCTCTGCAGGACCTGGTACAGGAAG
18S	CCATCCAATCGGTAGTAGCCG
	GTAACCCGTTGAACCCATT