Int J Clin Exp Med 2016;9(12):23329-23336
www.ijcem.com /ISSN:1940-5901/IJCEM0023961

Original Article

Targeting tissue inhibitor of metalloproteinase 1/2 using a shRNA lentiviral system offers a novel treatment strategy against hepatic fibrosis

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Received November 23, 2015; Accepted November 14, 2016; Epub December 15, 2016; Published December 30, 2016

Abstract: Hepatic fibrosis is a result of constant wound-healing response caused by repeated injury in liver. Up to now, there is still no standard treatment for this disease. Tissue inhibitors of metalloproteinase (TIMP) 1/2 have been described to be highly expressed in liver with hepatic fibrosis. However, whether the down-regulation of these two proteins would show protective or curative effect against hepatic fibrosis remains to be defined. In this study, using a shRNA lentiviral system, we investigated the effect of TIMP1/2 down-regulation against hepatic fibrosis on a rat model. Our results showed that specifically knock-down of TIMP1 or TIMP2 could significantly alleviate the severity of hepatic fibrosis. Further study also showed that matrix metalloproteinase (MMP)-1 and MMP-13 were significantly increased in rats with TIMP1/2 knock-down. Signaling pathway analysis by RT-PCR and western blot further revealed that the altered expression of TGF-β1-Smad signaling pathway proteins including TGF-β1, Smad3 and Smad7 was modulated back to normal levels in TIMP1/2 knock-down rats. Taken together, our study revealed that specifically knocking-down of TIMP1 or TIMP2 by shRNA lentiviral particles could increase the expression of MMP-1 and MMP-13 and alleviate the severity of hepatic fibrosis, probably via TGF-β1-Smad signaling pathway. The findings in this study imply a novel treatment strategy against hepatic fibrosis.

Keywords: Hepatic fibrosis, tissue inhibitor of metalloproteinase 1/2, shRNA lentiviral system

Introduction

Hepatic fibrosis is usually caused by chronic liver damage in conjunction with extracellular matrix (ECM) proteins accumulation [1, 2]. Factors causing hepatic fibrosis include alcohol abuse, chronic viral infection (e.g. hepatitis C virus), steatohepatitis and parasites [2]. Recent studies have proved that hepatic fibrosis is reversible, however, it, if without prompt treatment, could develop into cirrhosis, which is ranked as the sixth disease in mortality rate worldwide [3, 4]. Patients with cirrhosis usually have a 3-year survival rate less than 20% [4].

Hepatic fibrosis is a result of constant wound-healing response caused by repeated injury in liver [1]. During this process, inflammation response persists and ECM proteins constantly accumulate, which eventually distort the hepatic architecture and develop nodules of regenerating hepatocytes [5, 6]. The synthesis and degradation of ECM proteins are tightly regulated by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMPs) [7-10]. In liver, the predominantly expressed MMPs are MMP-1 and MMP-3 while the merely expressed TIMPs are TIMP-1 and TIMP-2. MMPs could degrade ECM proteins, whereas TIMP-1/2 could specifically bind to MMPs and inhibit such degradation, resulting in hepatic fibrosis [11, 12]. Previous studies have been reported that the expression of both TIMP-1 and TIMP-2 are significantly elevated in rats with hepatic fibrosis [13, 14].

Aside from ECM protein accumulation, overload inflammation response is also closely related to hepatic fibrosis [15]. A big variety of cytokines relating to inflammation response including CCL5, MCP-1, IL-10, IFN-γ and TGF-β1, have been participated in the regulation of hepatic

fibrosis [16, 17]. Among these cytokines, TGF-β1 has been believed to a key mediator in hepatic fibrosis [18, 19]. TGF-β1 could promote ECM protein synthesis and inhibit their degradation through TGF-β1-Smad signaling pathway [20, 21].

Although the mechanisms underlying hepatic fibrosis have been greatly advanced, the therapeutic approaches remain very limited. Up to now, no standard treatment for hepatic fibrosis is available. Therefore, the development of treatment strategies against hepatic fibrosis is still in need. Given the significance of TIMP-1/2 in hepatic fibrosis genesis, we in this study, on a rat model, investigated whether the down-regulation of TIMP-1/2 using a shRNA lentiviral system could be an effective treatment approach. Our results showed that down-regulated TIMP-1/2 in liver could significantly increase the activity of MMP-1 and MMP-13 as well as alleviate hepatic fibrosis severity through TGF-β1-Smad signaling pathway. The outcome of the study implies a novel treatment strategy against hepatic fibrosis.

Materials and methods

Animals

Male Sprague Dawley rats weighing 200±20 g were purchased from Shanghai slack laboratory animal co., LTD and hosted in specific-pathogen-free (SPF) conditions with water and food supplied. All protocols involving animals in this study were viewed by the Ethics Committee of The Second Affiliated Hospital of Nanchang University (No. 2014014) and performed in accordance with the guidelines of animal practice defined by provincial Laboratory Animal Science Association.

Animal experiment and sampling

Rats were divided into the following 5 groups: Group A: normal control, Group B: hepatic fibrosis control, Group C: hepatic fibrosis plus control shRNA lentivirus treatment, Group D: hepatic fibrosis plus TIMP-1 shRNA lentivirus treatment and Group E: hepatic fibrosis plus TIMP-2 shRNA lentivirus treatment.

Hepatic fibrosis was induced by carbon tetrachloride (CCl_4) injection as previously described [22]. In brief, rats were intraperitoneally received 20% CCl_4 for 1 ml/kg in olive oil twice a week for 6 weeks to induce hepatic fibrosis.

TIMP-1, TIMP-2 specific shRNA lentiviral vectors as well as control vector were purchased from Origene and corresponding lentiviral particles were produced and titrated using Lentivpak Packaging Kit (Origene) according to the manufacturer's instructions. For lentivirus transduction, $5*10^7$ transduction units of lentivirus were injected i.p. into rats twice a week for 2 weeks. Group A and B control rats received cell culture medium of the same volume. One week after the final injection, rats were sacrificed and serum and liver samples were harvested and stored at -80°C until use.

Hematoxylin and eosin (HE) staining of liver tissue

Liver samples were first fixed in 10% neutral buffered formalin for 24 h at room temperature and then embedded in paraffin and cut into 3-μm slides. For HE staining, slides were dewaxed in xylene, rehydrated in descendant ethanol and then stained with hematoxylin and eosin, respectively. Tissue fibrosis scale was graded as the following stages as previously described [23]: Stage 0: normal liver tissue; Stage I: slight collagenous fiber extending from central vein or portal area; Stage II: severe collagenous fiber extension but not cover the whole hepatic lobules; Stage III: collagenous fiber extension covers the whole hepatic lobules; Stage IV: hepatic lobule is separated by collagenous fiber and forms large square pseudolobules; Stage V: hepatic lobule structure is totally distorted and 50% of lobules as small circular pseudolobules; Stage VI: small circular pseudolobules scattered in the whole liver.

Determination of MMP-1 and MMP-13 activity

The MMP-1 and MMP-13 activity in serum samples were tested by ELISA (both from Elabscience) according to the manufacturer's instructions. The concentrations of the samples were calculated from the standard curves of each protein.

RT-PCR

The mRNA levels of TIMP1, TIMP2, TGF-β1, Smad3 and Smad7 were determined by RT-PCR using GAPDH as an internal control. First, total RNA was extracted from liver tissue samples using Trizol (Thermo Scientific) and then mRNA was reverse-transcribed into cDNA using Promega Reverse Transcription System (Promega) both according to the manufacturer's instructions. The genes of interest were

Table 1. PCR primer pairs used in the study

Target gene	Primer sequence (5'-3')
TIMP1	F: TTCGTGGGGACACCAGAAGTC
	R: TATCTGGGACCGCAGGGACTG
TIMP2	F: GTTTTGCAATGCAGATGTAG
	R: ATGTCGAGAAACTCCTGCTT
TGF-β1	F: CTGCTACCGCTGCTGCTGTGGCTACTG
	R: CGGTCGCGGGTGCTGTTGT
Smad3	F: GCAGTGGCAATCACAGAGAA
	R: AACAGCCTGGGAGAGACTCA
Smad7	F: TAGCCTTGTCAGATAAGGAAGGA
	R: CACCCACACACCATCCACG
GADPH	F: AGCCACATCGCTCAGACA
	R: TGGACTCCACGACGTACT

F: forward primer; R: reverse primer.

then amplified by PCR using specific primers listed in **Table 1**. The relative mRNA level of each gene was calculated using $2^{-\Delta\Delta t}$ using GAPDH as an internal control.

Western blot

The protein levels of TIMP1, TIMP2, TGF-β1, Smad3 and Smad7 were determined by western blot. Total protein from homogenized liver samples were first separated by 12% SDS-PAGE and then transferred on to a PVDF membrane (Millipore). Non-specific binding sites on the membrane were blocked with 5% non-fat milk. Membrane was then incubated with primary antibodies anti-TIMP1, TIMP2, TGF-β1, Smad3 and Smad7 and corresponding HRP-conjugated secondary antibodies at room temperature for 2 h and 1 h, respectively. After extensive washes, the immune-bands on the membrane werevisualized using ECL substrate (Pierce, Thermo Scientific) under a CCD camera (Bio-Rad). The grey scale of the immune-bands was quantified using Quantity One software (Bio-Rad).The following primary antibodies were used in the study: rabbit anti-rat TIMP1 (ab61224, Abcam), mouse anti-rat TIMP2 (ab1828, Abcam), rabbit anti-rat TGF-β1 (ab66043, Abcam), mouse anti-rat Smad3 (sc-101154, Santa Cruz) and rabbit anti-rat Smad7 (sc-11392, Santa Cruz).

Statistical analysis

Data were presented as mean ± SD. Student's *t* test and One-way ANOVA plus SNK post hoc were adopted for comparisons between two groups and among three or more groups, respectively. A p value < 0.05 was considered statistically significant. All statistical analysis was performed with Prism 4.0.3 (Graphpad).

Results

The knock-down efficiency of TIMP1/2 specific shRNA lentiviral particles

In this study, rats were divided into the following 5 groups: Group A: normal control, Group B: hepatic fibrosis control, Group C: hepatic fibrosis plus control shRNA lentivirus treatment, Group D: hepatic fibrosis plus TIMP-1 shRNA lentivirus treatment and Group E: hepatic fibrosis plus TIMP-2 shRNA lentivirus treatment.

First, we tested the knock-down efficiency of TIMP1/2 specific shRNA lentiviral particles in liver. As shown in **Figure 1**, TIMP1 and TIMP2 expression on both mRNA (**Figure 1A**) and protein (**Figure 1B** and **1C**) levels were significantly elevated in rats with hepatic fibrosis (Group B). The injections of control shRNA lentiviral particles did not show apparent impact on TIMP1/2 expression (Group C) while TIMP1 (Group D) and TIMP2 (Group E) specific shRNA lentiviral particles down-regulated the expression of TIMP1 and TIMP2 respectively on both mRNA and protein levels. These data herein indicated that TIMP1 and TIMP2 were specifically knocked down using TIMP1 and TIMP2 specific shRNA lentiviral particles.

TIMP1/2 knock-down alleviated hepatic fibrosis severity

Since TIMP1/2 expression was increased in hepatic fibrosis rats, we next investigated whether the down-regulation of these two proteins would show some impact on hepatic fibrosis prevention or alleviation. Liver samples from each group were harvested and stained by H&E and the fibrosis degree was graded. As shown in **Figure 2** and **Table 2**, Group B and C showed the most severe hepatic fibrosis with a mean score of 5.2 and 5.3, respectively. However, the TIMP1/2 knock-down significantly alleviated the severity of hepatic fibrosis in rats (**Figure 2D** and **2E**). Of note, between Group D and E, Group D showed slightly better efficacy in hepatic fibrosis alleviation.

MMP-1 and MMP-13 were elevated in the liver of rats with TIMP1/2 knock-down

MMP-1 and MMP-13, two main MMPs expressed in liver, regulate the degradation

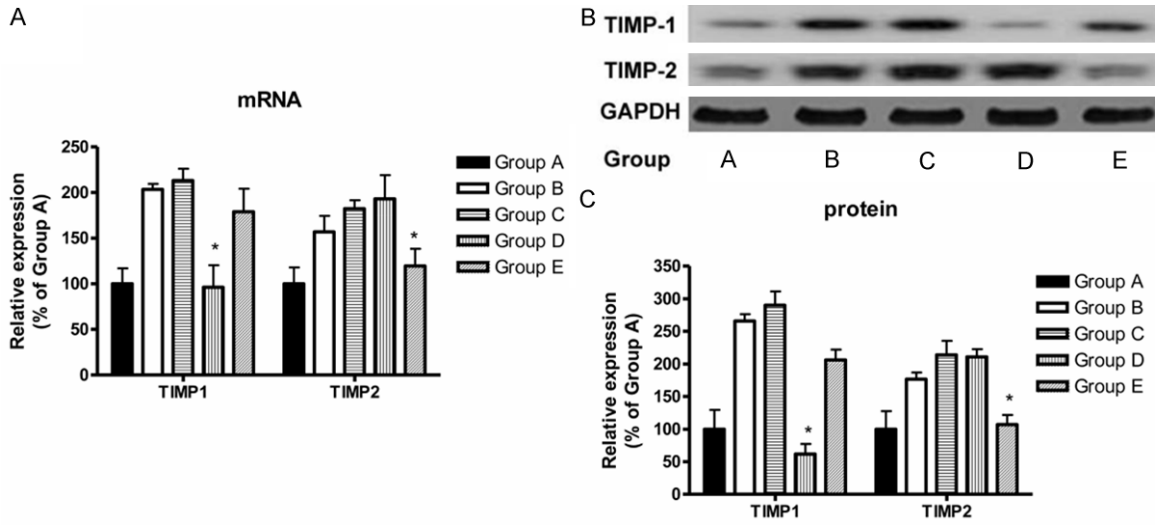

Figure 1. The efficiency and specificity of TIMP1/2 specific shRNA lentiviral particles in rat liver. Rats with or without hepatic fibrosis were injected with $5*10^7$ transduction units of lentivirus i.p. twice a week for 2 weeks, and then liver samples were harvested and the expression of TIMP1/2 on mRNA (A) and protein (B and C) levels were determined. (A) RT-PCR analysis of TIMP1/2 on mRNA levels. Data shown are mean ± SD of three independent experiments (n = 10). (B and C) Western blot analysis of TIMP1/2 on protein level. (B) Representative result is shown. (C) Gray scale of western blot was analyzed and graphed. Data shown are mean ± SD of three independent experiments (n = 10). *P < 0.05 vs. Group B.

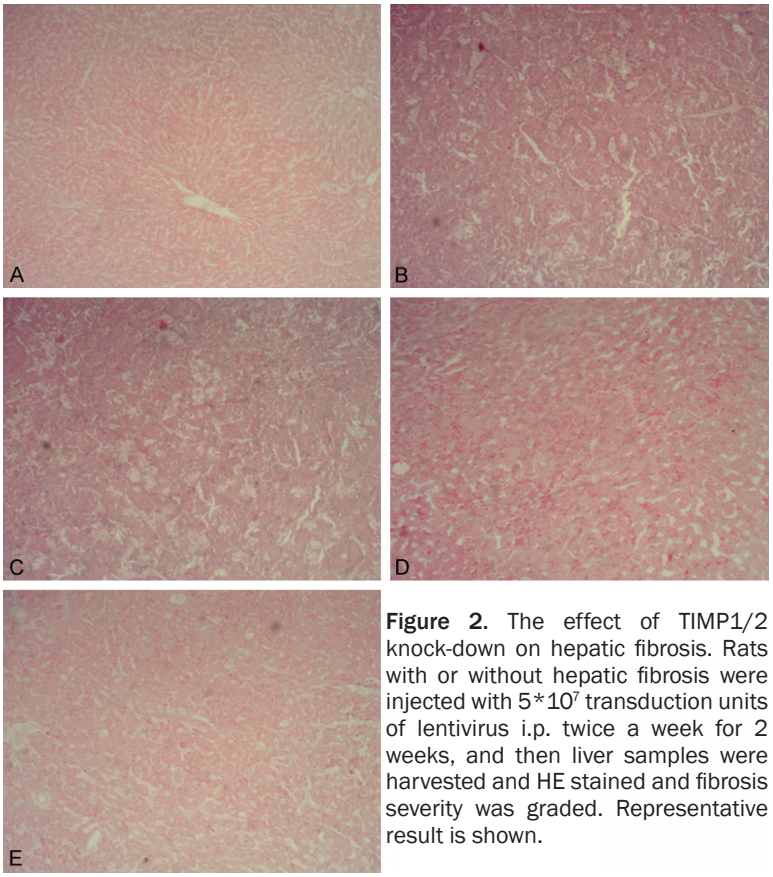

Figure 2. The effect of TIMP1/2 knock-down on hepatic fibrosis. Rats with or without hepatic fibrosis were injected with $5*10^7$ transduction units of lentivirus i.p. twice a week for 2 weeks, and then liver samples were harvested and HE stained and fibrosis severity was graded. Representative result is shown.

ECM proteins which account for the generation of hepatic fibrosis. Therefore, we further investigated whether increased MMP-1 and MMP-13 expression was associated with the TIMP-1/2 knock-down caused hepatic fibrosis alleviation. As shown in **Figure 3** and **Table 3**, both MMP-1 and MMP-13 were significantly decreased in Group B and C comparing to those in Group A. However, in Group D and E with TIMP1/2 knock-down, the levels of these two proteins, albeit still slightly lower than in Group A, were considerably higher than in Group B and C. These data indicated that TIMP1/2 knock-down might protect liver from fibrosis by increasing the expression of MMP1 and MMP-13.

TGF-β1-Smad signaling pathway was involved in TIMP1/2 knock-down regulated rat hepatic fibrosis

TGF-β1-Smad signaling pathway has been reported to be closely associated with ECM protein expression [20, 21]. Within TGF-β/Smads signaling path-

Table 2. The scoring of rat hepatic fibrosis

Group	n	Hepatic fibrosis grade							
		0	I	II	III	IV	V	VI	Mean score
A	10	0	0	0	0	0	0	0	0
B	10	0	0	0	0	2	4	4	5.2**
C	10	0	0	0	0	2	3	5	5.3**
D	10	0	1	4	4	1	0	0	2.5##
E	10	0	0	4	5	1	0	0	2.7##

**P < 0.01 (vs. Group A); ##P < 0.01 (vs. Group C).

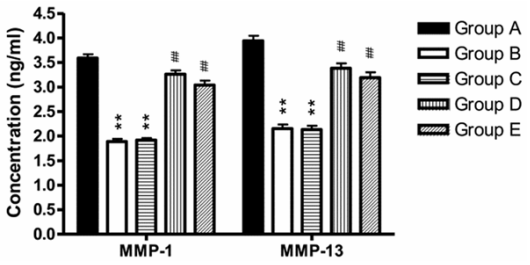

Figure 3. The effect of TIMP1/2 knock-down on MMP1 and MMP-13 expression. Rats with or without hepatic fibrosis were injected with $5*10^7$ transduction units of lentivirus i.p. twice a week for 2 weeks, and then serum samples were harvested and MMP-1 and MMP-13 levels were determined by ELISA. Data shown are mean ± SD of three independent experiments (n = 10). *P < 0.05; **P < 0.01; ***P < 0.001 (vs. Group A). #P < 0.05; ##P < 0.01; ###P < 0.001 (vs. Group B).

Table 3. The levels of MMP-1 and MMP-13 in liver

Group	MMP-1 (ng/ml)	MMP-13 (ng/ml)
A	3.59±0.24	3.94±0.33
B	1.89±0.17**	2.15±0.27**
C	1.92±0.13**	2.13±0.26**
D	3.26±0.25##	3.38±0.32##
E	3.04±0.28##	3.19±0.34##

**P < 0.01 (vs. Group A); ##P < 0.01 (vs. Group C).

way, Smad3 is the common-mediator participating in the pathway while Smad7 the inhibitory regulator which blocks the activation this pathway [24, 25]. Our study further investigated the involvement of TGF-β1-Smad signaling pathway in TIMP1/2 knock-down initiated hepatic fibrosis alleviation, by determining the expression of TGF-β1-Smad signaling pathway associated genes on both mRNA and protein levels. Our results showed that the mRNA level of TGF-β1 and Smad3 were significantly increased while that of Smad7 was decreased in Group B and C. Contrarily, these three proteins remained at similar levels in Group D and E as in Group A (**Figure 4A**). Similar results were observed when the protein levels of TGF-β1, Smad3 and Smad7 were determined (**Figure 4B** and **4C**). These data indicated that TGF-β1-Smad signaling pathway was altered in TIMP1/2 knock-down rats.

Taken together, our study revealed that specifically knocking-down of TIMP1 or TIMP2 by shRNA lentiviral particles could increase the expression of MMP-1 and MMP-13 and alleviate the severity of hepatic fibrosis, probably via TGF-β1-Smad signaling pathway. The findings in this study provide a novel treatment strategy against hepatic fibrosis.

Discussion

Repeated liver injury and constant wound-healing response is usually the cause of hepatic fibrosis. However, the direct cause of hepatic fibrosis is ECM accumulation induced hepatic architecture distortion [5, 6]. MMPs and TIMPs are two vital protein families that are responsible for ECM balance. The former causes ECM degradation while the latter inhibits such process [7-10]. There are two predominant MMPs (MMP-1 and MMP-13) and two TIMPs (TIMP1 and TIMP2) expressed in liver [13, 14]. Given the mechanisms underlying hepatic fibrosis genesis, there are consequently two principal means to treat this diseases, namely to increase MMP-1/13 expression or to down-regulate TIMP1/2 expression. Previous studies by others have shown that increase of MMP-1 expression by adenoviral introduction as well as inhibition of TIMP1 activity by TIMP1 antibody administration have both demonstrated effect in decreasing fibrosis degrees [26, 27]. In the current study, using a novel shRNA lentiviral system, we investigated the down-regulation of TIMP1 or TIMP2 in hepatic fibrosis. Our results showed that down-regulation of either TIMP could increase the expression of MMP-1 and MMP-13 and consequently attenuate the fibrosis degree. Of note, our results also revealed that TIMP1 knock-down possesses a better efficacy than TIMP2 knock-down. Our study not only shows consistent results as previous studies, but also demonstrates that knock-down of TIMP1/2 using shRNA lentiviral vectors has a better efficacy in liver protection against fibrosis. This is probably because that, compar-

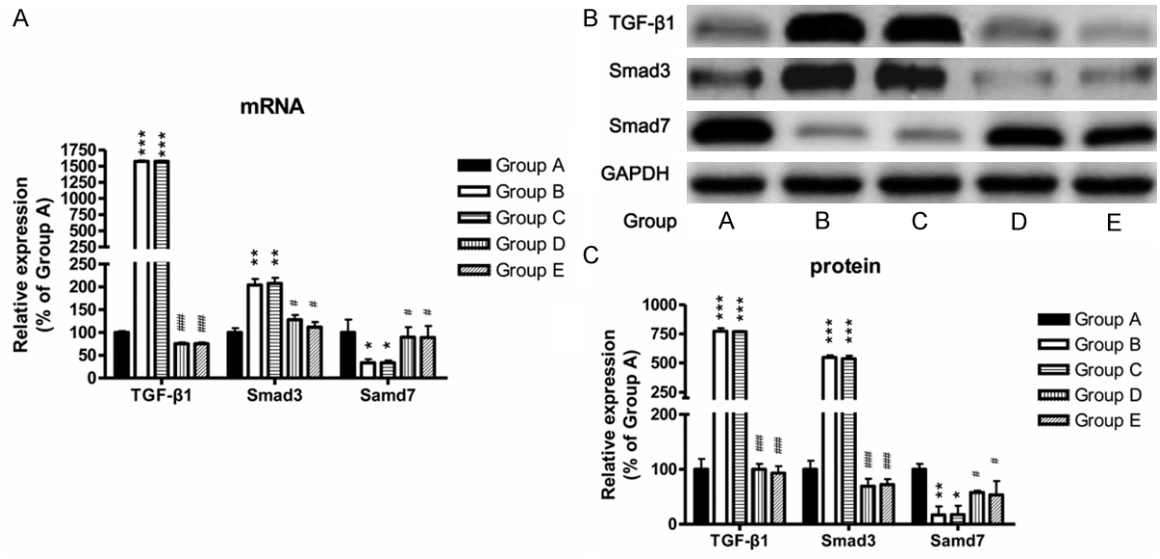

Figure 4. The effect of TIMP1/2 knock-down on TGF-β1-Smad signaling pathway. Rats with or without hepatic fibrosis were injected with $5*10^7$ transduction units of lentivirus i.p. twice a week for 2 weeks, and then liver samples were harvested and the expression of TGF-β1, Smad3 and Smad7 were tested on both mRNA and protein levels. A. RT-PCR analysis of TGF-β1, Smad3 and Smad7 on mRNA level was conducted. Data shown are mean ± SD of three independent experiments (n = 10). B and C. Western blot analysis of TGF-β1, Smad3 and Smad7 on protein level was conducted. B. Representative result is shown. C. Gray scale of western blot was analyzed and graphed. Data shown are mean ± SD of three independent experiments (n = 10). *P < 0.05; **P < 0.01; ***P < 0.001 (vs. Group A). #P < 0.05; ##P < 0.01; ###P < 0.001 (vs. Group B).

ing to MMP-1 ectopic expression, our current approach can elevate both MMP-1 and MMP-13 expression, while comparing to TIMP1 antibody injection, our approach has longer and more stable effect in suppression of TIMP1/2 expression.

Our study also took one step further and revealed that TGF-β1-Smad signaling pathway was involved in the TIMP1/2 knock-down modulated hepatic fibrosis alleviation. TGF-β1-Smad signaling pathway is an important pathway for ECM protein synthesis. Given the importance of the pathway to ECM protein expression, it would be interesting to investigate that whether direct modulation of this signaling pathway could also result in fibrosis attenuation, or even with better efficacy than the means adopted in our study or other previous studies.

Viral vectors have been receiving increasingly attentions in both clinical and non-clinical studies in recent years. There are many viral vectors available now, including vectors derived from retroviruses (lentiviruses), adenoviruses, adeno-associated viruses, herpes viruses and poxviruses [28]. Among these viral vectors, lentiviral vector constructs have been proven to be very efficient in siRNA and shRNA delivery both in vitro and in vivo, due to their infectivity among various cell types [29].

Taken together, our study revealed that specifically knocking-down of TIMP1 or TIMP2 by shRNA lentiviral particles could increase the expression of MMP-1 and MMP-13 and alleviate the severity of hepatic fibrosis, probably via TGF-β1-Smad signaling pathway. The findings in this study provide a novel treatment strategy against hepatic fibrosis.

Acknowledgements

This work is supported by the National Natural Science Foundation of China (81460118) and Science Technology Foundation of Jiangxi Province (No. 20151BBG70097 and No. 2014-1BBG70059).

Disclosure of conflict of interest

None.

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