Downregulation of S100A2 alleviates pulmonary fibrosis through inhibiting wnt/β-catenin signaling pathway

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Abstract

Background Pulmonary fibrosis (PF) is a progressive and lethal disease with poor prognosis. S100A2 plays an important role in the progression of cancer. However, the role of S100A2 in PF has not been reported yet. In this study, we explored the potential role of S100A2 in PF and its potential molecular mechanisms.

Methods: First, we analyzed S100A2 expression of patients with PF by retrieving RNA-sequencing datasets from Gene Expression Omnibus (GEO) database. Next, we detected the expression of S100A2 in patients with PF using quantitative real time PCR (qRT-PCR). Then, S100A2 expression was determined with or without the treatment of transforming growth factor-β1 (TGF-β1) in A549 cells. Epithelial-mesenchymal transition (EMT) biomarkers, including E-cadherin vimentin, and α smooth muscle actin (α-SMA), were identified using qRT-PCR and western blot. Finally, the relevant signalling pathway indicators were detected by western blot.

Results: Increased expression of S100A2 was first observed in lung tissues of PF patients. Meanwhile, we found that downregulation of S100A2 inhibited the TGF-β1-induced EMT in A549 cells. Mechanically, TGF-β1 up-regulated β-catenin and phosphorylation of GSK-3β, which was blocked by silencing S100A2 in vitro.

Conclusion: These findings demonstrate that downregulation of S100A2 alleviate pulmonary fibrosis via inhibiting EMT. S100A2 is a promising potential target for further understanding the mechanism and developing strategy for the treatment of PF and other EMT-associated disease.

Background

Pulmonary fibrosis (PF) is a common outcome of various interstitial lung diseases, including pneumoconiosis, drug-induced fibrosis and idiopathic pulmonary fibrosis[1, 2]. PF is characterized by alveolar epithelial cell injury, and the excessive proliferation of mesenchymal cells in the interstitium, which leads to the exaggerated accumulation of extracellular matrix and distorted lung architecture. Although we have made great progress in the diagnosis of and treatment for pulmonary fibrosis in recent years, the survival rate has not been improved[3].Therefore, it is crucial to explore the precise molecular mechanisms involved in the development of PF and to determine targets for the diagnosis and treatment of PF.

Epithelial-mesenchymal transition (EMT) is a process in which epithelial cells are transformed into a mesenchymal phenotype, with the loss of contact adhesion and apical-basal polarity, acquisition of mesenchymal property of invasion, migration and production of extracellular matrix (ECM)[4]. An increasing number of studies has shown that EMT plays an important role in the pathogenesis of PF[5]. Additionally, transforming growth factor-β1 (TGF-β1) is a key profibrotic factor that has been implicated to induce EMT during PF[6].

The S100 protein family is a group of high conserved elongation factor-hand calcium-binding proteins[7]. S100A2 is an important member of S100 protein family and has been involved in various cancers [8]. Sarwat et al reported that S100A2 promote the occurrence of cancer via regulating PI3K/Akt signaling and functional interaction with Smad3[9]. Additionally, a study indicated that the p53-S100A2 positive feedback loop negatively regulates epithelialization in cutaneous wound healing[10]. However, the role of S100A2 in fibrosis, including PF, has not been investigated.

In present study, we demonstrated that S100A2 was increased in lung tissues of patients with PF. Downregulation of S100A2 inhibited TGF-β1-indced EMT in human type II alveolar epithelial cells via blocking wnt/β-catenin pathway in vitro. These results improved the understanding of the pathological mechanisms of PF.

Methods

Data collection from GEO

We searched microarray data of S100A2 in the GEO database (http://www.ncbi.nlm.nih.gov/geo/). The following keywords were used: (lung fibrosis OR pulmonary fibrosis. The search results were further restricted as following: Series [Entry type] and Homo sapiens [Organism]. Inclusion criteria for microarrays were as follows: (1) dataset included fibrosis and non-fibrosis tissues; (2) the number of samples in fibrosis and non-fibrosis tissues was at least two; (3) dataset provided the expression values of S100A2 in fibrosis and non-fibrosis tissues.

Tissues, Cell line, and Cell culture

Twelve PF tissues were obtained from patients undergoing lung biopsy. A total of 17 control lung tissues were collected from the normal areas of the peripheral lung removed at lung cancer resection. All tissues were obtained at the First Affiliated Hospital of Chongqing Medical University.

The human type II alveolar epithelial cell line, A549, was purchased from the Cell Bank of the Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (PAN), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) at 37℃ in a 5% CO2 atmosphere. A549 cells were treated with recombinant human TGF-β1 (R&D Systems) at final concentration of 10 ng/ml for 48 h. Then, cells were collected for further analysis.

Lentivirus transfection

To establish stable genetic silencing of S100A2, lentivirus was utilized as a vector to carry the interference sequence. The lentivirus vectors inserted with the target gene or negative control were constructed by Hanbio Biotechnology Company (Shanghai, China). Then, lentivirus was added to 2 ml complete medium supplemented with a final concentration of 5 μg/ml polybrene. After 24hr, the medium was replaced with fresh complete medium without lentivirus and polybrene to culture for another 48hr.

Total RNA and protein were collected to confirm the transfection efficiency using qRT-PCR and western blot.

Quantitative RT-PCR

Total RNA was extracted from the lung tissues or A549 cells using TRIzol reagent (Invitrogen, Carlsbad, CA). The concentration of RNA was measured with the use of the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Then, 1000 ng of total RNA was reverse-transcribed using the PrimeScript RT reagent kit (TaKaRa). Subsequently, cDNA was amplified by SYBR Premix Ex Taq (TaKaRa, Dalian, China). The relative expression levels of mRNA were normalized to the levels of GAPDH and calculated by the $2^{-\triangle\triangle CT}$ method. The primers used in this study were listed as follows: α-SMA, forward, 5'-CCTGTATGCCAACAACGTCA-3', reverse, 5'- CTCGTCGTACTCCTGCTTGG-3'; S100A2, forward, 5'-ACCGACCC TGAAGCAGAACTC-3', reverse, 5'-CCTCATCTCCCAGCACTCCA-3';

E-cadherin, forward, 5'-CGATTCAAAGTGGGCACAGATG-3', reverse, 5'-GTAGG TGGAGTCCCAGGCGTAG-3'; vimentin, forward, 5'-TCTGGATTCACTCCCTCT GCTT-3', reverse, 5'-ATCGTGATGCTGAGAAGTTTCGT-3', and GAPDH, forward, 5'-CTTTGGTA TCGTGGAAGGACTC-3', reverse, 5'-GTAGAGGCAGG GATGATGTTCT-3'.

Western blot

Cells were lysed in RIPA lysis buffer containing protease inhibitor cocktail, 1% phenylmethanesulfonyl fluoride (PMSF) and 1% phosphatase inhibitor. Protein concentration was determined with a BCA kit (Beyotime, Biotechnology, China). Equal amounts of protein were separated with 10% SDS-PAGE gel and transferred to polyvinylidene fluoride membranes. After blocking in 5% non-fat milk for 2 h, the membranes were incubated with primary antibodies overnight at 4℃. The membranes were incubated in TBST supplemented with secondary antibody for 1 h at room temperature. In this experiment, the following antibodies were used: anti-p-GSK3β (1:1000, Affinity Biosciences), anti-GSK3β (1:500, Affinity Biosciences), anti-E-cadherin (1:10000, Abcam), anti-vimentin (1:3000, Abcam), anti-α-SMA (1:2000 Abcam), anti-S100A2 (1:5000, Abcam), anti-GAPDH (1:10000, Abcam), and anti-β-catenin (1:5000, Abcam).

Statistical analysis

Statistical analyses were performed with SPSS 24.0. All data was expressed as the mean ± standard deviation. Data was analyzed using independent-samples Student's t test between two groups and one-way analysis of variance for more groups with Dunnett's or LSD post hoc test. We carried out a comprehensive meta-analysis using STATA 12.0. Continuous outcomes were presented as standard mean difference (SMD) with 95% confidence interval (CI). The chi-squared test and I^2 were used to evaluate the heterogeneity among the studies. A random-effect model was applied if there was heterogeneity among the studies ($I^2 \geq 50\%$ or $P_{\text{heterogeneity}} < 0.05$). On the contrary, if $I^2 < 50\%$ or $P_{\text{heterogeneity}} > 0.05$, a fixed-effect model was employed. A value of $P < 0.05$ was considered statistically significant.

Results

S100A2 expression was increased in pulmonary fibrosis obtained from GEO database

A total of 14 GEO series (GSE) from Gene Expression Ominbus (GEO) database met the inclusion criteria. However, only 8 GSE provided the S100A2 expression data. As shown in Supplementary Table 1, the detail information of S100A2 expression data from PF and control groups were extracted based on GEO database. A total of 168 patients with PF were included. As can be seen in Fig. 1, PF groups had a significant higher expression level of S100A2 than control groups in GSE24206, GSE110147, GSE53845, GSE10667, and GSE72073. Although no statistical differences were observed in S100A2 expression level between PF groups and control groups in GSE21369, GSE2052 and GSE35145 (Fig. 1F, G, H), we found that PF groups also had a higher S100A2 expression than control groups.

Owing to different results existed about S100A2 expression in PF based on GEO database. Thus, we conducted a meta-analysis using the data of S100A2 expression level from GEO database. Owing to I^2 = 0.0% and $P_{heterogeneity}$ >0.05, a fixed model was applied. As shown in Fig. 1I, the combined results revealed that the expression of S100A2 was significantly higher in PF group than in control group (SMD = 1.06, 95% CI: 0.74 to 1.38, P<0.001).

Validation of differential S100A2 expression in pulmonary fibrosis in humans both in vivo and in vitro

To further confirm the differential expression of S100A2 during lung fibrosis, we compared its expression in lung fibrosis tissues from PF patients (n = 12) with control(n = 17). The detail of information for PF patients was listed in Supplementary Table 2. As shown in Fig. 2A, the expression of S100A2 in lung tissues was significantly increased in PF patients by means of qRT-PCR, compared with control groups (P<0.001). Additionally, we examined S100A2 expression in A549 cells following TGF-β1 treatment. As shown in Fig. 2B, the mRNA expression of S100A2 was significantly increased in TGF-β1-stimulated A549 cells. Furthermore, the S100A2 expression in A549 was also evaluated by western blot analysis (Fig. 2C and 2D). The protein expression of S100A2 was obviously overexpressed in A549 cells with TGF-β1 treatment. Taken together, these findings indicated that S100A2 was upregulated both in PF tissues and in A549 cells.

Downregulation of S100A2 inhibits TGF-β1-induced EMT in A549 cells

EMT is featured with the loss of the epithelial cell marker E-cadherin and the overexpressed expression of the mesenchymal markers vimentin and α-SMA. TGF-β1 is one of the key regulators of pulmonary fibrosis and induces EMT of alveolar epithelial cells (AECs) in vitro[11]. To investigate the effect of S100A2 on

TGF-β1-stimulate EMT in A549 cells, S100A2 gene expression knockdown was performed in A549 cells and then stimulated with TGF-β1. First, the knockdown efficiency of S100A2 was performed with the use of qRT-PCR and western blot assay. As shown in Supplementary Fig. 1, the mRNA and protein expression of S100A2 was dramatically downregulated in the shRNA-S100A2 group compared with the shRNA-NC group. Downregulation of S100A2 obviously increased TGF-β1-induced expression of E-cadherin and reduced the expression of vimentin and α-SMA in A549 cells both at the mRNA and protein level (Fig. 3A-3F),suggesting that S100A2 knockdown inhibited TGF-β1-induced EMT.

Deletion of S100A2 suppresses TGF-β1-induced EMT in A549 cells via wnt/β-catenin signaling pathway

To determine whether S100A2 mediated TGF-β1-induced EMT via wnt/β-catenin pathway,control and silencing S100A2 A549 cells were treated with TGF-β1 and β-catenin/GSK-3β protein expression levels were detected using western blot. Knockdown of S100A2 suppressed TGF-β1-induced β-catenin and phosphorylation of GSK-3β, and the protein levels of non-phosphorylated GSK-3β remained unchanged in each group (Fig. 4). These results demonstrated that S100A2 blocked TGF-β1-induced EMT through downregulation of β-catenin pathway.

Discussion

Pulmonary fibrosis is characterized by progressive dyspnea, and patients eventually died from respiratory failure. The pathologic mechanisms of PF is not completely clear and effective therapy has not been well developed. Therefore, it is crucial to explore the precise molecular mechanisms involved in the development of PF and to determine target for treatment of PF.

In this study, we explored the expression, regulation, and potential role of S100A2 in PF. First, we studied and combined the S100A2 expression of PF patients from GEO database. The pooled results from the GEO database showed that the expression of S100A2 in PF lung tissues was significantly higher than in normal lung tissues, which demonstrated that S100A2 may be involved in PF. Furthermore, we confirmed the increased expression of S100A2 in patients with PF using the qRT-PCR assay. Further studies indicated that knockdown of S100A2 suppressed TGF-β1-induced EMT in A549 cells. Moreover, our study shown that downregulation of S100A2 inhibited EMT via wnt/β-catenin pathway. These findings indicated that S100A2 was correlated with the development of PF and may be serve as a new target for the prevention and treatment of PF.

S100A2 is widely studied in various cancers. Many studies indicate that there is a certain similarity between tumor metastasis and fibrotic diseases. For instance, dysregulated TGF-β1 pathway and activated EMT process are involved in these two diseases. Downregulation of S100A2 was detected in gastric cancer, and oral squamous cell cancer[8]. However, increased S100A2 expression was observed in ovarian cancer[12], bladder cancer[13], and non-small cell lung cancer[14]. Here, our data showed the first evidence that the expression of S100A2 was increased in PF lung tissue.

EMT is involved in the pathogenesis of fibrotic diseases in many organs including lung. TGF-β1 is consider as a key profibrotic cytokine and a regulator of EMT. Here, we employed TGF-β1 to build an EMT model using A549 cell lines. A549 is usually used as a replacement for primary AECs because of AECs are difficult to obtain and maintain in culture ex vivo. In our study, we demonstrated that downregulation of S100A2 inhibited the TGF-β1-induced EMT.

Wnt/β-catenin signaling pathway plays an important role in the pathological processes involving pulmonary fibrosis, and suppression of wnt/β-catenin signaling could inhibit the development of pulmonary fibrosis[15–17]. GSK3β is a serine/threonine protein kinase, which promotes fibrogenic activity as a partaker in execution of wnt/β-catenin pathway[18–20]. In our current study, we discovered that inhibition of S100A2 could restrain EMT by inhibiting the wnt/β-catenin pathway.

Conclusion

In conclusion, we demonstrate that the expression of S100A2 was increased in the lung tissues in the patients with PF. Inhibition of S100A2 can attenuate TGF-β1-induced EMT. S100A2 is a promising potential target for further understanding the mechanism and developing strategy for the treatment of PF and other EMT-associated disease.

Abbreviations

PF, pulmonary fibrosis; GEO, Gene Expression Omnibus; qRT-PCR, quantitative real time PCR; TGF-β1, transforming growth factor-β1; EMT, epithelial-mesenchymal transition; ECM, extracellular matrix; SMD, standard mean difference; CI, confidence interval; GSE, GEO series; α-SMA, α-smooth muscle actin; Ctrl, control; T, TGF-β1; shRNA-NC, short harpin RNA-negative control; AECs, alveolar epithelial cells.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Guichuan Huang performed the experiments and wrote the manuscript; Jing Zhang and Daishun Liu conducted the statistical analysis; Gang Qing, Xin Wang, Yi chen, and Yishi Li collected the samples of patients with pulmonary fibrosis. Shuliang Guo conceived and designed the experiments. All authors have read and approved the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article

Ethics approval and consent to participate

The study protocol was approved by the Institutional Review Board of the first affiliated hospital of Chongqing Medical University. Each patient provided a written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Supplementary Files

Supplementary Figure 1. The knockdown efficiency of S100A2 was determined by qRT-PCR (A) and western blot (B and C). *P<0.05 versus control; ** P<0.01 versus control.

Abbreviations: Ctrl, control; TGF-β1, transforming growth factor-β1; T, TGF-β1; shRNA-NC, short harpin RNA-negative control.

Supplementary Table 1. Characteristics of S100A2 expression profile datasets obtained from GEO

Abbreviations: GEO, Gene Expression Omnibus; PF, pulmonary fibrosis; SD, standard deviation.

Supplementary Table 2. Clinical features of patients and control people

Abbreviation PF, pulmonary fibrosis.

Figures

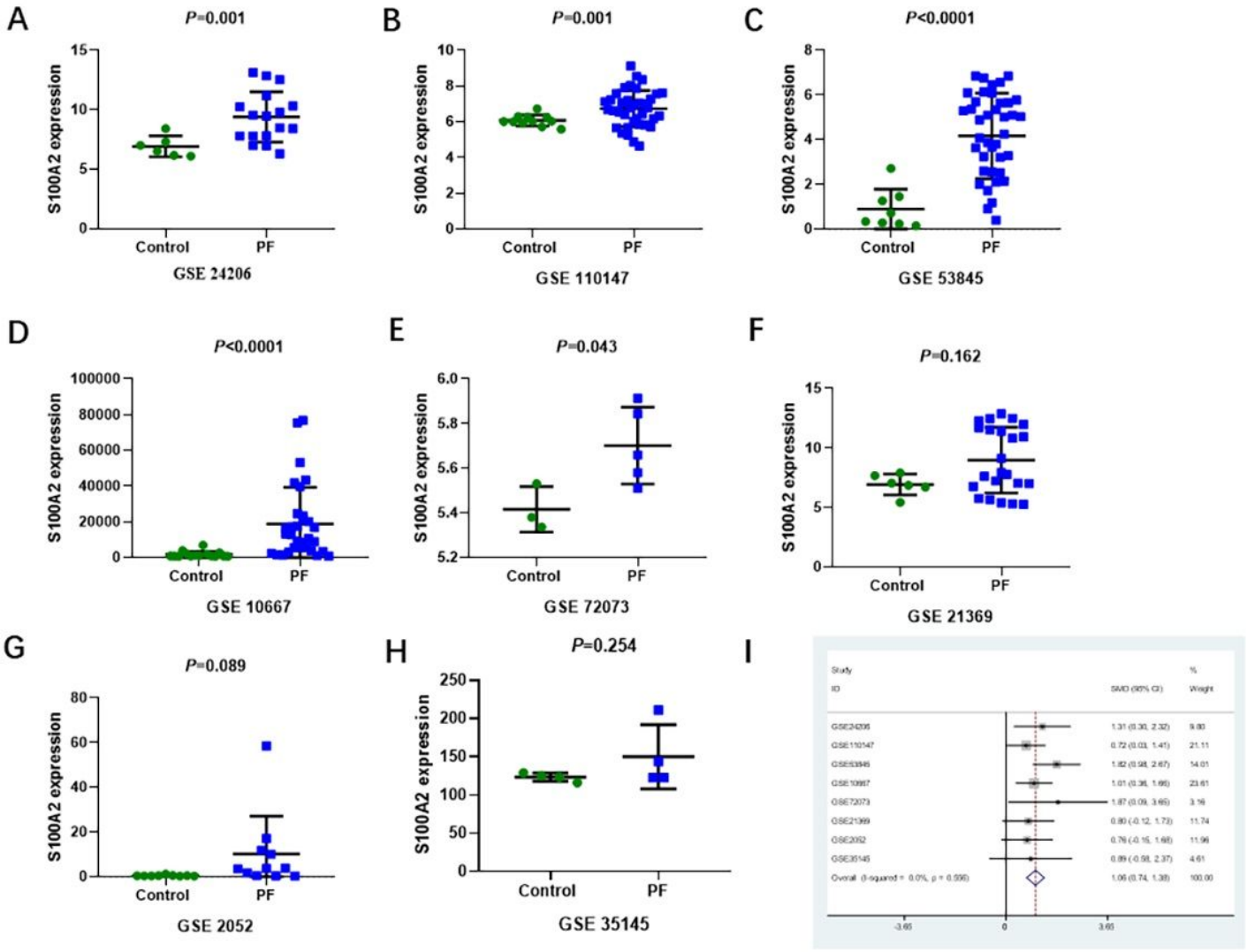

Figure 1

Expression levels of S100A2 in PF and control lung tissues from the GEO. Notes: (A) GSE24206, (B) GSE110147, (C), GSE53845, (D), GSE10667, (E), GSE72073, (F), GSE21369, (G), GSE2052, (H), GSE35145, (I) meta-analysis for all datasets. Abbreviations: PF, pulmonary fibrosis; GEO, Gene Expression Omnibus.

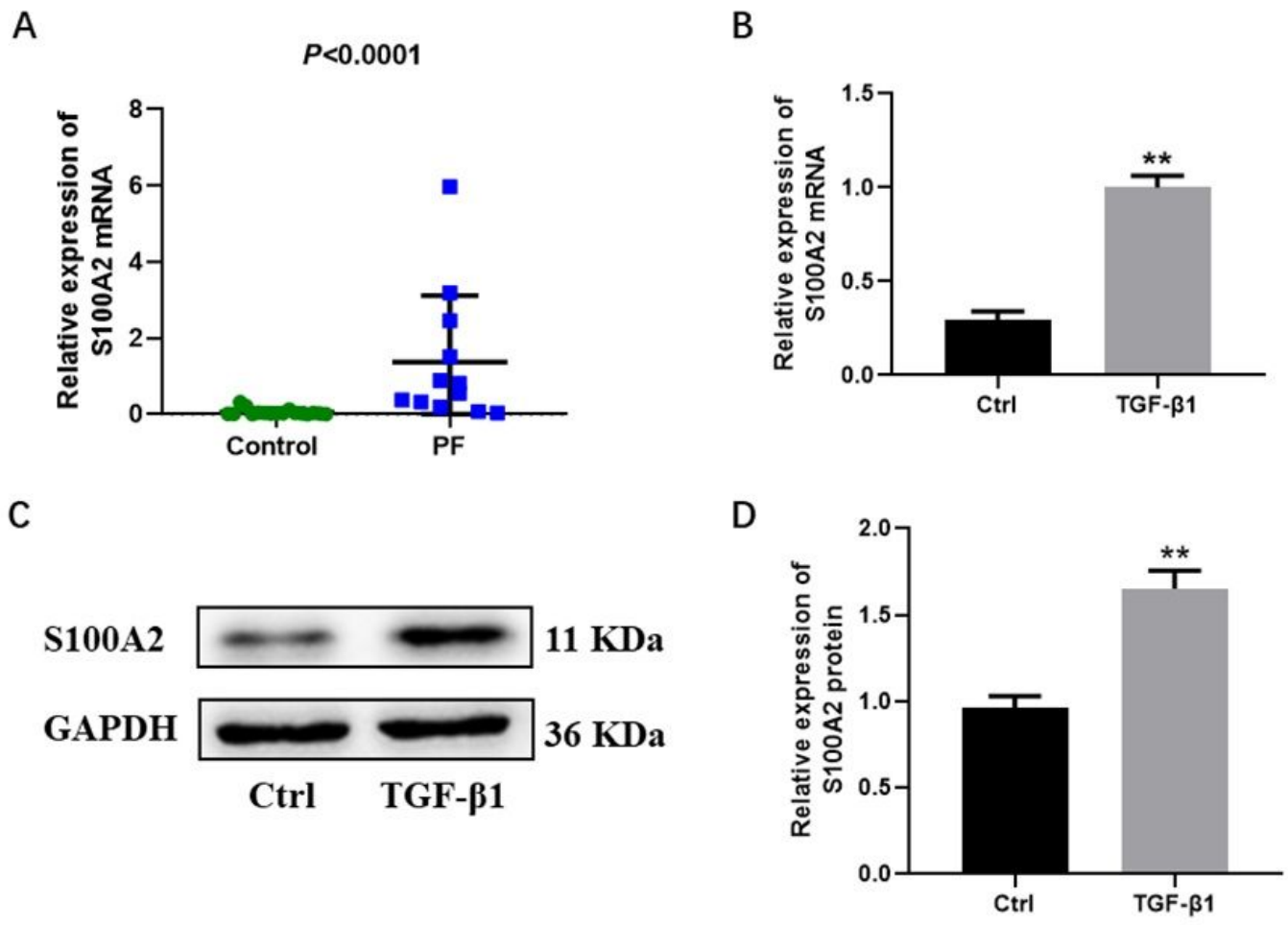

Figure 2

Expression of S100A2 in human PF tissues and human pulmonary epithelial cells (A549). Notes: (A) The mRNA expression of S100A2 in human PF tissues and in normal human lung tissues detected by qRT-PCR. A549 cells were treated with TGF-β1 for 48h. (B) The mRNA expression of S100A2 was determined by qRT-PCR. (C, D) The protein expression of S100A2 was measured by western blot. *P<0.05 versus Ctrl; ** P<0.01 versus Ctrl Abbreviations: PF, pulmonary fibrosis; TGF-β1, transforming growth factor-β1; Ctrl, control.

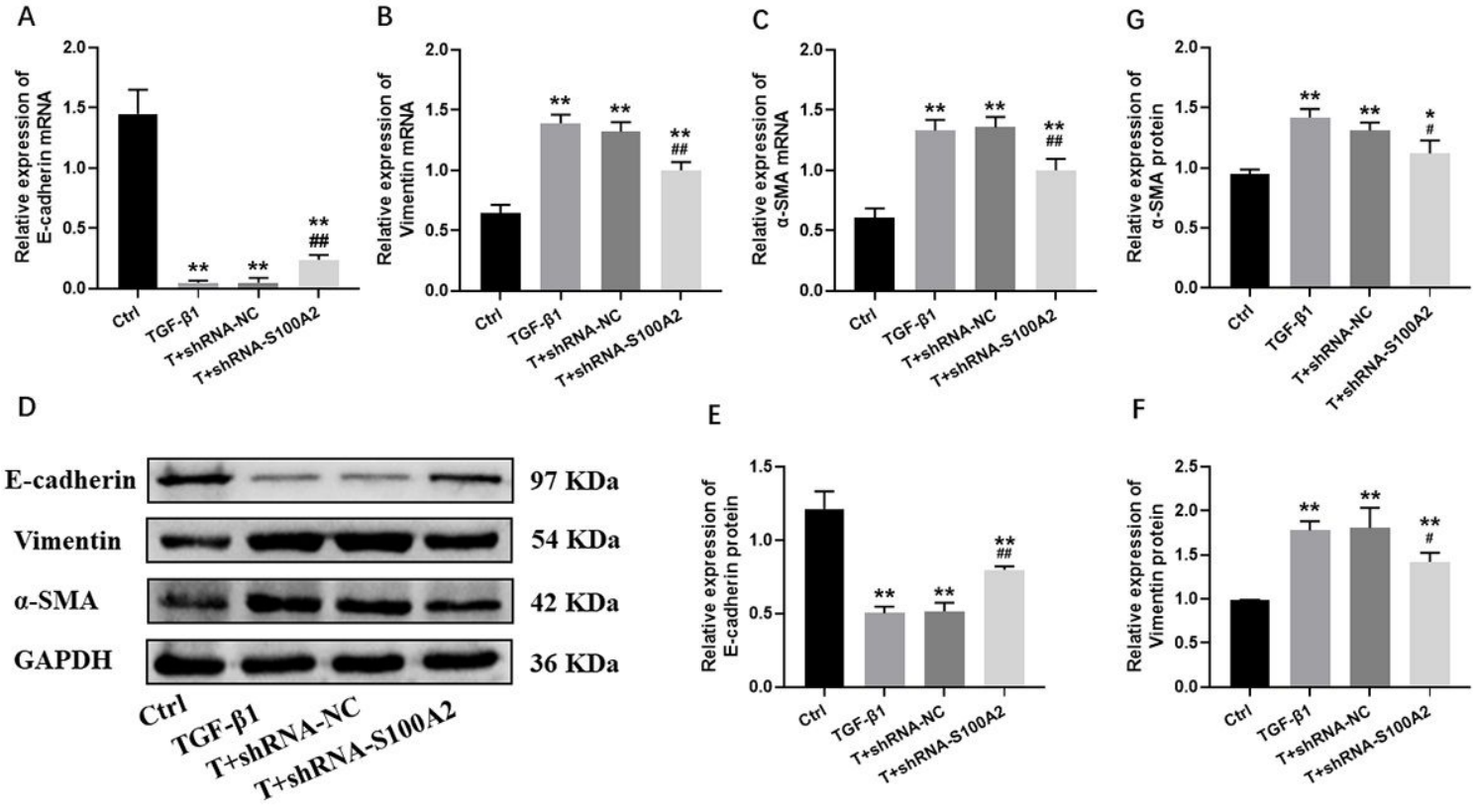

Figure 3

Effect of S100A2 on TGF-β1-induced EMT. A549 cells were exposed to 10ng/ml TGF-β1 stimulation for 48h. The mRNA of E-cadherin (A), vimentin (B), and α-SMA (C) was measured using qRT-PCR. The protein of E-cadherin (E), vimentin (F), and α-SMA (G) was detected by western blot (D). *P<0.05 versus Ctrl; ** P<0.01 versus Ctrl. # P<0.05 versus T+shRNA-NC; ## P<0.01 versus T+shRNA-NC. Abbreviations：TGF-β1, transforming growth factor-β1; α-SMA, α-smooth muscle actin; EMT, epithelial mesenchymal transition; Ctrl, control; T, TGF-β1; shRNA-NC, short harpin RNA-negative control.

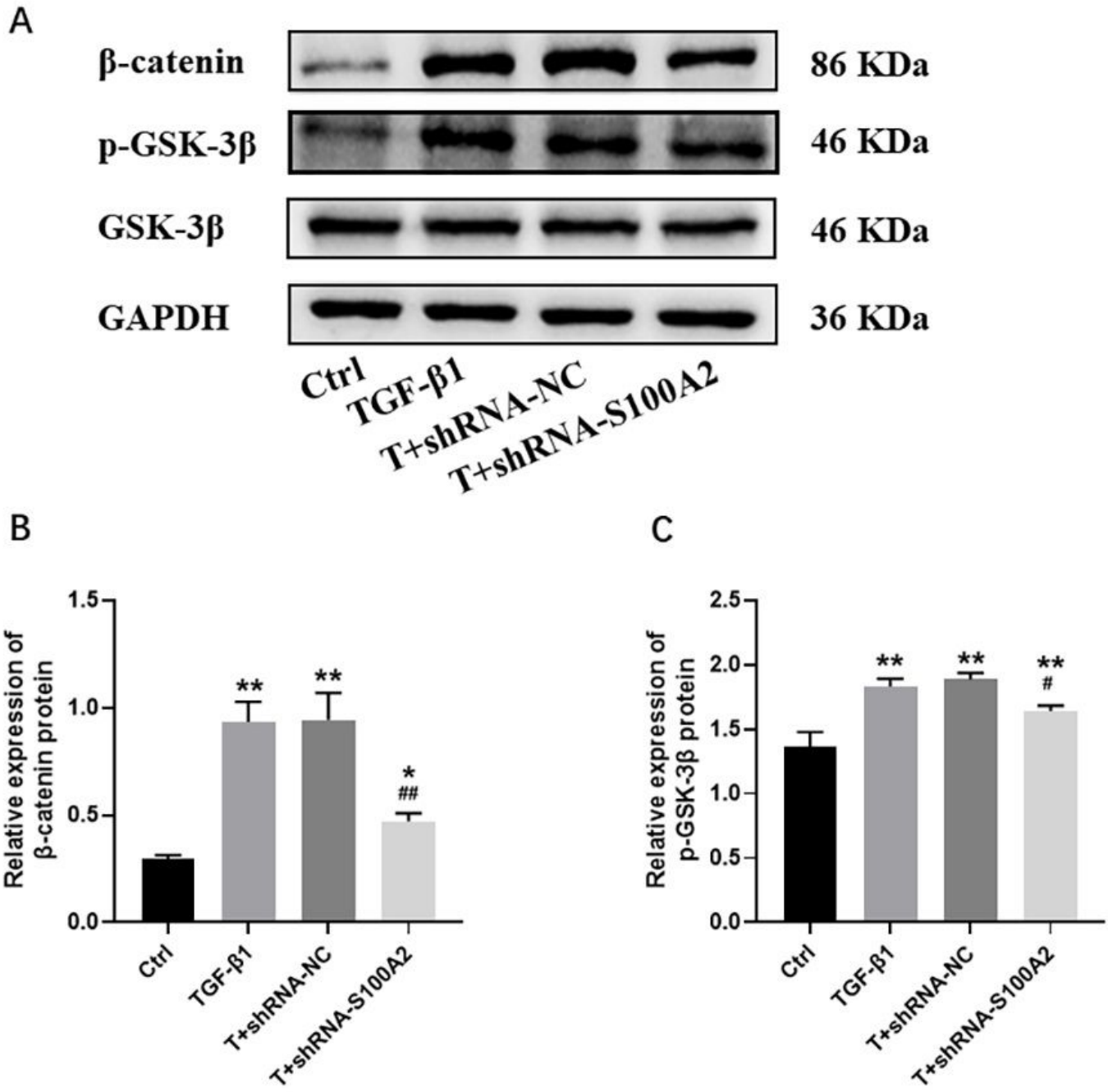

Figure 4

Downregulation of S100A2 suppresses the activation of wnt/β-catenin signaling pathway. The relative protein expression of β-catenin (B) and p-GSK-3β (C) was determined by western blot (A). *P<0.05 versus Ctrl; ** P<0.01 versus Ctrl. # P<0.05 versus T+shRNA-NC; ## P<0.01 versus T+shRNA-NC. Abbreviations TGF-β1, transforming growth factor-β1; Ctrl, control; T, TGF-β1; shRNA-NC, short harpin RNA-negative control.

Supplementary Files

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