MicroRNA-146a modulates TGF-beta1-induced hepatic stellate cell proliferation by targeting SMAD4

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A B S T R A C T

Activation of hepatic stellate cells (HSC) plays a pivotal role in the development of hepatic fibrosis. Transforming growth factor-β1 (TGF-β1) is considered to be the main stimuli factor responsible for the activation of HSC. MicroRNAs (miRNAs) have recently been shown to regulate cell proliferation, differentiation, and apoptosis. The involvement of miRNAs and their roles in TGF-β1-induced HSC activation remains largely unknown. Our study found that the expression of miR-146a was downregulated in HSC in response to TGF-β1 stimulation in dose-dependent manner by one-step real-time quantitative PCR. Moreover, we sought to examine whether miR-146a became dysregulated in CCL4-induced hepatic fibrosis in rats. Our study revealed that miR-146a was downregulated in liver fibrotic tissues. In addition, the HSC transfected with miR-146a mimics exhibited attenuated TGF-β1-induced α-smooth muscle actin (α-SMA) expression compared with the control. Furthermore, overexpression of miR-146a suppressed TGF-β1-induced HSC proliferation, and increased HSC apoptosis. Bioinformatics analyses predict that SMAD4 is the potential target of miR-146a. MiR-146a overexpression in TGF-β1-treated HSC did not decrease target mRNA levels, but significantly reduced target protein expression. These results suggested that miR-146a may function as a novel regulator to modulate HSC activation during TGF-β1 induction by targeting SMAD4.

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1. Introduction

Liver fibrosis is a chronic scarring process of the liver that is associated with an excessive accumulation of extracellular matrix (ECM). Hepatic stellate cells (HSCs), the major mesenchymal cells in liver, are widely accepted as playing a critically important role in liver fibrosis [1,2]. Following multiple injuries and/or exposure to inflammatory cytokines, activated HSCs lose their lipid droplets, migrate to injured sites and are transformed into myofibroblast-like cells that secrete large amounts of ECM leading finally to liver fibrosis [3]. The transdifferentiation of fibroblasts to myofibroblasts can be caused by different inflammatory cytokines, where transforming growth factor-β1 is recognized as the main profibrogenic mediator [4,5]. TGF-β1 mainly activates HSC through TGF-β1/Smad signal pathway, thus causing hepatic fibrosis [6,7]. Smads are intracellular signal transductive molecules of the TGF-β superfamily. However, that SMAD4 plays a key role in transduction of TGF-β1 signals comes from multiple lines of biochemical and genetic evidence [8]. While it has been reported that both in vivo and in vitro TGF-β1 trigger the fibroblast phenotype to myofibroblasts including increased expression of alpha-smooth muscle actin (α-SMA), so far, less is known about the regulatory mechanism of this process.

MicroRNAs (miRNAs) are endogenous, small, non-coding RNAs ranging in size from 20 to 25 nt [9,10]. And miRNAs work as posttranscriptional regulators of gene expression through their interaction with the 3′untranslated region (3′UTR) of target mRNAs [11,12]. In the past few years, miRNAs are well known to participate in cell proliferation, differentiation, and apoptosis. Regarding HSCs, recent studies have demonstrated that overexpression of miR-29b markedly attenuated the expression of Col1a1 and Col1a2 mRNAs and led HSCs to remain in a quiescent state [13]. MiR-15b and miR-16 may be essential for apoptosis by targeting Bcl-2 and the caspase signaling pathway [14]. MiR-150 and miR-194 were also reported to inhibit HSC activation and ECM production, at least in part, via inhibition of c-myc and rac1 expression [15]. These studies suggest that miRNAs are HSC regulators that play an important role in liver fibrosis.

MiR-146a, located in the second exon of the LOC285628 gene on human chromosome 5, is a negative regulator of immune and inflammatory responses and is believed to be associated with the pathogenesis of various autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis [16–18]. But the roles of miR-146a during TGF-β1-induced HSC proliferation and function remain limited at the present. In this study, we show the effects of miR-146a on TGF-β1-induced HSC activation. Overexpression of miR-146a suppressed TGF-β1-induced HSC activation.

Abbreviations: ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSC, hepatic stellate cell; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor-β1; 3′UTR, 3′untranslated region; SMAD4, signaling effectors (mothers against decapentaplegic protein) 4.

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HSC proliferation, increased HSC apoptosis, and the expression of α-SMA in addition to its involvement in the well-known TGF-β/smads pathway. We further found that miR-146a is involved in TGF-β-induced HSC proliferation by targeting SMAD4.

2. Materials and methods

2.1. Animals, CCL4 liver injury model

Male Sprague–Dawley rats were obtained from the Experimental Animal Center of Anhui Medical University were used for CCL4 liver injury model. The animal experimental procedures were reviewed and approved by the University Animal Care and Use Committee. Liver fibrosis was generated by 12-week treatment of rats (200–220 g) with CCL4 (CCL4/olive oil, 1:1 (vol/vol) per kg body weight by intraperitoneal injection twice weekly) as previously described [19]. Control animals were treated intraperitoneally with 1 ml/olive oil/kg body weight at the same time intervals. 24h after the last CCL4 injection rats were sacrificed and liver tissues were harvested for the further analysis. The liver tissues were used for hematoxylin and eosin (H&E) staining and masson staining by following the manufacturer’s instructions at a 10% formalin.

2.2. Cell culture and transient transfection of miR-146a mimics

The HSC-T6 cell line was obtained from Shanghai Fumeng Gene Biological Corporation (Shanghai, China). The HSC-T6 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, USA) supplemented with 10% fetal bovine serum, 100U/ml penicillin, and 100 mg/ml streptomycin, and 2 mM L-glutamine and incubated at 37°C at an atmosphere of 5% CO2. On the day of transfection, the cells were plated in DMEM supplemented with 10% FBS at a density of 2×105 cells/ml and were transfected with the miR-146a mimics and the non-specific (NS)-miRNA (GenaPharma, China) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s instructions at a final concentration of 60 nM for 24h. The culture medium was changed 6h after transfection, and TGF-β1 (Peprotech, USA) was added at a concentration of 10 ng/ml. The sequences of oligonucleotides used are as follows:

miR-146a mimics: 5′-UGAGAACUGAUUCUAGGGUU-3′
5′-CACAUGGAUAUCUAGCACAUAU-3′
NS-miRNA: 5′-UUCUCGCAACGUCAGCGUTT-3′
5′-ACUGACACCUUGGAGAATT-3′

2.3. One-step quantitative real-time PCR

To confirm expression of miR-146a, one-step real-time qPCR was performed. Total RNA was extracted from HSC-T6 using RISO RNA Isolation Reagent (Bios, USA). MiR-146a expression was measured using EzOms miRNA qPCR Detection Primer Set (Bios, USA) and EzOms One-Step qPCR Kit (Bios, USA) in an ABI Prizm 7500 PCR machine. PCR was performed at 42°C for 30 min; 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 20 s, 62°C for 30 s, 72°C for 30 s. The melt curve stage was performed at 95°C for 30 s, 60°C for 30 min, 95°C for 30 s. The relative miRNA expression was calculated from three different experiments. The fold-change for miRNA relative to U6 was determined by the formula 2−ΔΔCt.

2.4. Quantitative real-time PCR and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from HSC-T6 using TRIzol reagents (Invitrogen, USA), and the first-strand cDNA was synthesized using ThermoScript RT-PCR synthesis kit (Fermentas, USA) according to the manufacturer’s instructions. Quantitative real-time PCR analyses for miRNA of SMAD4, α-SMA, and GAPDH were performed by using ABI RT-PCR kits (ABI, USA). The mRNA level of GAPDH was used as an internal control. Primer sequences were listed in Supplemental Table 1. RT-PCR was carried out under standard protocol using the following primers: β-actin (forward: 5′-TCAGCTTGGTGTGCCCCCTGAG-3′; reverse: 5′-GGGCGATCCGACTGCTGCTATCTTTAT-3′), α-SMA: (forward: 5′-TGGCCACTGCTGCCCTGCTCTCTTCTT-3′; reverse: 5′-GGTCGATCTCCTGCTATCTTCTT-3′), SMAD4: (forward: 5′-GGGCTGTCGGACACGAGGTA-3′; reverse: 5′-CGAGTGTCCTGATCCCTGAGT-3′). PCR was performed at 94°C for 5 min, followed by 30–35 cycles of amplification at 94°C for 40 s, 51°C for 40 s and 72°C for 1 min by using ABI 7900. The band intensities were measured by adensitometer and the results were normalized with β-actin. The results were repeated by at least three times independently from three different pools of templates, while each pool of template was extracted from at least eight ventricles.

2.5. Cell proliferation assay

Cell proliferation assay was determined by standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, the cells were seeded at a density of 5×104 cells per well in 96-well culture plates and transfected with miR-146a mimics and negative control mimics as described above. Cell proliferation was assessed 24h later. After culture, 5 μg/ml MTT (Sigma, USA) was added and incubated at 37°C for another 4h; thereafter, the medium was replaced and the formazan crystals were dissolved in 150 μl dimethyl sulphoxide (DMSO). The optical density (OD) was determined with Thermomax microplate reader (Bio-Tek EL, USA) at 490 nm wavelength. All experiments were performed in triplicate and repeated at least three times.

2.6. Cell cycle analysis

For cell cycle analysis, we performed Cell Cycle and Apoptosis Analysis Kit (Beyotime, China). Cells were washed three times by cold PBS, and then cells were fixed in 70% ethanol in PBS at −20°C for 12 h. After fixation, cells were washed with cold PBS and stained with 0.5 ml of propidium iodide (PI) staining buffer, which contains 200 ng/ml RNase A, 50 μg/ml PI, at 37°C for 30 min in the dark. Analyses were performed on BD LSR flow cytometer (BD Biosciences). The experiments were repeated three times.

2.7. Apoptosis analysis

For apoptosis analysis, quantification of apoptotic cells was performed with Annexin-V-FITC Apoptosis Detection Kit (BestBio, China) according to the manufacturer’s instruction. Early apoptotic cells were defined as Annexin-V-positive, PI-negative cells. Analyses were performed on BD LSR flow cytometer (BD Biosciences). The experiments were repeated three times.

2.8. Western blot analysis

Rat liver tissues and cells were lysed with RIPA lysis buffer (Beyotime, China). Whole extracts were prepared, and protein concentration was detected using a BCA protein assay kit (Boster, China). Total protein (30 or 50 mg) from samples was separated by SDS-PAGE and blotted onto a PVDF membrane (Millipore Corp, Billerica, MA, USA). After blocking, nitrocellulose blots were incubated for 1 h with primary antibodies diluted in PBS/Tween20 (0.075%) containing 3% Marvel. Mouse polyclonal anti-α-SMA (Proteintech, USA) was diluted 1:600, mouse monoclonal antibody directed against SMAD4 (Santa Cruz, USA) was used at 1:600 as was anti-β-actin (Santa Cruz, USA). Horse-radish peroxidase conjugated anti-mouse and anti-rabbit antibodies were used as secondary antibodies correspondingly. After four washes in PBS/Tween-20, the membranes were developed with distilled water for detection of antigen using the enhanced chemiluminescence system. Proteins were visualized with ECL-Chemiluminescent kit (ECL-plus, Thermo Scientific).
Liver tissues were fixed in 10% neutral buffered formalin solution, embedded in paraffin, and stained for routine histology. The sections were dewaxed in xylene and dehydrated in alcohol, antigen retrieval was achieved by microwaving in citric saline for 15 min. Thin sections were deparaffinized and treated with 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. The sections were further blocked by 2% bovine serum albumin followed by incubation with primary antibody against α-SMA (1:400) for 16 h at 4°C. After rinsing, the sections were incubated with biotinylated secondary antibody for 60 min at room temperature. α-SMA expression was visualized by 3,3′-diaminobenzidine tetrahydrochloride (DAB) staining. Slides were counterstained with hematoxylin before dehydration and mounting. α-SMA positive areas within the fibrotic region were then observed. Quantitative analysis was calculated from five fields for each liver slice.

2.10. Statistical analysis

All results are expressed as Mean± SE. Statistical significance was performed by either the Student’s t-test for comparison between means or one-way analysis of variance with a post hoc Dunnett’s test. P<0.05 was considered statistically significant.

3. Results

3.1. Regulation of α-SMA expression by TGF-β1 in HSC

Stimulation of HSCs by TGF-β1 is known to be the key fibrogenic response in liver fibrosis because of the evidence: potency of TGF-β1 to upregulate ECM expression [20]. At first, we found that the myofibroblast marker α-SMA mRNA expression increased dose-dependently by TGF-β1 (Fig. 1a).

3.2. MiR-146a is downregulated in TGF-β1-treated HSC and liver fibrotic tissues

We investigated the involvement of miR-146a in TGF-β1-treated HSC conversion. We showed that TGF-β1 induced the decreased expression of miR-146a was dose-dependent in the range from 5 to 15 ng/ml by one-step quantitative RT-PCR (Fig. 1b). These dose-dependent data convincingly showed that miR-146a was downregulated in TGF-β1-treated HSC. In order to identify the expression of miR-146a in liver fibrotic tissues, the degree of liver fibrosis was determined by hematoxylin and eosin (H&E) staining and masson staining. Histopathological analysis revealed that in liver fibrotic tissue sections stained with H&E and masson compared with sections from livers in the control rat, CCl4 caused prominent hepatic steatosis, nerosis, and formation of regenerative nodules and fibrotic septa between CCl4 groups (Fig. 2a). Immunohistochemical results demonstrated that α-SMA was stained to investigate the cellular events in rats and the levels of α-SMA in CCl4 rats were the higher compared with the control rats (Fig. 2b). The one-step real-time qPCR result showed that miR-146a was downregulated in liver fibrotic tissues compared with the control liver tissues (Fig. 2c).

3.3. Overexpression of miR-146a causes inhibitor of TGF-β1-induced HSC proliferation and increases cell apoptosis

In order to investigate the roles of miR-146a in regulating TGF-β1-induced HSC proliferation and apoptosis, we tested the effect of miR-146a overexpression on the proliferation of TGF-β1-treated HSC. The cells transfection with miR-146a mimics significantly increased mature miR-146a expression (Fig. 3a). The MTT assay showed that introduction of miR-146a caused a significant inhibition of cell proliferation in TGF-β1-treated HSC (Fig. 3b). Next, to understand whether the reduced cell proliferation was due to cell cycle arrest or apoptosis, we used Flow cytometric analyses to measure the effect of miR-146a on induction of cell cycle and apoptosis. We found that the proportion of apoptotic cells induced by transfection of miR-146a mimics was greater than that induced by transfection of the NS-miRNA mimics, but overexpression of miR-146a did not have significant effect on cell cycle distribution (Fig. 3c). These results indicated that overexpression of miR-146a caused inhibitor of TGF-β1-induced HSC proliferation, at least in part, via influencing cell apoptosis but not cell cycle arrest.

3.4. Overexpression of miR-146a inhibits α-SMA expression in TGF-β1-treated HSC

Next, the effects of overexpression of miR-146a on TGF-β1-induced HSC activation and ECM protein production were studied. α-SMA is the marker of activated HSC. First, the effect of miR-146a on α-SMA mRNA expression was analyzed using qRT-PCR and RT-PCR, respectively. The results demonstrated that transfection of miR-146a mimics significantly inhibited α-SMA mRNA expression in TGF-β1-treated HSC (Fig. 4a). Additionally, western blot was performed to evaluate the protein expression of α-SMA. We determined that overexpression of miR-146a resulted in a significant decrease in α-SMA protein levels compared with NS-miRNA-transfected TGF-β1-treated HSC (Fig. 4b).

3.5. SMAD4 is a target of miR-146a in HSC and miR-146a mediates SMAD4 expression via posttranslational suppression

To determine the potential role of miR-146a in mediating TGF-β1-induced HSC proliferation, potential targets that are components
of TGF-β1 signaling pathway were identified by using miRBase Targets, miRanda, and TargetScan 5.1. Among the candidate miR-146a targets (Table 1), we paid more attention to SMAD4, because SMAD4 is well known to function as one of the downstream effectors of TGF-β1 and occupies a central role in transduction of TGF-β1 signals [8,21] (Fig. 5a).

![Image](image1.png)

**Fig. 2.** The miR-146a expression in control liver tissues and liver fibrotic tissues. a. Pathology observation of the experimental rat liver sections stained with hematoxylin and eosin (H&E) staining and massion staining (×200). H&E staining of control and CCL4-treated liver tissue is shown in (A) and (B), respectively. Massion staining of control and CCL4-treated liver tissue is shown in (C) and (D), respectively. b. The level of the α-SMA was analyzed by immunohistochemistry in control liver tissue (A) and liver fibrotic tissue (B). Representative views from each group are presented (original magnification, ×20). The results are expressed as relative expression against control expression without treatment. *P<0.05, **P<0.01 vs control. c. Downregulation of miR-146a expression in liver fibrotic tissues compared with control liver tissues. The miR-146a expression was analyzed by one-step quantitative real-time PCR. The results are expressed as relative expression against control expression without treatment. *P<0.05 vs control.
To determine whether SMAD4 gene is a true target of miR-146a, miR-146a mimics/control was transfected into TGF-β1-treated HSC. Western blot and real-time PCR analysis revealed that SMAD4 mRNA and protein were upregulated in response to TGF-β1 stimulation, and SMAD4 protein but not SMAD4 mRNA expression was significantly lower in miR-146a-transfected HSC compared with the control.

**Fig. 3.** Effect of miR-146a on cell proliferation, cell cycle and apoptosis in TGF-β1-treated HSC. 

- **a.** Upregulation of miR-146a expression in transfected HSCs. Light microscope of HSC transfected with fluorescently labeled miR-146a after 24h. The miR-146a expression of HSC was analyzed by one-step quantitative real-time PCR. Control: The cells were transfected with 60nM NS-miRNA for 24h. The results are expressed as relative expression against control expression. *P<0.05. **P<0.01 vs NS-miRNA.

- **b.** MiR-146a overexpression significantly inhibited proliferation of TGF-β1-induced HSC. The role of miR-146a in regulating TGF-β1-treated HSC proliferation was tested by MTT assay. The data represent the mean±SD of three different experiments. *P<0.05 vs control, **P<0.01 vs NS-miRNA.

- **c.** Effect of miR-146a on cell cycle and apoptosis in TGF-β1-treated HSC. Cell cycle analysis showed no difference between TGF-β1-treated HSC transfected with miR-146a mimics and NS-miRNA. Overexpression of miR-146a significantly increased cell apoptosis in TGF-β1-treated HSC. One representative experiment of the three independent experiments is demonstrated.
NS-miRNA-transfected cells (Fig. 5b and c). These results suggest that SMAD4 gene is a miR-146a target. MiR-146a regulated SMAD4 gene expression at the posttranscriptional level. Above data suggest that miR-146a inhibits TGF-β1-induced HSC proliferation and ECM expression, at least in part, via inhibiting SMAD4.

### 4. Discussion

Hepatic stellate cells (HSC) are unequivocally the main cells shown to play a key role in liver fibrosis. As a result of injury, HSCs undergo “activation” or transdifferentiation, from a quiescent, vitamin A-storing cell to a myofibroblast-like cell, with several new phenotypic characteristics, such as enhanced cell migration and adhesion, expression of α-SMA [2,3,22]. Accumulating studies have demonstrated that miRNAs play an important role in regulating HSC functions such as cell proliferation, differentiation, and apoptosis. For instance, miR-27a and 27b allowed culture-activated rat HSCs to switch to a more quiescent HSC phenotype, with restored cytoplasmic lipid droplets and decreased cell proliferation [23]. MiR-29b can bind directly to Col1A1 3′UTR to suppress the mRNA and protein expression [24]. Transduction of miR-16 significantly inhibits HSC proliferation and increases the apoptosis index [25]. Restoring miR-335 expression significantly inhibited cell migration and reduced α-SMA and collagen type I level, at least in part, via decreasing the expression of TNC [26].

Recently the microarray analysis revealed that miR-146a was identified to be downregulated in activated HSCs as compared to that in quiescent HSCs [17,27]. G. Maubach et al. [27] has shown that miR-146a is involved in HSC activation and targets TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1), two key adaptor molecules in the Toll-like receptor and IL-1 receptor signaling pathways. Our understanding of the role of miR-146a dysregulation in the development of hepatic fibrosis remains limited. The present study was destined to determine whether miR-146a might play a role in regulating HSC activation in response to TGF-β1 stimulation and show the expression of miR-146a in liver fibrotic tissues induced by CCl4. In our study, miR-146a expression was downregulated dose-dependently under TGF-β1 stimulation, correlating with a dose-dependent increase in α-SMA expression, and there was a significant decrease in the expression of miR-146a in liver fibrotic tissues compared with the control liver tissues. Surprisingly, miR-146a was not downregulated significantly in vivo activated pHSCs (CDE diet), and the expression of miR-146a was downregulated at day 3, and recovered subsequently until day 10 during the in vitro activation of pHSCs [27]. In our findings, dose-dependent downregulation of miR-146a is associated with the stimuli of TGF-β1.

Activated HSCs are the principal cell type promoting synthesis and deposition of ECM proteins. Hence, in order to study the overexpression

### Table 1

**Analysis of target regions in predicted target genes of miR-146a.**

<table>
<thead>
<tr>
<th>miR-146a Target</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF receptor-associated factor 6 (TRAF6)</td>
<td>Interleukin-1 receptor-associated kinase 1 (IRAK1)</td>
</tr>
<tr>
<td>Zinc and ring finger 3 (ZNRF3)</td>
<td>SMAD family member 4 (SMAD4)</td>
</tr>
<tr>
<td>BCL6 co-repressor-like 1 (BCORL1)</td>
<td>Neuronal PAS domain protein 4 (NPAS4)</td>
</tr>
</tbody>
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**Fig. 4.** Effect of miR-146a mimics on the expression of α-SMA in TGF-β1-treated HSC. a. The α-SMA mRNA expression was analyzed by Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR. The results of RT-PCR and real-time PCR were shown in (A) and (B), respectively. b. The α-SMA protein expression was analyzed by Western blots. The results are expressed as relative expression against control expression. *P<0.05 vs control, †P<0.05 vs NS-miRNA.
Fig. 5. a. The region of the rat SMAD4 mRNA 3′UTR predicted to be targeted by miR-146a. Three different bioinformatics approaches (miRbase, miRANDA, and Targetscan 5.1) were used for the target prediction. b. The SMAD4 mRNA expression was analyzed by RT-PCR and real-time PCR. The results of RT-PCR and real-time PCR were shown in (A) and (B), respectively. c. The SMAD4 protein expression was analyzed by Western blots. The results are expressed as relative expression against control expression. *P<0.05 vs control, **P<0.05 vs NS-miRNA.
of miR-146a on the expression of α-SMA, one of the markers of stellate cell activation, we transfected miR-146a into HSC followed by exposure to 10ng/ml TGF-β1. Our data showed that transient transfection of miR-146a mimics completely suppressed the upregulation of α-SMA mRNA and protein under TGF-β1 stimulation. Moreover, the cell proliferation assay showed that transfection of miR-146a mimics caused a significant inhibition of cell proliferation in TGF-β1-treated HSC. Overexpression of miR-146a correlated with decreased cell growth rates, increased cell apoptosis, and had no significant effect on the cell cycle distribution. These data suggested that miR-146a was involved in the inhibition of TGF-β1-induced HSC proliferation, activation, and affected cell apoptosis but not cell cycle arrest.

TGF-β1 mainly activates hepatic stellate cells (HSC) through TGF-β1/Smad signal pathway, thus causing hepatic fibrosis [6]. Smads can be divided into three distinct subclasses: receptor-activated Smads (R-Smads), common-partner Smads (Co-Smads) and inhibitory Smads (anti-Smads). In TGF-β1/Smad signaling pathway, Smad2 and Smad3 function as R-smads, SMAD4 functions as Co-smad, and Smad7 functions as an anti-smad. SMAD4 is the only identified Co-smad in mammals. When TGF-β1 binds to its receptor, Smad 2/3 is phosphorylated and binds with SMAD4 to form multimers and together they are transported into the nucleus and combine with a co-activator or a co-repressor to regulate the translation and expression of the target gene [7,28]. In this process, SMAD4 plays a key role in the TGF-β1/signal pathway. D. Maurice et al. [29] also found that cell lines that lack SMAD4 fail to respond to TGF-1 stimulation, transfection of wild-type SMAD4 restores the signaling capabilities of these cells. In addition, reducing the abundance of SMAD4 can delay the progression of liver fibrosis and inhibit HSC activation [30]. In our study, both mRNA and protein expressions of SMAD4 were remarkably upregulated in response to TGF-β1 stimulation. Recently it has been reported that miR-146a might influence proliferation of APL cells through TGF-β1/Smad signal transduction pathway during ATRA induction by targeting SMAD4 [31]. MiR-146a regulates dendritic cells (DCs) maturation and antigen presentation function during BxPC-3-conditioned medium (BxCM) induction by targeting SMAD4 [34]. Z. Liu et al. [35] also found that miR-146a can directly target SMAD4 and modulates TGF-β1-induced phenotypic differentiation in human dermal fibroblasts. All these publications showed that SMAD4 is the target of miR-146a. The HSCs, which undergo a transdifferentiation process when cultured in vitro, were a principal target of the profibrogenic agent TGF-β1. During the transdifferentiation of HSC following TGF-β1 stimulation, SMAD4 implicated in downstream signal transduction was upregulated. Our experiments demonstrated that miR-146a overexpression resulted in no significant difference in SMAD4 mRNA levels, but significantly decreased SMAD4 protein expression, suggesting that the inhibition might be at a posttranslational level. On the basis of our findings, as well as those of previous study by others, we now propose the following framework that miR-146a functions as a negative feedback in the TGF-β1 pathway during HSC transdifferentiation, probably to inhibit hepatic fibrosis. This is supported also by the finding that miR-146a overexpression decreased α-SMA expression after TGF-β1 stimulation. In conclusion, we demonstrated in the present study that miR-146a might play a critical role in hepatic fibrogenesis. It appears that miR-146a regulates TGF-β1-induced HSC differentiation, at least in part, via decreasing the expression of SMAD4. But the function of miR-146a in HSC may be more complex than we imagine, further functional analysis to determine the precise role of miR-146a in HSC could provide a novel therapeutic approach for treating hepatic fibrosis.

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2012.06.003.

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References