Original Research

Targeting transforming growth factor βRII expression inhibits the activation of hepatic stellate cells and reduces collagen synthesis

Rongquan Fu[1], Jinguo Wu[1], Jiguang Ding[1], Jifang Sheng[2], Liang Hong[1], Qingfeng Sun[1], Hui Fang[2] and Dairong Xiang[1]

[1]Department of Infectious Diseases, the Third Affiliated Hospital of Wenzhou Medical College, 108 Wan song Road, Rui'an, Zhejiang 325200; [2]Department of Infectious Diseases, the First Affiliated Hospital of Medical School of Zhejiang University, Hangzhou, Zhejiang 310003, China
Corresponding author: Jinguo Wu. Email: WuJinguo126@126.com

Abstract

Abnormal production of extracellular matrix (ECM) components significantly contributes to the development of liver fibrosis. This study aimed at examining the effects of short-hairpin RNA (shRNA)-mediated transient knockdown of transforming growth factor βRII (TGFβRII) expression on the proliferation and activation of hepatic stellate cells (HSCs) and synthesis of fibrogenic ECM components in HSC cells. Three different shRNA-expressing plasmids were constructed for the expression of shRNA-(a, b, c) targeting to the rat TGFβRII mRNA beginning at nucleotide position 339, 444 and 528 and they were transfected into a rat stellate cell line, HSC-T6 cells, respectively. The levels of TGFβRII, α-smooth muscle actin (α-SMA), and type I and III collagen expressions were characterized by reverse transcription polymerase chain reaction and Western blot assays. The concentrations of hyaluronic acid (HA) and type IV collagen in the supernatants of cultured cells were measured by enzyme-linked immunosorbent assay. Transfection with the TGFβRII-specific shRNAs resulted in varying levels of inhibition in the expression of TGFβRII in HSC-T6 cells, and transfection with the potent shRNA-c inhibited the expression of TGFβRII in a dose-dependent manner. Knockdown of TGFβRII expression significantly reduced the levels of α-SMA, type I, III and IV collagen, and HA expression in HSC-T6 cells ($P < 0.01$). In conclusion, our data indicated that knockdown of TGFβRII expression inhibited the activation of HSCs and the production of fibrogenic ECM components in HSC-T6 cells. Therefore, our findings support the notion that TGFβRII is an important factor of the pathogenic process of liver fibrosis.

Keywords: TGFβ, liver fibrosis, collagen, α-SMA, hyaluronic acid

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Introduction

Liver fibrosis can lead to liver cirrhosis and is a major health problem with high morbidity and mortality worldwide.[1,2] Liver fibrosis usually results from continuous wound-healing responses to chronic hepatic injuries, such as chronic hepatitis, long-term alcohol abuse and non-alcoholic steatohepatitis.[1] Histologically, liver fibrosis is characterized by excessive deposition of extracellular matrix (ECM) constituents of different types of collagens, glycoproteins and proteoglycans in the liver, which forms a fibrous scar and distorts the hepatic architecture.[3] However, regulation of the production of collagen and the fibrosis process is still poorly understood. Discovery of the mechanisms underlying the process of liver fibrosis should be of great significance in the design of new therapy for human liver fibrosis.

A previous study has revealed that hepatic stellate cells (HSCs), also known as lipocytes, Ito cells or perisinusoidal cells, are primarily responsible for collagen production in normal liver as well as during fibrogenesis.[4] Following liver injury, HSCs usually undergo an activation process to contractile myofibroblasts, characterized by the up-regulation of specific markers, such as α-smooth muscle actin (α-SMA).[5,6] In clinical practice, liver biopsy has been the gold standard for the diagnosis of liver fibrosis. However, its invasive nature has scared many patients. Recently, numerous studies have found that high levels of serum N-terminal propeptide of type III collagen (ColIII), hyaluronic acid (HA), tissue inhibitor of metalloproteinase type 1 (TIMP-1) and YKL-40 are biomarkers for the development of liver fibrosis and have been used for the diagnosis of liver fibrosis.[7] However, how the expression of these proteins is up-regulated following HSC activation and during the process of liver fibrosis remains unclear. Previous studies have shown that a variety of intracellular signaling

cascades regulate the expression of the HSC activation-related target genes and form a regulatory network during the process of liver fibrosis. Hence, dissection of each pathway and understanding its role in the process of liver fibrosis may provide a framework for the design of new therapies for the treatment of human liver fibrosis, In particular, the pathway that is initiated following HSC activation plays an essential role in controlling target gene expression and contributes to the fibrogenic responses of HSCs. In the current study, we focused on one pathway, the transforming growth factor β (TGFβ) signaling.

The TGFβ and TGFβ receptor-related signal pathway are critical regulators of liver fibrosis. The TGFβ superfamily consists of a large number of structurally related polypeptide growth factors that regulate many aspects of cellular processes, such as embryogenesis, proliferation, differentiation, adhesion, apoptosis, migration and fibrosis.[8] Engagement of type II (TGFβRII) and type I (TGFβRI) serine/threonine kinase receptors on the plasma membrane by TGFβ promotes a heteromeric receptor complex formation and receptor activation. The activated TGFβRI recruits and phosphorylates the receptor-regulated Smad proteins (R-Smads). Subsequently, the phosphorylated R-Smads dissociate from the cell membrane receptors and form a complex with a common mediator Smad, Smad4. These Smad complexes then translocate into the nucleus, where they regulate the transcription of target genes.[9] TGFβ is a potent and prominent fibrogenic cytokine, and regulates the function of HSCs in both autocrine and paracrine manners during the process of liver fibrosis.[10] Consequently, TGFβ signaling can stimulate the synthesis of ECM components, such as type I collagen (ColI), ColIII, type IV collagen (ColIV), fibronectin, tenascin, elastin and HA; up-regulate the expression of antiproteases such as TIMP-1 and plasminogen activator inhibitor; and down-regulate interstitial collagenase and other matrix metalloproteinases. Therefore, TGFβ signaling has become an attractive target for antifibrotic therapy.[11,12]

In the current study, we used the rat HSC line, HSC-T6, as a model and investigated the *in vitro* effects of targeting TGFβRII with short-hairpin RNA (shRNA) on the proliferation and activation of HSCs and the production of ECM components in HSC-T6 cells.

Materials and methods

Cell culture

The immortalized rat liver stellate cell line, HSC-T6, was obtained from the Cell Bank of Xiangya School of Medicine (Changsha, China) and cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco, USA), at 37°C, in a humidified atmosphere of 5% CO_2 in air.

Transfection of shRNA targeting TGFβRII

Three target sequences for rat TGFβRII gene (NM_031132) were chosen, according to the siRNA Design Guidelines (www.ambion.com), and these sequences were non-homologous to any other rat genes, analyzed by BLAST (Basic Local Alignment Search Tool) analysis (www.ncbi.nlm.nih.gov/BLAST). These target sequences were designed and synthesized as (1) 5′AAGTCGGTTAACAGCGATCTG 3′; (2) 5′AAGTCTTGCATGAGCAACTGC 3′; and (3) 5′ AAGA ACATTACTCTGGAGACC 3′. These three target sequences were then cloned into the shRNA-expressing plasmid pSilencer2.1-U6 (Ambion, Austin, TX, USA) and named shRNA-a, -b and -c, respectively (Figure 1). As a control, a scrambled sequence (5′AAGCTTCATAAGGCGC ATAGC3′) without targeting any known rat genes was also constructed into pSilencer2.1-U6 (shRNA-Ctrl). These shRNA-expressing plasmids were identified by DNA sequencing.

To knockdown TGFβRII expression, HSC-T6 cells in 12-well plastic plates were transfected in duplicate with shRNA-a, shRNA-b, shRNA-c or shRNA-Ctrl separately for varying periods using Metafectene, according to the manufacturer's instruction (Invitrogen, Gaithersburg, MD, USA).

Reverse transcription followed by semiquantitative polymerase chain reaction

Total RNA was extracted from HSC-T6 that had been transfected with individual types of plasmids using Trizol (Invitrogen), and 3 μg of total RNA was reversely transcribed into cDNA using RT-PCR kit (Takara, Shiga, Japan), according to the manufacturer's instructions. The relative levels of target mRNA transcripts to control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined by polymerase chain reaction (PCR) using the cDNA as a template and specific primers. The PCR primers were synthesized by Takara, and their sequences were forward 5′-TGGAGGAAGAACGACAAGAAC-3′, reverse 5′-CGGTGGACACGGTAACAGTAG-3′ for TGFβRII (NM_031132) with the amplicon size of 317 base pairs (bps); forward 5′-AGGGTCATCGTGGCTTCTCT-3′, reverse 5′-CAGGCTCTTGAGGGTAGTGT-3′ for type I collagen alpha 1 (Col1a1, NM_053304.1) with the amplicon size of 392 bp; forward 5′-AGCGGAGAATACTGGGTTGA-3′, reverse 5′-GATGTAATGTTCTGGGAGGC-3′ for type III collagen alpha 1 (Col3a1, AJ005395.1) with the amplicon size of 290 bp; and forward 5′-GTGCTGAGTATGTCGTGGAG-3′, reverse 5′-GTCTTCTGAGTGGCAGTGAT-3′ for internal control GAPDH (AF106860.2) with the amplicon size of 289 bp. The PCR reactions were performed at 95°C for five minutes, and subjected to 30 cycles of 94°C for 45 s, 56°C for 45 s and 72°C for one minute, followed by a final extension at 72°C for 10 min. The PCR products were analyzed by 2% agarose gel electrophoresis and visualized with ethidium bromide, followed by imaging. The relative levels of each target gene mRNA transcripts were analyzed by densimetric scanning using ImageTool software (version 3.0) and expressed as the ratio of target to control GAPDH.

Western immunoblot

To examine the expression of target proteins, the plasmid-transfected HSC-T6 cells were harvested and lysed in buffer containing 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L

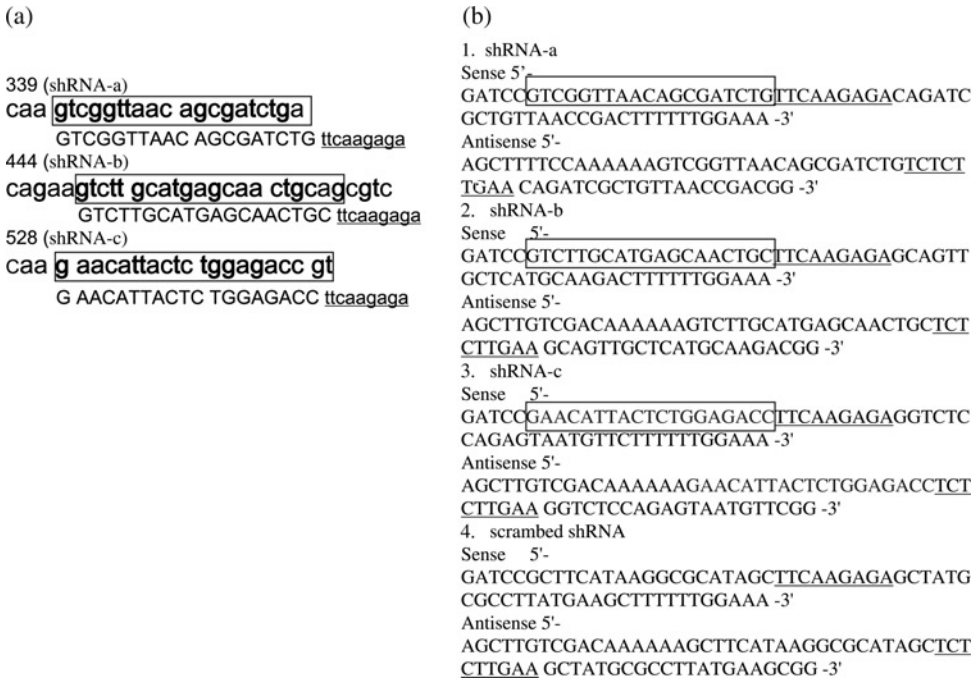

Figure 1 Construction of short-hairpin RNA (shRNA)-expressing plasmids. (a) A schematic map of the targeted sequences of these small interfering RNAs (siRNAs). The specific sequences in mRNA targeted by siRNA were boxed and the loop sequence was underlined. The numbers reflect the nucleotide positions. (b) The sequences of individual shRNAs were cloned into the *Bam*HI and *Hin*dIII sites of the vehicle and the loop sequences were underlined

NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 2 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L PMSF, 1 mmol/L leupeptin, 0.1 mol/L Pepstatin A and 30 μg/mL aprotinin. After quantification of the protein concentration using the BCA kit (Pierce, Rockford, IL, USA), the lysate proteins (60 μg/lane) were separated by 8% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred onto a poly(vinylidene fluoride) membrane, followed by being blocked with 5% non-fat milk in TBST (0.2% Tween-20, 20 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.4) for two hours. The target proteins were probed with individual antibodies anti-Col1a1 (1:200), anti-Col3a1 (1:200, Oncogene), anti-TGFβRII (1:500), anti-α-SMA (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin (internal control) (1:200; Santa Cruz Biotechnology) at 4°C overnight. Subsequently, the bound antibodies were detected by horseradish peroxidase-conjugated secondary antibody (1:2000, Santa Cruz Biotechnology) and visualized with ECL substrate (Amersham, Buckinghamshire, UK).

MTT assay

HSC-T6 cells at 10^5/well were cultured in 96-well plates and transfected with, or without, 1 μg of each type of plasmid (six wells per group), as described above for 48 h. The cells were exposed to 5 mmol/L of MTT (3-4,5-dimethylthiazol-2,5 diphenyl tetrabromide; Sigma, St Louis, MO, USA) during the last four hours of incubation. The generated formazan was dissolved in dimethyl sulfoxide and measured for the absorbance at 570 nm.

Enzyme-linked immunosorbent assay

The levels of HA and ColIV secreted in cell culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using specific kits, according to the manufacturer's instructions (Haiyan Biotech, Shanghai, China).

Statistical analysis

Quantitative data are presented as mean $\pm$ SD from three independent experiments. The difference among different groups of cells was calculated by Student's *t*-test using SPSS software (version 13.0). A value of $P < 0.05$ was considered statistically significant.

Results

Knockdown of TGFβRII expression in HSC-T6 cells

To investigate the role of TGFβ signaling in regulating liver fibrosis, we designed three TGFβRII-specific shRNAs that targeted different sequences in the TGFβRII gene. After cloning these sequences into the shRNA-expressing plasmid pSilencer2.1-U6, three plasmids, shRNA-a, shRNA-b and shRNA-c, were generated and confirmed by DNA sequencing (data not shown). A similar approach was used for the generation of control plasmid containing a scrambled sequence that did not target any of the known rat genes.

To determine the efficacy of TGFβRII knockdown, HSC-T6 cells were transfected with individual types of plasmids for 48 h, and the relative levels of TGFβRII expression

to control GAPDH were determined by reverse transcription (RT)-PCR and Western blot assays using untransfected HSC-T6 cells as control (Figures 2a and b). As expected, transfection of HSC-T6 cells with control shRNA did not modulate the levels of TGFβRII expression because similar levels of TGFβRII mRNA transcripts and proteins were detected in control shRNA-transfected and untransfected blank HSC-T6 cells. In contrast, transfection with different TGFβRII-specific shRNAs resulted in varying levels of inhibition in the TGFβRII expression. Transfection with shRNA-a, shRNA-b and shRNA-c reduced the levels of TGFβRII expression by 10–15%, 50–55% and 75–77%, respectively, as compared with that in control cells. Apparently, shRNA-c was the most potent in silencing TGFβRII expression in our experimental system. More importantly, transfection with different doses of shRNA-c displayed dose-dependent inhibition in reducing the expression of TGFβRII in HSC-T6 cells (Figure 2c).

Targeting TGFβRII inhibited the activation and proliferation of HSC-T6

Following transfection of shRNA-expressing plasmids, we first measured the cell viability 48 h after transfection by an MTT assay. As shown in Figure 3a, while transfection with control plasmid did not affect the proliferation and survival of HSC-T6 cells, the optical density value of HSC-T6 cells that had been transfected with the plasmid shRNA-c was significantly less than that in the other two groups ($P < 0.05$). Hence, knockdown of TGFβRII expression by the shRNA-c significantly inhibited the proliferation of HSC-T6 cells, which may be associated with the inhibition of endogenous TGFβ-mediated signaling, activation and proliferation in HSC-T6 cells.

Since the activation of HSCs plays a significant role in promoting the development of liver fibrosis, we investigated the effects of TGFβRII silencing on the activation of HSCs. We examined the relative levels of α-SMA expression, a marker for activated HSCs, and found that following transfection with shRNA-c for 48 h, the relative levels of α-SMA expression were significantly reduced by approximately 65%, as compared with that in unmanipulated and shRNA-Ctrl-transfected cells ($P < 0.01$; Figure 3). These data indicate that knockdown of TGFβRII expression inhibited the activation of HSC-T6 cells.

Knockdown of TGFβRII inhibited intracellular synthesis of type I and type III collagen

Next, we examined the impact of TGFβRII knockdown on the intracellular synthesis of ECM proteins, ColI and ColIII by RT-PCR and Western blot assays. As shown in Figure 4a, the levels of ColI mRNA transcripts and proteins in the cells transfected with the shRNA-c were significantly reduced by approximately 64% and 70%, respectively, as compared with that in control cells ($P < 0.01$). A similar pattern of inhibition in the ColIII mRNA transcripts and proteins was observed in the cells transfected with the shRNA-c ($P < 0.01$; Figure 4b). Therefore, knockdown of

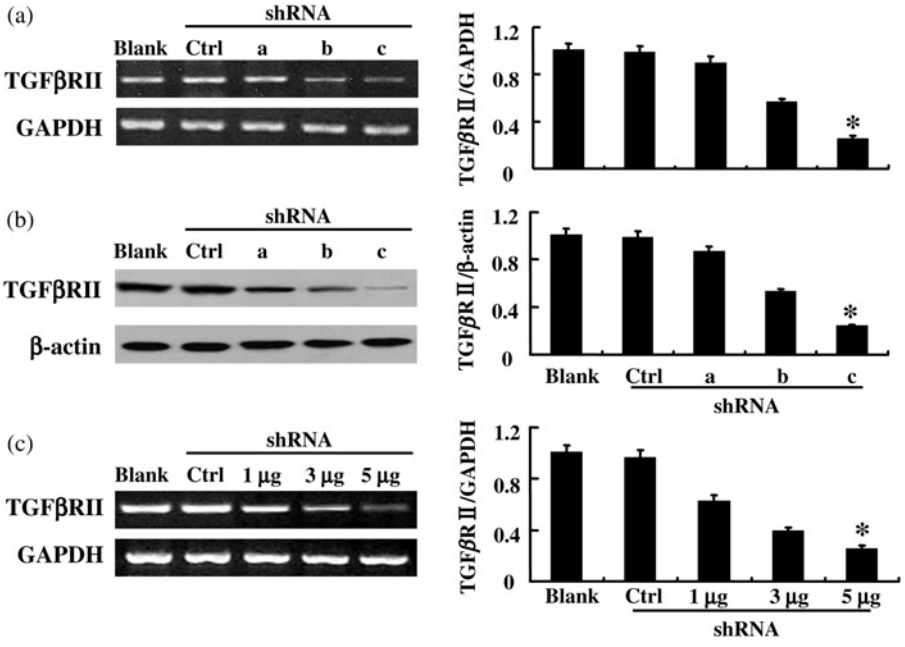

Figure 2 Knockdown of transforming growth factor βRII (TGFβRII) expression by specific short-hairpin RNAs (shRNAs). HSC-T6 cells were transfected with 5 μg of control (Ctrl) or one of the three TGFβRII-specific shRNA-expressing plasmids (lanes a, b and c) for 48 h and the relative levels of TGFβRII mRNA transcripts and proteins to control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined by reverse transcription polymerase chain reaction (RT-PCR) and Western blot assays. (a) RT-PCR analysis of the TGFβRII mRNA transcripts. (b) Western blot analysis of the relative levels of TGFβRII proteins. (c) Dose-dependent effects of shRNA-c on the levels of TGFβRII mRNA transcripts. Data shown are representative images and expressed as mean $\pm$ SD of each group of cells from three independent experiments. The relative levels of TGFβRII mRNA transcripts and proteins in unmanipulated (blank) control cells were arbitrarily designated as 1. $^*P < 0.01$ versus controls

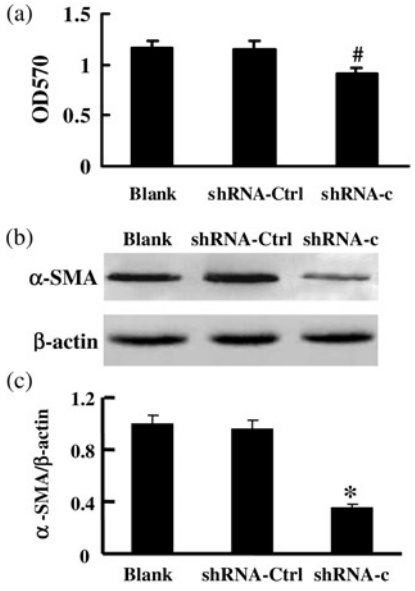

Figure 3 Knockdown of transforming growth factor βRII (TGFβRII) expression inhibited the proliferation and activation of HSC-T6 cells. HSC-T6 cells were cultured in medium alone (blank) or transfected with the indicated short-hairpin RNA (shRNAs) for 48 h and the impact of TGFβRII silencing on the proliferation and activation of HSC-T6 *in vitro* was determined by MTT (3-4,5-dimethylthiazol-2,5 diphenyl tetrabromide) and Western blot assays, respectively. (a) MTT assay analysis of HSC-T6 cell proliferation. Data are expressed as mean ± SD of each group of cells from two separate experiments. $^{\#}P < 0.05$ versus controls. (b) The relative levels of α-smooth muscle actin (α-SMA) expression were determined by Western immunoblot and densimetrically scanning. Data shown are representative images from three independent experiments. (c) Quantitative analysis of the expression of α-SMA by densimetric scanning. Data are expressed as mean ± SD of each group of cells from three separate experiments, and the level of α-SMA expression in control cells was designated as 1. $^{*}P < 0.01$ versus controls

TGFβRII expression inhibited intracellular synthesis of ColI and ColIII in HSC-T6 cells.

Knockdown of TGFβRII expression reduced the secretion of HA and type IV collagen

Next, we measured the levels of ECM components, HA and ColIV, secreted in the cell culture supernatants by ELISA. While similar concentrations of HA and ColIV were detected in the supernatants from unmanipulated and control shRNA-transfected HSC-T6 cells, the concentrations of HA and ColIV in the supernatants from the shRNA-c-transfected HSC-T6 cells were significantly reduced by 50–67%, as compared with controls ($P < 0.01$; Figures 5a and b). However, transfection with the shRNA-c for varying periods did not cause significant difference in inhibition of HA and ColIV secretion in HSC-T6 cells. These data clearly indicated that knockdown of TGFβRII expression significantly and rapidly inhibited the secretion of fibrogenic HA and ColIV in HSC-T6 cells.

Discussion

In the present study, we examined the impact of knockdown of TGFβRII expression, the cell-surface receptor essential for initiating downstream TGFβ signaling, with shRNA on the endogenous TGFβ-mediated activation, proliferation and synthesis of fibrogenic ECM components in HSC-T6 cells *in vitro*. We found that knockdown of TGFβRII expression inhibited the activation and proliferation of HSC-T6 cells *in vitro* and decreased the synthesis/secretion of fibrogenic ECM components, such as type I, III, IV collagens and HA.

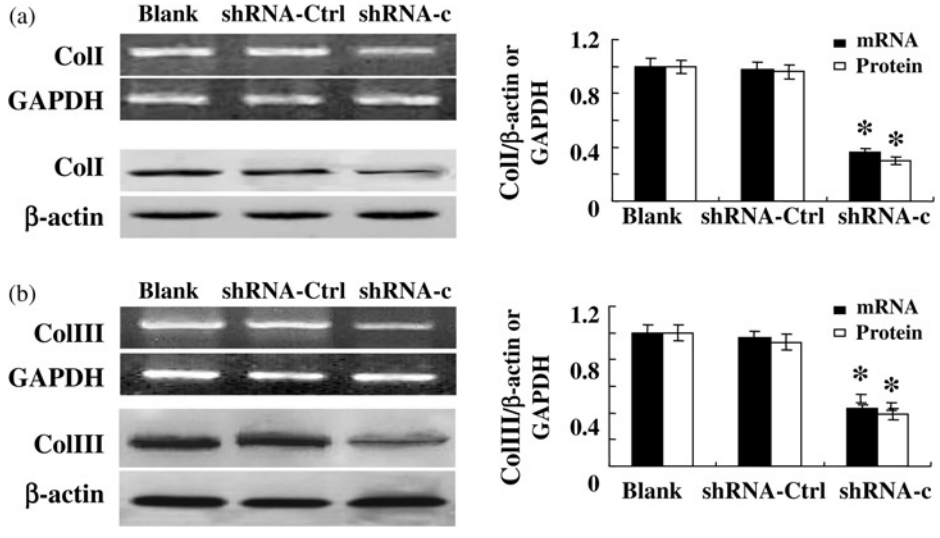

Figure 4 Knockdown of transforming growth factor βRII (TGFβRII) expression inhibited the expression of type I and III collagens. HSC-T6 cells were transfected with the indicated plasmids, respectively. The levels of ColI (a) and ColIII (b) mRNA transcripts and proteins to controls were determined by RT-PCR and Western blot analysis, respectively, followed by densimetric analysis. Data shown are representative images (the left panels) or expressed as mean ± SD (the right panels) of each group of cells from three independent experiments. The levels of ColI and ColIII expression in Blank cells were arbitrarily defined as 1. The upper panels: RT-PCR; the bottom panels: Western blot analysis. $^{*}P < 0.01$ versus control cells. GAPDH, glyceraldehyde 3-phosphate dehydrogenase

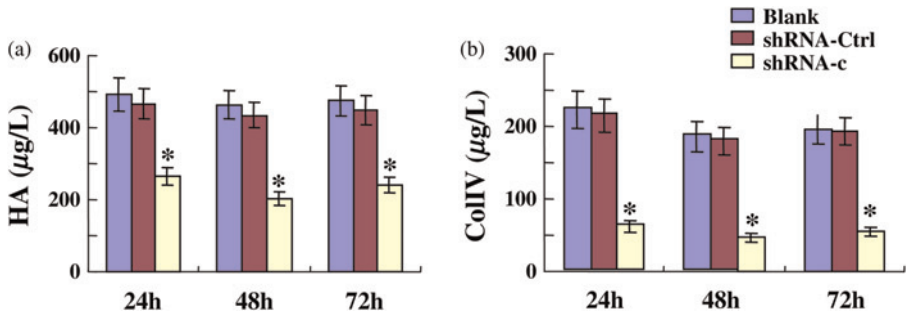

Figure 5 Knockdown of transforming growth factor βRII (TGFβRII) expression inhibited the secretion of hyaluronic acid (HA) and type IV collagen. HSC-T6 cells were transfected with the indicated plasmids for varying periods, and the concentrations of HA (a) and ColIV (b) secreted in the supernatants of cultured cells were measured by enzyme-linked immunosorbent assay. $^{*}P < 0.01$ versus controls

The RNA interference (RNAi) has been shown to be a powerful tool to analyze the function of mammalian genes and a potential therapeutic approach for human disease. The small interfering RNA (siRNA) duplexes can bind to and promote the degradation of target mRNAs in a sequence-specific manner, by silencing the expression of specific genes.[13] Two general approaches have been used for silencing the selective gene. Cytoplasmic delivery of short dsRNA oligonucleotide mimics an active intermediate of the endogenous RNAi mechanism to silence the expression of the target gene. Furthermore, nuclear delivery of gene cassettes expresses shRNA that can be subsequently processed to siRNA by the cellular machinery.[14] Therefore, design of the siRNA/shRNA sequence for a specific gene will be crucial for effective knockdown of the gene expression.[14] We targeted the expression of TGFβRII in HSC-T6 cells because TGFβ is an important regulator of human liver fibrosis and TGFβRII is the essential cell-surface receptor responsible for ligand binding and signal transduction. We designed three shRNA sequences that targeted the rat TGFβRII mRNA beginning at nucleotide 339, 444 and 528, respectively. After cloning these sequences into the vehicle, three TGFβRII-specific shRNA-expressing plasmids were generated. We found that transfection with these plasmids into HSC-T6 cells resulted in varying levels of inhibition in the expression of TGFβRII, and the shRNA-c was the most potent, inhibiting the TGFβRII expression in a dose-dependent manner in HSC-T6 cells. Apparently, validation of the efficacy of shRNA-mediated knockdown of specific gene expression *in vitro* is crucial for effectively silencing it *in vivo*.

High levels of TGFβ production are associated with the pathogenesis of liver fibrosis.[15] TGFβ can activate HSCs and promote the deposition of collagen in perisinusoidal space, enhancing the process of liver fibrosis.[16] Following activation, HSC cells can convert into myofibroblasts, which express high levels of TGFβ1.[11,17] Subsequently, TGFβ1 further induces the transdifferentiation of quiescent HSCs into myofibroblasts that undergo proliferation, express α-SMA and produce an excess of ECM molecules in autocrine and paracrine manners.[18] Hence, TGFβ1 is a critical regulator of the pathogenesis of liver fibrosis. We found that transient knockdown of TGFβRII expression by the shRNA approach significantly reduced the viability of HSC-T6 and inhibited the expression of α-SMA, suggesting that silencing TGFβRII expression inhibited the proliferation and activation of HSCs *in vitro*. Furthermore, knockdown of TGFβRII expression dramatically reduced the expression of ColI and ColIII, and the production of ColIV and HA in HSCs. Our data provide additional evidence to demonstrate that TGFβ signaling is critical for regulating the production of multiple ECM components by HSCs. Our findings are similar to that from a 'dominant-negative' strategy or soluble TGFβRII treatment.[19–23] Apparently, blocking the TGFβ signaling by targeting TGFβRII expression can effectively inhibit the process of liver fibrosis. Conceivably, the shRNA-based knockdown of TGFβRII expression may be used for the treatment of liver fibrosis in clinical practice.

In summary, we provide evidence that transient knockdown of TGFβRII expression by shRNA-mediated gene silencing inhibits the proliferation and activation of HSCs and reduces the synthesis and secretion of fibrosis-related ECM components in HSCs *in vitro*. We are interested in further investigating the impact of stable transfection with the shRNA-expressing plasmid for persistent knockdown of TGFβRII expression on the exogenous and endogenous TGFβ-mediated signaling and the synthesis of fibrosis-related ECM components in HSCs. Therefore, our findings support the notion that *TGFβRII* is an important factor of the pathogenic process of liver fibrosis.

Author contributions: RF helped in designing the experiments and interpreting the data; JW was involved in interpreting the data and writing the manuscript; JD, JS, LH, HF performed experiments and collected data; QS was involved in searching for information and discussing the manuscript; and DX helped in analysis of data.

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