Downregulation of S100 calcium-binding A4 (S100A4) ameliorates hepatic fibrosis *via* regulating Wnt/β-catenin signaling pathway

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ABSTRACT

S100 calcium-binding protein A4 (S100A4), a fibrosis-associated calcium-binding protein, has been implicated in fibrotic progression across multiple organs. Activation of the Wnt/β-catenin signaling pathway is a critical driver of hepatic fibrosis, yet the mechanistic role of S100A4 in this context remains poorly defined. This study investigated the regulatory role of S100A4 in hepatic fibrosis *in vitro* and *in vivo*. Hepatic stellate cells (HSCs) were treated with TGF-β to induce fibrotic activation, and S100A4 expression was silenced using shRNA. A carbon tetrachloride (CCl_4)-induced murine hepatic fibrosis model was employed for *in vivo* validation. Fibrotic markers, including collagen I, fibronectin, and α-smooth muscle actin (α-SMA), were assessed *via* qRT-PCR, Western blotting, immunofluorescence, and immunohistochemistry. Liver histopathology and function were evaluated using Masson trichrome staining, hematoxylin-eosin staining, and serum ALT/AST assays. *In vitro* experiments demonstrated that TGF-β treatment upregulated S100A4 expression in HSCs, while S100A4 silencing suppressed HSC activation, extracellular matrix (ECM) deposition, and Wnt/β-catenin signaling. *In vivo*, S100A4 downregulation attenuated CCl_4-induced hepatic fibrosis, reduced collagen accumulation, improved liver histology, and normalized serum ALT/AST levels. These findings indicate that S100A4 promotes hepatic fibrosis by activating the Wnt/β-catenin pathway, highlighting its potential as a therapeutic target.

Key words: S100 calcium-binding A4; liver fibrosis; Wnt/β-catenin; HSCs.

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Availability of data and materials: the data used to support the findings of this study are available from the corresponding author upon reasonable request.

Introduction

Liver fibrosis is a common disease with high morbidity and mortality worldwide. As a consequence of the wound healing process, liver fibrosis is defined by the accumulation of fiber cells and ECM extracellular matrix (ECM) deposition synthesized by activated stellate cells.[1] A variety of pathogenic factors, such as hepatitis induced by viral, drug-induced liver disease and other continuous action on the liver can cause long-term sustained damage to the liver and progressive fibrosis, eventually leading to the occurrence of cirrhosis.[2,3] Numerous researches have shown that the risk of patients with liver fibrosis turning into hepatocellular carcinoma is greatly enhanced.[4] Therefore, further exploring the occurrence and development mechanism of liver fibrosis is of great significance. The activation of stellate cells is a pivotal factor that can induce liver fibrosis among various etiological factors. Under normal conditions, retinoids are almost metabolized and stored in hepatic stellate cells (HSCs), but are mobilized toward sites leading to fibrosis and cirrhosis when injured hepatocytes produce reactive oxygen species and inflammatory mediators. The loss of retinoids storage results in the activation of HSCs, in which undergoes significant phenotypic and functional transformations, manifested by the mass synthesis of ECM components, the high expression of α-smooth muscle actin (α-SMA).[5,6] The Wnt/β-catenin signal transduction pathway is involved in biological evolution, regulating pluripotency of stem cells and determining the fate of cell differentiation during development.[7] Multiple studies have confirmed that this pathway plays a critical role in the process of fibrosis.[8] Wnt/β-catenin signaling pathways evoke HSCs proliferation and activation along with the increase transformation of epithelial cell and synthesis of ECM.[9] Wnt/β-catenin signaling pathway is widely reported to be activated during the process of liver fibrosis and numerous components such as nuclear β-catenin are downregulated and implicated in this process.[10] S100A4 is one of the S100 protein family, known as fibroblast specific protein 1 (FSP1), is important to the differentiation and growth of cells and tissues.[11] S100A4 has also been reported to be involved in promoting the motility and invasion of tumor cells.[12] Upregulation of S100A4 expression promotes proliferation of hepatocellular carcinoma cells and potentially triggers the release of cytokines and inflammatory processes.[13,14] We speculate that these cytokines may increase HSCs differentiation to myofibroblast and promote hepatic fibrosis process. Recent studies have highlighted the critical role of S100A4 in diverse fibrotic conditions beyond hepatic fibrosis, such as pulmonary and cardiac fibrosis. For example, Zhang *et al*.[15] demonstrated that S100A4 is implicated in lung fibroblast activation and ECM remodeling, suggesting its potential as a cross-organ fibrotic regulator. Interestingly, a recent finding indicated that it has a beneficial effect on cardiac fibrosis.[16] However, the function of S100A4 in hepatic fibrosis is still unclear. Hence, we explored the association between S100A4 expression and hepatic fibrosis progression based on the Wnt/β-catenin signaling pathway.

Materials and Methods

Cell culture and transfection

HSCs were inoculated in flasks. Dulbecco's modified Eagle medium (DMEM, Gibco, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Gibco), 1% penicillin, and 1% streptomycin is routinely used to culture the cells. When the cells reached approximately 80% confluence, we transfected S100A4-shRNA into HSCs using adenovirus sh-RNA technology and further induced myofibroblast differentiation by transforming growth factor-β (TGF-β, 20 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) for 24 h. All *in vitro* experiments were performed in triplicate to ensure reproducibility. For shRNA transfection, 2 μg of S100A4-shRNA was mixed with 5 μL of Lipofectamine 3000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in serum-free DMEM and incubated for 6 h before replacing the medium.

Hepatic fibrosis model

C57BL/6J mice (male, 6-8 weeks, 20-22 g) were selected for the liver fibrosis model. All animals are obtained from Zhengzhou University Animal Center and all procedures are approved by the Animal Ethics Committee of Zhengzhou University Animal Center (2021-KY-02-91). The mice were divided into three groups randomly: the control, CCl4 and CCl4+ S100A4-shRNA groups (n=6). Control group was injected with the same volume of saline as CCl4 group intraperitoneally. CCl4 group was injected intraperitoneally with CCl4 dissolved in olive oil (0.15 mL/kg, 20%) twice a week. CCl4+ S100A4-shRNA group was injected with a volume of 5×10^5 pfu/g (2.5 μL/g, 2×10^7 pfu/mL) S100A4-shRNA post CCl4 treatment *via* intraperitoneal injection for 3 days.

Quantitative RT-PCR (qRT-PCR)

TRIzol solution was used to harvest cells and tissues in the total RNA extraction kit following the manufacturer's protocol. The reverse transcription was conducted using the qScript Flex cDNA Synthesis Kit (Quanta Biosciences, USA). The mRNA detection was measured using ABI using the SYBR1 Taq™ kit (TaKaRa, Tokyo, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was applied for standardization. Primers were designed as follows: S100A4: 5′-GATGAGCAACTTGGACAGCAA-3′ (F), 5′-CTGGGCTGCTTATCTGGGAAG-3′ (R); Fibronectin: 5′-CGGTGGCTGTCAGTCAAAG-3′ (F), 5′-AAACCTCGGCTTCCTCCATAA-3′ (R); collagen I: 5′-TGGTCCTGCTGGTCCTGCTG-3′ (F), 5′- CTGTCACCTTGTTCGCCTGTCTC-3′ (R); GAPDH: 5′-AGAAGGCTGGGGCTCATTTG-3′ (F), 5′-AGGGGCCATCCACAGTCTTC-3′ (R).

Western blotting

Western blotting was performed to isolate whole cell lysates (50 μg) by SDS polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membrane (0.25 μm, Arkema, Colombes, France). The blocked-off 5% BSA was left at room temperature for 1 h, and the membrane was incubated overnight at 4°C with corresponding primary antibody: β-catenin (1:1000; Cell Signaling Technology, Danvers, MA, USA); p-β-catenin (1:1000; Cell Signaling Technology); GAPDH (1:2000, Cell Signaling Technology) Then the membrane was incubated with secondary antibodies at 4°C for 2 h. Then enhanced chemiluminescence method was utilized to measure protein.

Immunohistochemical staining

The liver tissue was fixed in 4% paraformaldehyde at 4°C for 24 h and embedded in 5 μm paraffin sections. The sections were incubated overnight at 4°C with the primary antibody. After rinsing with PBS, they were incubated with the corresponding secondary antibody, followed by DAB staining. The nuclei were counterstained with hematoxylin for 5 seconds. Finally, images were captured using a microscope. The primary antibodies used were α-SMA (1:200 dilution, Abcam, Cambridge, UK) and β-catenin (1:200 dilution, Cell Signaling Technology), while the secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:500

dilution, Abcam). Antigen retrieval was performed by heating sections in citrate buffer (10 mM, pH 6.0) at 95°C for 15 min. Negative controls were conducted by omitting the primary antibody to ensure specificity of the staining. Images were captured using an Olympus BX53 fluorescence microscope equipped with a 40× objective. For each sample, three randomly selected fields were analyzed to ensure representative data. Image analysis was conducted using ImageJ software to quantify the percentage of positively stained cells. Three randomly selected fields per sample were analyzed, and the average percentage of immunopositive cells was reported.

Immunofluorescence technique

Paraffin sections of hepatic stellate cells were subjected to antigen blocking with 1% BSA in PBS at room temperature for 1 h to prevent non-specific binding. The sections were then incubated overnight at 4°C with the primary antibody (anti-α-SMA, 1:500 dilution, A2547, Sigma). After washing with PBS, the sections were treated with a fluorescent secondary antibody (Alexa Fluor 488, 1:1000 dilution, SY0683, Keygen, Nanjing, China) and incubated in the dark at room temperature for 1 h. The nuclei were stained with DAPI (1 μg/mL, Keygen) for 10 min to visualize cellular structures. Negative control sections were treated with PBS instead of the primary antibody to assess the specificity of staining. Images were captured using a fluorescence microscope (Olympus, Tokyo, Japan) with a 40× oil immersion objective. Image analysis was performed using ImageJ software (National Institutes of Health) to quantify fluorescence intensity and localization of α-SMA in hepatic stellate cells.

Liver function

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by Deary CS-T300 chemical analyzer for acid (TBA) kit (Deary Medical Technology, Jiangsu China) in accordance with the manufacturers' protocol.

Histology assessment

Hematoxylin-eosin (H&E) staining and Masson's trichrome staining were used to visualize the extent of damage and fibrosis in liver tissue. Staining was performed using the H&E staining kit and Masson's Trichrome staining kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. The sections were observed under a Nikon ECLIPSE Ti2 microscope with a 20× objective to assess the histological features. The percentage of fibrosis-positive areas was quantified using ImageJ software (National Institutes of Health). Fibrosis-positive areas were identified by blue staining in Masson's trichrome, and the percentage of fibrosis-positive areas was calculated as the ratio of blue-stained regions to the total tissue area.

Statistical analysis

All experiments were performed by three independent studies. The data are described in terms of mean ± standard deviation (mean ±SD). The student's t-test conducts statistical analysis of differences. One-way analysis of variance (ANOVA) was utilized to compare the two groups. A p-value ≤0.05 is considered statistically significant.

Results

S100A4 was up-regulated in HSCs after TGF-β treatment

Firstly, we examined the expression levels of collagen I and fibronectin at 6, 8, and 10 h following TGF-β treatment. Both proteins showed a significant increase, reaching their peak expression at 10 h (Figure 1 A,B). These results indicate that TGF-β treatment

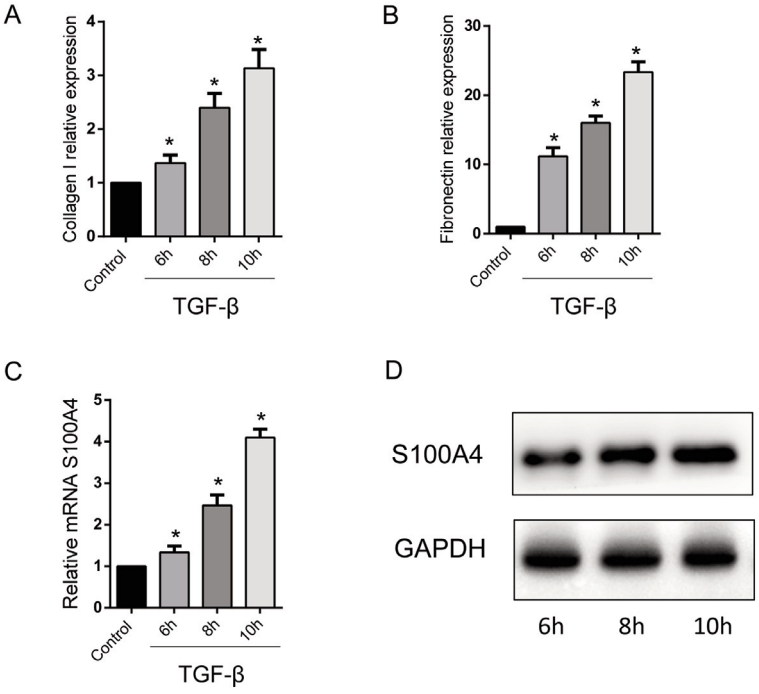

Figure 1. S100A4 was up-regulated in HSC cells after TGF-β treatment. The relative RNA expression of collagen I (**A**) and fibronectin (**B**) in control, 6 h, 8 h and 10 h. **C**) The relative mRNA of S100A4 in control, 6 h, 8 h and 10 h. **D**) Protein levels of S100A4 in 6 h, 8 h and 10 h.

markedly enhances fibrosis-related markers. Similarly, qRT-PCR analysis revealed that S100A4 expression was highest at the 10-h time point (Figure 1C). Western blot analysis confirmed this trend, showing that S100A4 protein levels peaked at 10 h compared to 6 and 8 h (Figure 1D). Therefore, subsequent experiments were conducted under the 10-h treatment condition. Collectively, these findings suggest that S100A4 plays a crucial role in liver fibrotic changes.

Downregulation of S100A4 inhibits HSCs differentiation to myofibroblast *via* regulating Wnt/β-catenin signaling pathways

To further validate the above findings, p-β-catenin levels were measured using Western blotting. The p-β-catenin expression was increased under the TGF-β treatment and total β-catenin protein levels did not alter, indicating the activation of Wnt/β-catenin signaling pathways, while the S100A4 downregulation reversed the trend (Figure 2 A,B). Moreover, silencing S100A4 contributed to the decrease of the level of S100A4 (Figure 2C). The accumulations of collagen I and fibronectin were significantly increased following TGF-β treatment, but decreased after the downregulation of S100A4 (Figure 2 D,E). A subsequent immunofluorescence analysis showed that downregulation of S100A4, confirmed by quantitative RT-PCR and western blotting, inhibited the expression of α-SMA (Figure 2F). In conclusion, the above experiments proved that the downregulation of S100A4 had a positive alleviating effect on the process of hepatic fibrosis *via* decreasing HSCs differentiation efficiently and inhibiting Wnt/β-catenin signaling pathways.

Downregulation of S100A4 regulates hepatic fibrosis *via* Wnt/β-catenin signaling pathway degrading in the livers of fibrotic mice

Considering its potency in fibrosis inhibition *in vitro*, it is rational to speculate the capabilities of S100A4 against hepatic fibrosis *in vitro*. Western blotting, PCR and immunohistochemistry were used to detect the changes of liver tissue expression. The

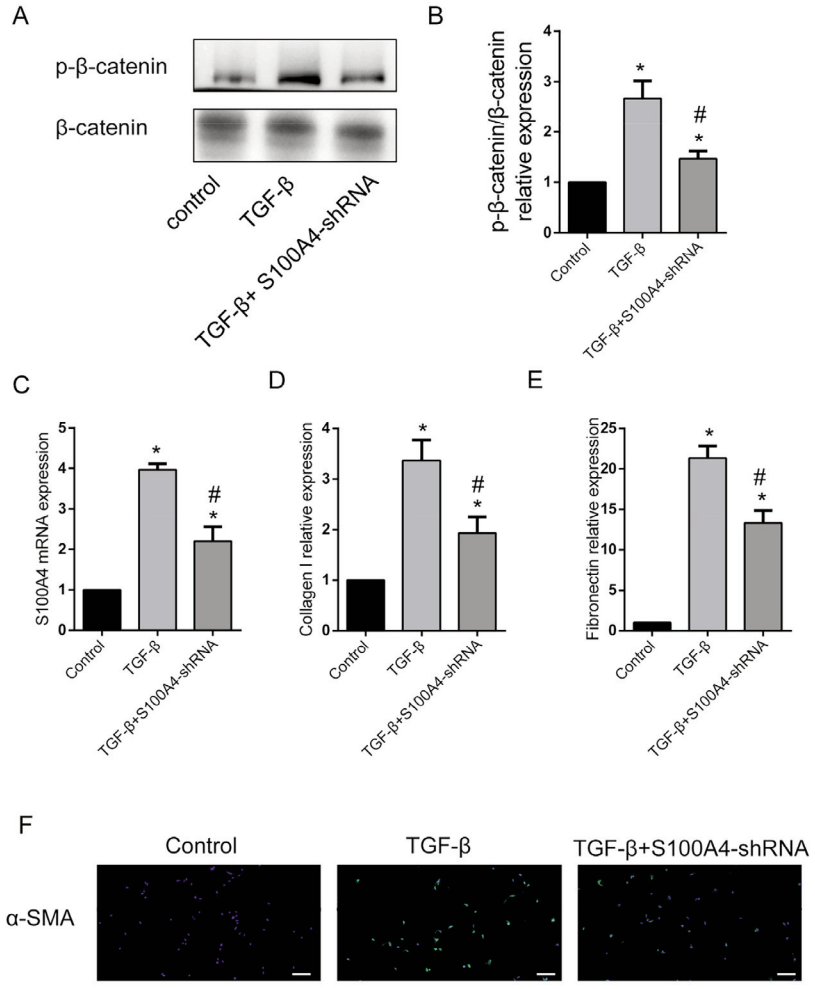

Figure 2. Downregulation of S100A4 inhibits HSCs differentiation into myofibroblasts *via* Wnt/β-catenin signaling pathways. **A**) Representative western blot showing protein bands of phosphorylated β-catenin (p-β-catenin) and total β-catenin in HSCs under different conditions: control, TGF-β treatment, and TGF-β + S100A4-shRNA transfection. **B**) Quantitative analysis of the p-β-catenin/β-catenin ratio across experimental groups, indicating the attenuation of Wnt/β-catenin activation due to S100A4 downregulation. **C**) Relative mRNA expression of S100A4 in Control, TGF-β-treated, and S100A4-shRNA-treated groups. **D**) Relative RNA expression of collagen I and (**E**) fibronectin as markers of extracellular matrix synthesis in HSCs under different conditions. **F**) Immunofluorescence staining images showing α-SMA expression in HSCs under control, TGF-β-treated, and TGF-β + S100A4-shRNA conditions; downregulation of S100A4 reduces α-SMA expression, indicating inhibition of HSC differentiation into myofibroblasts. Scale bars: 100 μm.

results reflected that the expression of p-β-catenin declined significantly following S100A4 downregulation in fibrotic mice (Figure 3 A,B). Meanwhile, S100A4 expression decreased in the liver by qRT-PCR under the downregulation of S100A4 (Figure 3C). Compared with the CCl4-treated group in which CCl4 led to a significant elevation of collagen I and α-SMA, the expressions of both decreased in S100A4-inhibited mice (Figure 3 D,E). Consistently, immunohistochemistry staining and image analysis results displayed that the sporadic α-SMA immunopositive cells in liver (Figure 3F). In conclusion, S100A4 knockout modulates liver fibrosis in fibrotic mice through degradation of the Wnt /β-catenin signaling pathway.

Downregulation of S100A4 ameliorates CCl4-induced liver fibrosis in mice

Finally, we explored the therapeutic effect of decreasing S100A4 on mice with liver fibrosis. Masson trichrome staining revealed that downregulation of S100A4 attenuated liver fibrotic lesions in mice, as quantified by measuring the percentage of fibrosis-positive areas using ImageJ software (Figure 4A). Consistent with this observation, H&E staining showed that severe tissue destruction was in CCl4-treated mice, with extensive size of hepatocytes swelling and necrosis, intense neutrophilic infiltration. However, the histologic disruption of liver

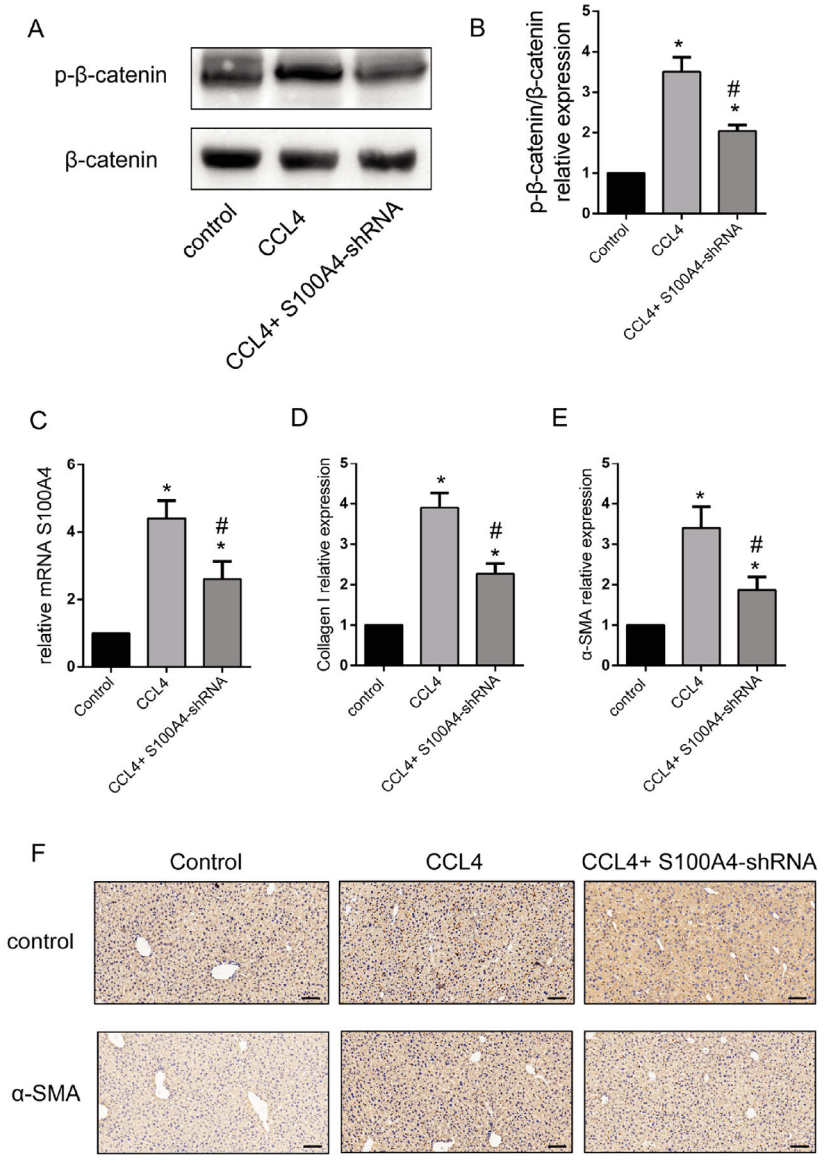

Figure 3. Downregulation of S100A4 inhibits hepatic fibrosis by degrading Wnt/β-catenin signaling pathways in fibrotic mice. **A**) Western blot showing p-β-catenin and total β-catenin protein levels in liver tissues from Control, CCl4-treated, and CCl4 + S100A4-shRNA-treated mice. **B**) Quantitative analysis of the p-β-catenin/β-catenin ratio in liver tissues, highlighting the inhibition of Wnt/β-catenin signaling in the S100A4-downregulated group. **C**) Relative mRNA expression of S100A4 in liver tissues from Control, CCl4-treated, and CCl4 + S100A4-shRNA-treated mice. **D**) Relative RNA expression of collagen I and (**E**) α-SMA in liver tissues, indicating reduced fibrogenesis due to S100A4 downregulation. **F**) Immunohistochemical staining of α-SMA in liver tissues showing reduced α-SMA-positive cells in the CCl4 + S100A4-shRNA group compared to the CCl4 group. Quantitative image analysis confirmed significant reduction in α-SMA-positive areas, reflecting the antifibrotic effect of S100A4 downregulation; scale bars: 50 μm.

tissue was mitigated *via* the downregulation of S100A4 (Figure 4B). Subsequently, ALT and AST were detected for liver function assessment, showing that ALT and AST levels in serum were decreased after downregulation of S100A4 (Figure 4 C,D). Hence, the results suggest that downregulation of S100A4 significantly alleviates CCl4-induced liver fibrosis in mice and is conducive to improving liver function.

Discussion

Several studies have shown that S100A4 is considered as a criterion in the various fibrotic organs and tissues. S100A4 has been regarded as a necessity during pulmonary fibrosis *via* promoting lung fibroblasts.[17] S100A4 can active lung fibroblasts in pulmonary fibrosis.[18] Additionally, the activity of p53 can be regulated by S100A4, mediating cardiac fibrosis potentially.[19] In the pathogenesis of fibrosis-associated hepatocellular carcinoma, Li *et al*. found that S100A4 affects liver fibrosis and increase fibrosis-related liver cancer stemness.[18] However, previous studies were mainly focused on its role among various organs. In our experiment, we gave a detail description on the expression of S100A4 in pathologic process of hepatic fibrosis and clarified the efficacy and safety of S100A4 downregulation. S100A4 mRNA expression levels and protein contents are highly upregulated in HSCs after TGF-β treatment but the downregulation of S100A4 has an antagonistic effect on the process of liver fibrosis. Increased TGF-β-induced liver fibrosis which is characterized by extensive ECM synthesis and secretion and inhibition of extracellular matrix degradation, promotes massive myofibroblast activation and proliferation.[20] Following TGF-β administration, S100A4 was positively correlated with treatment time in HSCs, significantly contributing to fibrosis progression. Besides, we used shRNA interference technology to downregulate the expression of S100A4 in HSCs and observed that silencing S100A4 inhibits TGF-β-induced HSCs differentiation manifested by the decreased level of collagen I, fibronectin and α-SMA. Tissue fibrosis in various diseases is closely related to the activation of Wnt/β-catenin signaling pathway. A report demonstrated that Galangin ameliorates hepatic fibrosis by regulating HSCs apoptosis *via* Wnt/β-Catenin signaling pathway in LX-2 Cells.[21,22] Recent studies have further emphasized the critical role of Wnt/β-catenin signaling in liver fibrosis.[23-25] Above results suggest that Wnt/β-catenin signaling pathway plays a significant role in liver fibrosis. In the present experiment, we found that the expressions of p-β-catenin are up-regulated in TGF-β-treated HSCs and in CCl4 induced hepatic fibrosis mice. However, knockout of S100A4 decreases the Wnt/β-Catenin signaling pathway and reduces the synthesis of fibrogenic factors in TGF-β-treated HSCs and mice with hepatic fibrosis. Interestingly, we found that when S100A4 was downregulated, less p-β-catenin were quantified. These results imply that Wnt/β-Catenin signaling pathway is activated by S100A4 in the liver fibrosis and the downregulation of S100A4 plays an ameliorative role in liver fibrosis by blocking

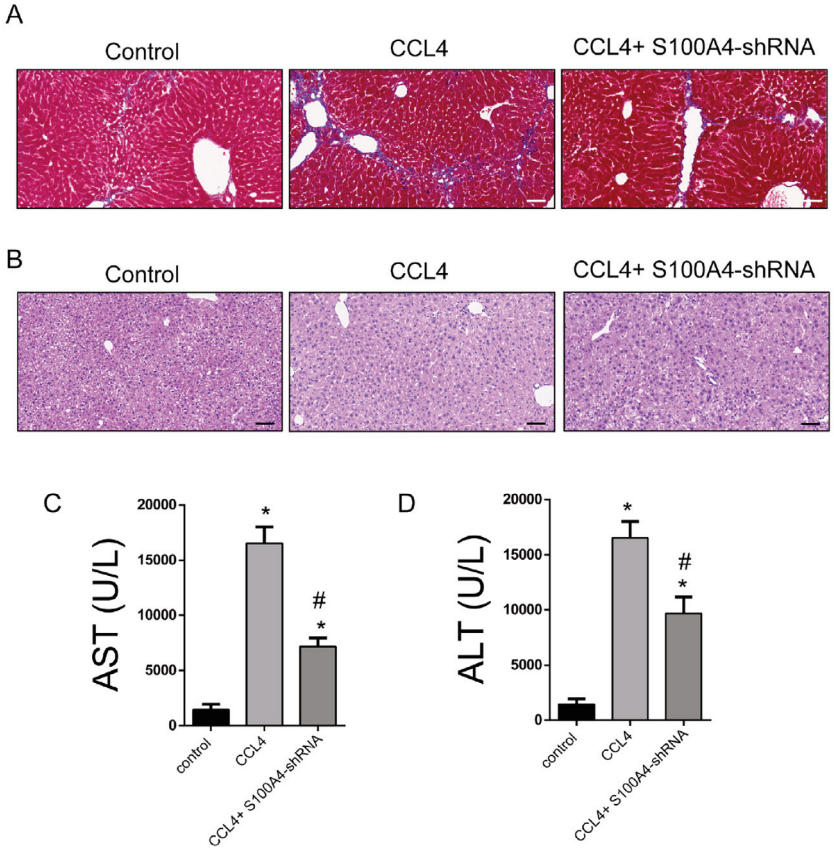

Figure 4. Downregulation of S100A4 ameliorates CCl4-induced liver fibrosis in mice. **A**) The Masson images of liver in control, CCl4 and CCl4+S100A4-shRNA group; scale bars: 50 μm. **B**) H&E images of liver in control, CCl4 and CCl4+S100A4-shRNA group; scale bars: 50 μm. The qualification of ALT(**C**) and AST(D) in control, CCl4 and CCl4+S100A4-shRNA group post CCl4 treatment.

Wnt/β-Catenin signaling pathway. S100A4 is implicated in promoting liver fibrosis *via* activation of HSCs.[26] Our results suggest that downregulating S100A4 may regulate Wnt/β-Catenin signaling pathway to inhibit hepatic fibrosis and further verify the biocompatibility and safety of the knockout of S100A4, complementing the previous findings.

Importantly, previous studies have shown many S100 calcium-binding protein family members have potent inflammation and apoptosis properties.[27] For example, S100A12 can regulate vascular inflammation in smooth muscle cells and S100A8/A9 increases the mobilization of pro-inflammatory monocytes in osteoarthritis.[28] Meanwhile, Wnt/β-Catenin signaling pathway is indispensable to inflammation and apoptosis.[29] Hence, S100A4 may be also closely associated with inflammation and apoptosis *via* regulating Wnt/β-Catenin signaling pathway, which needs to be explored in further investigation. Taken together, downregulation of S100A4 ameliorates the process of liver fibrosis, improves histological and functional recovery of liver, and inhibits TGF-β-mediated fibrosis in HSCs by inhibiting Wnt/β-Catenin signaling pathway. Our study has proved that S100A4 is a new potential direction to treat liver fibrosis in future. Our study, while demonstrating the antifibrotic potential of S100A4 downregulation, has several limitations. Primarily, the experiments were conducted using animal models, which, despite their relevance, do not fully replicate the complexity of human liver fibrosis. Therefore, further studies involving human liver samples and clinical trials will be essential to validate our findings and assess their translational potential.

Targeting S100A4 holds significant therapeutic potential due to its key role in regulating fibrosis. However, clinical applications require careful consideration of potential limitations, including the possibility of off-target effects and the need for tissue-specific delivery methods. For instance, nanoparticle-based siRNA systems or antibody-based therapies may provide a promising approach to selectively downregulate S100A4 in hepatic tissues while minimizing systemic risks. Furthermore, comprehensive preclinical testing is essential to evaluate long-term safety and efficacy.

We acknowledge that while downregulation of S100A4 demonstrates promising antifibrotic effects, its clinical application may involve challenges. Potential side effects, including unintended immune responses and off-target effects on other tissues expressing S100A4, require further investigation. Future studies should focus on developing targeted delivery methods, such as nanoparticle-based siRNA systems, to ensure tissue-specific downregulation and minimize systemic risks.

In summary, this experiment shows that downregulation of S100A4 can effectively reduce liver fibrosis *via* the regulation of Wnt/β-catenin signaling pathway. This may provide a promising auxiliary therapy for liver fibrosis. More experiments and evaluations are needed to further strengthen our research results.

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