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S100A4 amplifies TGF- β -induced epithelial–mesenchymal transition in a pleural mesothelial cell line

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ABSTRACT

Pleural fibrosis can dramatically lower the quality of life. Numerous studies have reported that epithelial–mesenchymal transition (EMT) regulated by transforming growth factor- β (TGF- β) is involved in fibrosis. However, the molecular mechanism is inadequately understood. Fibroblast-specific protein-1 (S100A4) is a target of TGF- β signaling. In our previous study, we have reported that S100A4 is highly expressed in pleural fibrosis. Thus, we suggest that S100A4 took part in the TGF- β -induced EMT in pleural fibrosis. In this study, we determined the expression of S100A4 and EMT-related markers in Met-5A cells (pleural mesothelial cells) treated with TGF- β or TGF- β inhibitor by real-time PCR and western blot. In order to explore the role of S100A4, we used siRNA to knock down the expression of S100A4 in cell model. We found that the expression of epithelial cell marker was decreased and the mesenchymal cell marker increased with S100A4 upregulation after treatment with TGF- β . Moreover, the changes of EMT-related event were restricted when the expression of S100A4 was knocked down. Conversely, S100A4 can partially rescue the EMT-related expression changes induced by TGF- β inhibitor. These findings suggest that S100A4 expression is induced by the TGF- β pathway, and silencing S100A4 expression can inhibit the process of TGF- β -induced EMT.

INTRODUCTION

Pleural fibrosis can cause serious restrictive ventilatory functional disturbance and dramatically lower the quality of life. However, the pathogenesis of pleural fibrosis is inadequately understood. Recently, certain studies have demonstrated that transforming growth factor- β (TGF- β) signaling pathway is involved in fibrosis of some organs, such as liver, kidney and lung.^{1–3} TGF- β regulates cell apoptosis, cell proliferation, cell migration, cell differentiation and extracellular matrix production, and so on. Also, TGF- β can induce pleural fibrosis through the generation of extracellular matrix and reduce its degradation.⁴ Reports have shown that in the process of fibrosis, epithelial cells can be transformed into fibroblasts, a process called epithelial–mesenchymal transition (EMT).⁵ TGF- β is a powerful mediator of EMT.

Significance of this study

What is already known about this subject?

- ▶ Epithelial–mesenchymal transition (EMT) is involved in fibrosis.
- ▶ Transforming growth factor- β (TGF- β) is a key mediator of EMT.
- ▶ S100A4 is a target of TGF- β signaling.

What are the new findings?

- ▶ S100A4 is highly expressed in pleural fibrosis.
- ▶ S100A4 takes part in TGF- β -induced EMT in Met-5A cells.
- ▶ S100A4 regulates ECM in Met-5A cells.

How might these results change the focus of research or clinical practice?

- ▶ These results told us that S100A4 might be a key factor in TGF- β -induced EMT. The finding might pave the way for new and effective treatment for pleural fibrosis.

EMT is an essential developmental process. It participates in mature tissue healing, remodeling and recovery from injury, through which the epithelial cells lose their polarity and acquire mesenchymal phenotypes, including fibroblast-like morphology and increased potential for motility. EMT also takes part in many pathologic processes, such as inflammation, rheumatoid arthritis, tumor metastasis and chronic organ fibrosis, including lung, liver and kidney.

TGF- β can induce the expression of fibroblast-specific protein-1 (S100A4) in renal tubular epithelial cells, while the expression of mesenchymal cell markers is upregulated and epithelial cell markers downregulated.⁶ S100A4, a kind of Ca²⁺-binding proteins with the double helix structure of EF hand, is thought to be specifically expressed in fibroblast in the past years. However, the latest researches report S100A4 is widely expressed in normal cells, such as monocytes, macrophage, T cells, neutrophil and endothelial cells.⁷ S100A4 was related to various diseases, including tumor, rheumatoid arthritis, lung disease, vascular disorder, nerve injury, myocardial hypertrophy and regeneration of repaired cornea. It affects



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the function of numerous intracellular molecular through protein–protein interaction. Numerous studies have shown that S100A4 plays an important role in tumor metastasis. S100A4 maintains cancer-initiating cells in head and neck cancers.⁸ Also previous research reported S100A44 was closely related to renal and liver fibrosis through EMT.⁹ But the role of S100A4 in TGF- β -mediated pleural fibrosis remains unknown. Since S100A4 was highly expressed in fibrotic pleural tissue,¹⁰ we sought to examine the association between TGF- β and S100A4 in phenotype transition of pleural mesothelial cells (PMCs) and pleural fibrosis. In the present study, we demonstrated the expression changes of S100A4, and EMT-related markers in Met-5A cells treated with TGF- β and its inhibitor. Also we observed the effect of S100A4 on EMT-related marker expression. Our data suggested that the S100A4 expression is induced by the TGF- β pathway, and silencing the expression of S100A4 can inhibit the process of TGF- β -induced EMT. This finding might pave the way for new and effective treatment for pleural fibrosis.

MATERIALS AND METHODS

Cell line, culture conditions and treatment

The human PMC line, Met-5A, was purchased from the American Type Culture Collection (ATCC). The cells were cultured in Medium 199 (Hyclone) supplemented with 10% fetal calf serum (Gibco), 3.3 nM epidermal growth factor (Abcam), 400 nM hydrocortisone (Sigma), 870 nM zinc-free bovine insulin (Novo Nordisk), 20 mM HEPES (Gibco), 3.87 μ g/L selenious acid (H₂SeO₃, Sigma) and trace elements B (Biodee, Beijing, China) liquid used at 1:100 dilution in a 5% CO₂ and 95% air incubator at 37°C. Cells were cultured in the absence of serum overnight prior to the treatment with LY2157299 (30 ng/mL, Selleck Chemicals) for the indicated periods.

Cell proliferation and viability

Met-5A cells were plated at a density of 3 \times 10⁴ cells/mL in 96-well plates (100 μ L medium per well). Twenty-four hours after seeding, the cells were treated with TGF- β and six replicates were included for each concentration (1.0 ng/mL, 5.0 ng/mL, 10.0 ng/mL, 20.0 ng/mL). Cell proliferation and viability were measured using the Cell Counting Kit (CCK-8) (Dojindo, Kumamoto, Japan) and a microplate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, California, USA) at a wavelength of 450 nm.

Transfection

SiRNA targeting S100A4 was chemically synthesized by Hangzhou Hibio Technologies. The target sequence for S100A4-siRNA was 5'-UCAACAAGUCAGAAC-UAAA-3'. Twenty-four hours before transfection, cells were seeded onto 6-well culture plates. When cells grew to 80%–90% confluence, transfection was performed using Lipofectamine 2000 Reagent (Life Technologies) according to manufacturer's instructions. The expressions of TGF- β , S100A4, α -smooth muscle aorta (SMA) and cytokeratin were confirmed by real-time RCR and western blot after 48 hours.

Real-time PCR

Total RNA of the cells was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Primers for the target gene were designed by Hangzhou Hibio Technologies (table 1). cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). All PCR reactions were carried out using a SuperReal PreMix Plus (with SYBR Green I) (Tiangen) according to the manufacturer's instructions. Amplification was performed using a CFX Connect Real-Time PCR System. The expression levels of the target mRNAs were directly normalized to GAPDH.

Western blotting analysis

The Met-5A cells were lysed in phenylmethanesulfonyl fluoride at 4°C for 30 min, and then centrifuged at 11 000 rpm for 5 min at 4°C. The supernatant was collected for total cellular protein, and its concentration was determined using the Bradford assay (Sigma Chemicals, Bangalore, India). Equivalent amounts of total cellular protein (30 μ g) were fractionated on a reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (8%–12%), followed by blotting on a polyvinylidene difluoride membrane. Membranes were blocked in 5% non-fat dry milk for 2 hours, and then incubated with the following antibodies overnight at 4°C: anti-TGF- β (Millipore), anti-S100A4 (Santa Cruz Biotechnology), anti- α -SMA (Santa Cruz Biotechnology) and anticytokeratin (CST). An horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) was applied, and a chemiluminescent substrate system (Amersham Biosciences) was used to detect the signals. Images were documented by scanner

Table 1 Sequences of primers for TGF- β , S100A4, α -SMA, keratin 7 and GAPDH

	Sequence	Expected lengths
TGF- β	Forward: 5'-AACATGATCGTGCCTCTGCAAGTGCAGC- 3' Reverse: 5'-AGGACGGACAGACGTGATA-AGGAA- 3'	200 bp
S100A4	Forward: 5'-AGGGACAACGAGGTGGACTT- 3' Reverse: 5'-CTTCCTGGGCTGCTTACTCTGG- 3'	105 bp
α -SMA	Forward: 5'-AGCGTGGCTATTCCTTCGTTA-3' Reverse: 5'-GCCCATCAGGCAACTCGTAA-3'	153 bp
Cytokeratin	Forward: 5'-CTTGGCAGCAGCAGCCTCTA-3' Reverse: 5'-TCCAGAAACCGCACCTTGTG-3'	239 bp
GAPDH	Forward: 5'-AGAAGGCTGGGGCTCATTTG-3' Reverse: 5'-AGGGCCATCCACAGTCTTC-3'	258 bp

α -SMA, smooth muscle aorta; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TGF- β , transforming growth factor- β .

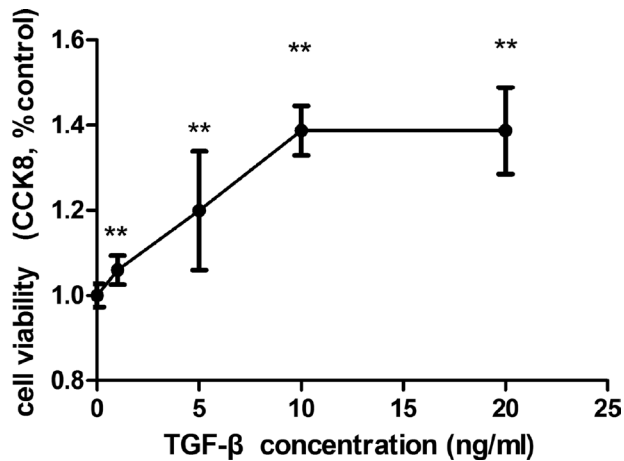


Figure 1 Effect of TGF- β on the proliferation of Met-5A cells. Met-5A cells were treated with 1.0 ng/mL, 5.0 ng/mL, 10.0 ng/mL and 20.0 ng/mL TGF- β , respectively. Cell proliferation was measured by CCK-8. TGF- β induced a clear dose-dependent increase in the viability of Met-5A cells after 48-hour exposure. Data are presented as average \pm SD for three independent experiments. ** p <0.01 indicates significant difference between exposed and untreated Met-5A cells. CCK-8, Cell Counting Kit; TGF- β , transforming growth factor- β .

and analyzed by Quantity One software. β -actin expression was used as a loading control.

Immunohistochemical staining

Cells were seeded on poly-L-lysine-coated glass coverslips in 6-well plates and received different treatments. After being fixed with 4% paraformaldehyde for 15 min, cells were permeabilized in 0.5% Triton X-100 for 20 min, and subsequently incubated with 0.3% hydrogen peroxidase for 10 min in order to block endogenous peroxidase activity. Slides were incubated with normal goat serum for 1 hour to block unspecific labeling. Subsequently, cells were incubated with the following antibodies overnight at 4°C: anticytokeratin (CST) and α -SMA (Abcam). Slides were then washed in

phosphate buffer saline (PBS) followed by incubation with secondary antibody (Wuhan Boster Biological Engineering) for 1 hour at 37°C, followed by incubation with streptavidin-peroxidase (Dako) for 15 min at 37°C. 3,3'-diaminobenzidine (DAB, Dako) was applied as the chromogenic agent. Then slides were counterstained with hematoxylin, dehydrated in graded ethanol and coverslipped. Cells were observed under a fluorescent microscope.

Statistical analysis

Statistical analysis of data was performed using SPSS V.22.0 for Windows software. All data from experiments are presented as the mean \pm SE. One-way analysis of variance or two-tailed Student's t -tests were used for comparisons between groups. p <0.05 was considered to be statistically significant.

RESULTS

Effect of TGF- β on the proliferation of Met-5A

First, we detected the effect of TGF- β on the proliferation of Met-5A cells using CCK-8 method. TGF- β induced a clear dose-dependent increase in the viability of Met-5A cells after 48-hour exposure. Significant increase of cell viability was observed when TGF- β concentration was higher than 1.0 ng/mL, and the cell viability reached plateau phase at the concentration of 10.0 ng/mL (figure 1).

Effect of TGF- β on the expression of S100A4

It is obvious that TGF- β participates in pleural fibrosis, but the mechanism was not known by now. In our previous study, we have reported that S100A4 is highly expressed in pleural fibrosis. In order to check whether S100A4 was involved in the TGF- β -mediated pleural fibrosis, we first examined the mRNA and protein expression of S100A4 in Met-5A cells treated with different expression levels of TGF- β . Our results showed that TGF- β treatment led to an increase in the expression of S100A4, and the S100A4 level was decreased when Met-5A cells were treated with LY2157299, an inhibitor of TGF- β receptor. However, the expression of S100A4 in Met-5A cells treated with

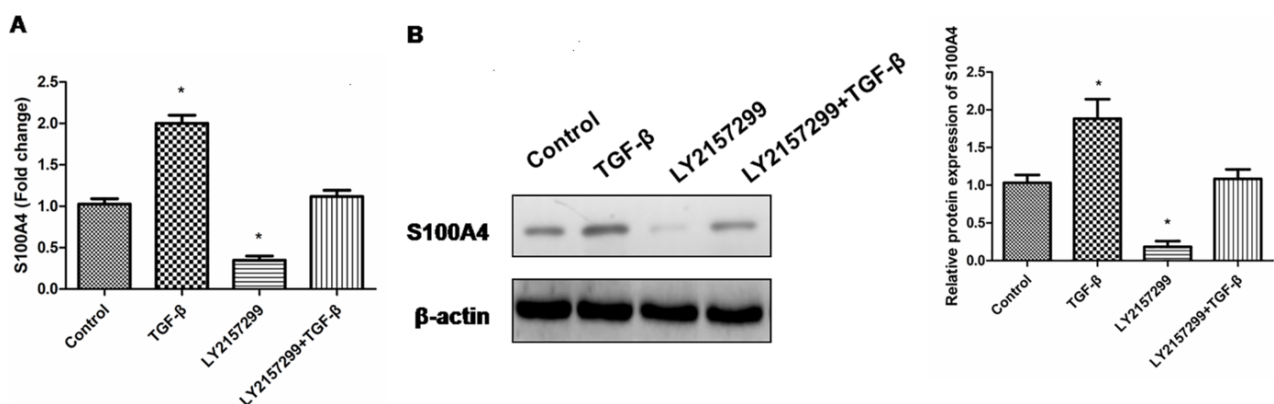


Figure 2 Effect of TGF- β on the expression of S100A4 in Met-5A cells. Met-5A cells were treated with TGF- β and/or LY2157299. S100A4 mRNA (A, real-time PCR) and protein (B, western blot) levels were upregulated after 48 hours of TGF- β treatment, while downregulated 48 hours after LY2157299 treatment. TGF- β can rescue the expression changes induced by LY2157299. The bar graph shows the relative mRNA or protein expression levels among groups. GAPDH and β -actin were used as an endogenous control for normalization. Data are presented as average \pm SD for three independent experiments. * p <0.05 indicates significant difference between treated and control groups. TGF- β , transforming growth factor- β .

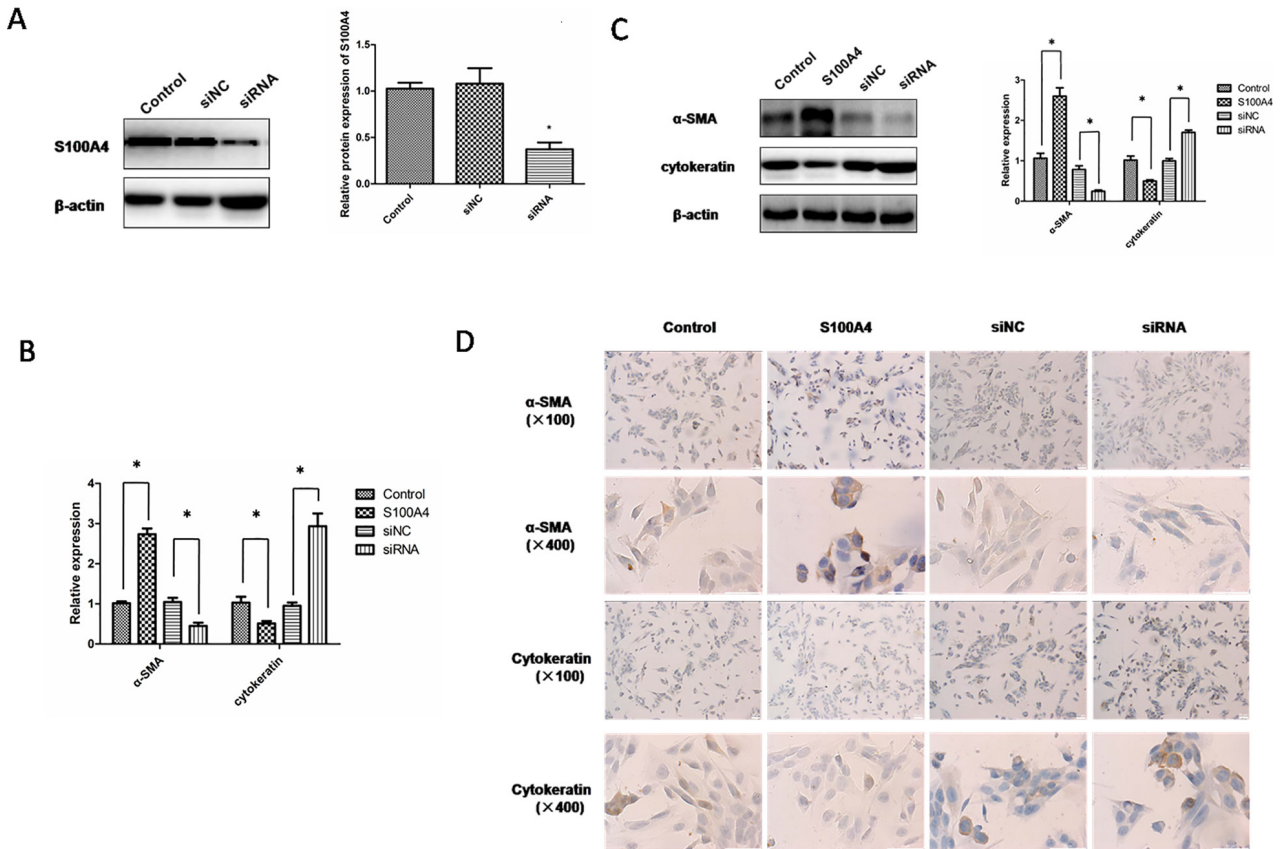


Figure 3 Effect of S100A4 in regulating EMT in Met-5A cells. (A) Western blot analysis of S100A4 expression in Met-5A cells transfected with S100A4-siRNA. S100A4 expression was significantly decreased. Real-time PCR (B), western blot (C) and immunohistochemical (D) analyses of α -SMA and cytoke- ratin in Met-5A cells with different expression levels of TGF- β and S100A4. GAPDH and β -actin were used as an endogenous control for normalization. The bar graphs show the relative expression of mRNA or proteins among each treatment groups. Data are presented as average \pm SD for three independent experiments. * p <0.05 indicates significant difference between treated and control groups. EMT, epithelial–mesenchymal transition; TGF- β , transforming growth factor- β .

LY2157299 was significantly increased after treatment with TGF- β for 48 hours to activate TGF- β , in both the mRNA and protein levels (figure 2).

Effect of S100A4 in regulating EMT in Met-5A cells

As previously described, EMT plays an important role in chronic organ fibrosis. To investigate whether S100A4 took part in EMT in Met-5A cells, we examined the expression of α -SMA (mesenchymal cell marker) and cytoke- ratin (epithelial cell marker) using real-time PCR, western blot and immunohistochemical analysis. As expected, we found that S100A4 treatment resulted in an increase in the expression of mesenchymal cell marker and a decrease in the expression of epithelial cell marker in Met-5A cells. Conversely, downregulation of S100A4 reversed these expression changes (figure 3).

Effect of S100A4 on the expression of EMT-related markers in Met-5A cells treated with LY2157299

It has been widely reported that TGF- β was a powerful EMT regulator. Consistent with previous reports, TGF- β induced an increase in the expression of α -SMA and a decrease in cytoke- ratin in Met-5A cells, in both the mRNA and protein levels. As we reported above, S100A4 was a

molecular marker that can initiate EMT. In order to explore the role of S100A4 in TGF- β -induced EMT, we analyzed the expression of EMT-related markers when Met-5A cells were treated with LY2157299, and found the expression of cytoke- ratin was enhanced and α -SMA decreased, at both the mRNA and protein levels. And as expected, S100A4 could partially reverse the expression changes. Meanwhile, when the expression of S100A4 was downregulated, the expression of α -SMA was partially decreased and cytoke- ratin partially increased in Met-5A cells treated with TGF- β , compared with cells exposed to TGF- β only. In other words, downregulation of S100A4 could partially prevent the EMT initiated by TGF- β . Thus, these results indicated that S100A4 played a pivotal role in TGF- β -induced EMT in a PMC line (figure 4).

DISCUSSION

Any disease-infected pleura with inadequacy treatment can cause pleural fibrosis, which manifests as pleural thickening and adhesion. Pleural fibrosis can cause different levels of respiratory dysfunction. However, there is no effective method to cure pleural fibrosis at present. Understanding the molecular mechanisms under- lying the progression of pleural fibrosis may provide

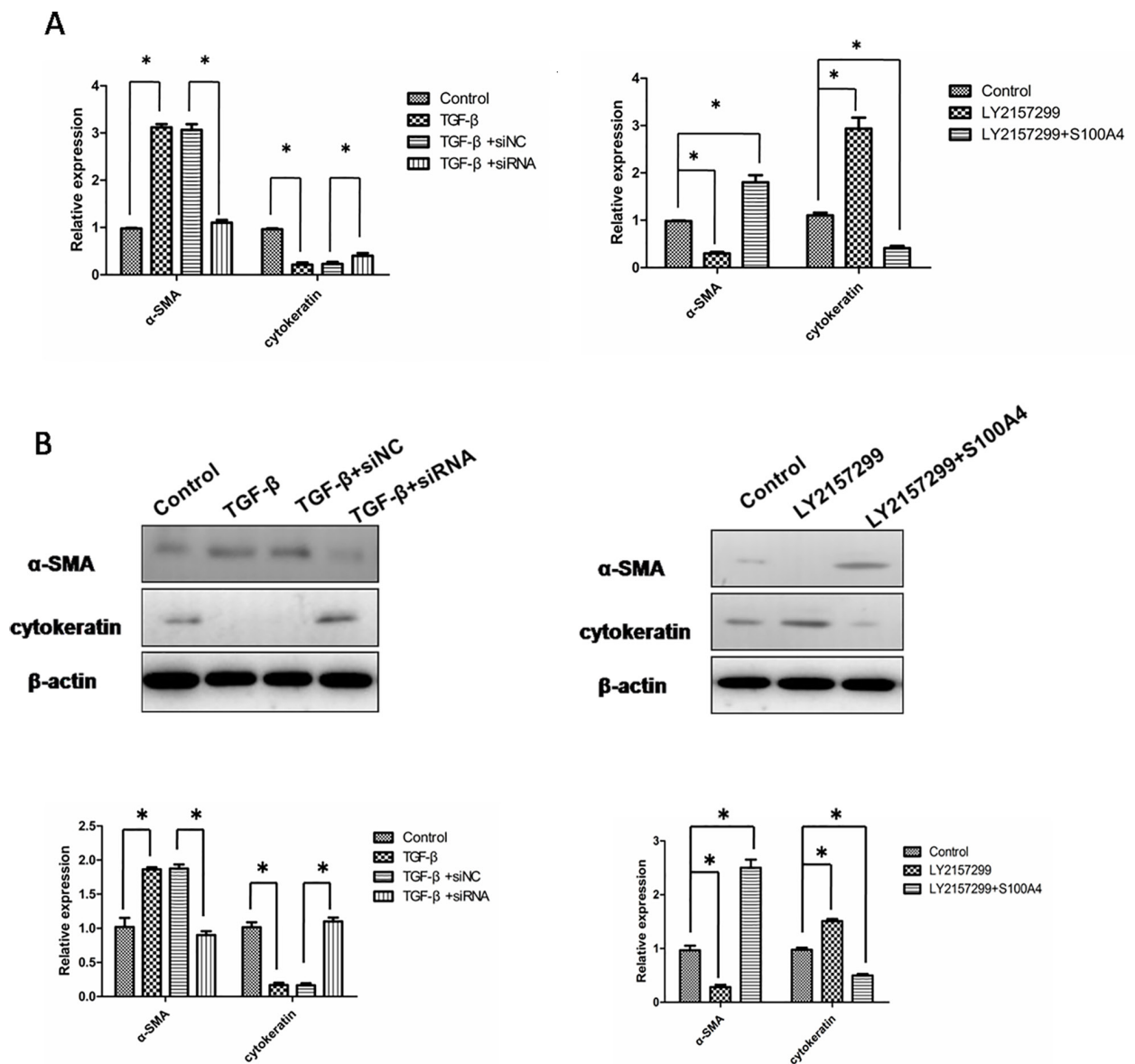


Figure 4 Effect of S100A4 on the expression of EMT-related markers in Met-5A cells treated with LY2157299. Met-5A cells were transfected with S100A4 siRNA to inhibit TGF- β -induced EMT, or treated with S100A4 to activate the TGF- β -EMT pathway. The mRNA and protein expression of EMT-related markers were determined by real-time PCR (A) and western blot (B), respectively. GAPDH and β -actin were used as an endogenous control for normalization. The bar graphs show the relative expression of mRNA or proteins among each treatment groups. Data are presented as average \pm SD for three independent experiments. * p <0.05 indicates significant difference between treated and control groups. EMT, epithelial–mesenchymal transition; TGF- β , transforming growth factor- β .

ways for the development of antipleural fibrosis therapies. In this study, we investigated the role of S100A4 in TGF- β -induced fibrosis.

PMCs play a significant role in the pathogenesis of pleural fibrosis, and their response to inflammation is an important element in this condition. Huggins and Sahn¹¹ reported that the reaction of mesothelial cells and its basement membrane to pleural injury and inflammation, along with their ability to self-repair, determines the prognosis, in other words whether there is normal healing or pleural fibrosis. Nathalie *et al* transferred TGF- β into the PMCs of rats, which induced the occurrence of pleural fibrosis and

caused serious lung capacity limits.¹² Also we chose Met-5A cell line as our cell model.

Existing reports have demonstrated that the first step of fibrosis is tissue damage caused by a chronic inflammation. Then activated myofibroblasts migrated to the inflammatory sites to induce wound healing. Finally, fibrosis forms due to excessive deposition of extracellular matrix (ECM).^{5,13} In this process, epithelial cells can be transformed into myofibroblasts, called EMT. TGF- β is the key factor in organ fibrosis and tumor metastasis, and also involved in EMT. Gaudie J *et al*¹⁴ reported upregulation of TGF- β in lung tissue can induce severe and progressive fibrosis in rodent

lung. The expression level of TGF- β in pleural effusion is positively correlated with pleural thickening in experimental empyema, and intrapleural injection of TGF- β antibody will inhibit pleural fibrosis.^{15 16} TGF- β regulates cell proliferation in numerous cell lines. Consistent with the previous report, our data showed that TGF- β promotes the proliferation of PMCs.

S100A4 played important role in organ fibrosis. In our previous study, we have reported that S100A4 is highly expressed in pleural tuberculosis.¹⁰ Xie R *et al*¹⁷ found S100A4 mediated endometrial cancer invasion and was a target of TGF- β signaling. Matsuura *et al*¹⁸ reported functional interaction between Smad3 and S100A4 became important regulator for TGF- β -mediated cancer cell invasion. However, there was no evidence showing the relationship between TGF- β and S100A4 in pleural fibrosis. In the present study, we found that TGF- β regulates the expression of S100A4 in Met-5A cells. This is direct evidence supporting the theory that S100A4 is a target of TGF- β signaling in PMCs.

Kim *et al*¹⁹ found PMCs in pleural tuberculosis participated in pleural fibrosis through EMT. In the present study, we found S100A4 treatment led to an increase in the expression of mesenchymal cell marker and a decrease in the expression of epithelial cell marker in Met-5A cells. This is consistent with previous studies showing that S100A4 participates in the process of EMT. In order to check whether S100A4 was involved in the TGF- β -induced EMT, we examined the S100A4 expression in Met-5A cells when treated with TGF- β for 48 hours. The mRNA and protein expressions of S100A4 were increased, and also TGF- β treatment led to an increase in the expression of mesenchymal cell marker and a decrease in the expression of epithelial cell marker in Met-5A cells. Interestingly, down-regulation of S100A4 partially inhibited the TGF- β -mediated expression changes. These data told us that S100A4 might be a key factor in TGF- β -induced EMT.

Contributors QN, FL, LW, HL, YY and TH carried out the experiments, ZS designed the study, and QN and ZS prepared the manuscript. All authors read and approved the final manuscript.

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Competing interests None declared.

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