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Original Paper

Long Noncoding RNA HOTTIP Promotes Mouse Hepatic Stellate Cell Activation via Downregulating miR-148a

Zhiqin Li   Jia Wang   Qinglei Zeng   Chunling Hu   Jiajia Zhang
Hongyan Wang   Jingya Yan   Hua Li   Zujiang Yu

Department of Infectious Diseases, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

Abstract
Background/Aims: HOTTIP is a critical modulator in human diseases including liver cancer, but its role and molecular biological mechanisms in liver fibrosis are still unclear. *Methods:* The expression profile of HOTTIP during the progression of liver fibrosis was detected in human liver samples and in CCl_4-treated mice using qRT-PCR. The expressing sh-HOTTIP adenoviral vector was used to reduce HOTTIP levels *in vivo*. Dual-Luciferase Reporter Assay was performed to validate the interaction between miR-148a and HOTTIP, TGFBR1, or TGFBR2. *Results:* HOTTIP expressions in fibrotic liver samples and cirrhotic liver samples were significantly upregulated compared with healthy liver controls, and cirrhotic samples exhibited the highest levels of HOTTIP. Moreover, HOTTIP expressions were substantially induced in the liver tissues and hepatic stellate cells (HSC) of CCl_4-treated mice. Ad-shHOTTIP delivery could alleviate CCl_4-induced liver fibrosis in mice. Down-regulation of HOTTIP inhibited the viability and activation of HSCs *in vitro*, and HOTTIP negatively regulated miR-148a expression in HSCs. miR-148a had a negative effect on HSC activation by targeting TGFBR1 and TGFBR2. *Conclusion:* HOTTIP is involved in the progression of liver fibrosis by promoting HSC activation. The high level of HOTTIP downregulates miR-148a, thus to increase the level of TGFBR1 and TGFBR2 and contribute to liver fibrosis.

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Introduction

Liver fibrosis, characterized by a diffuse extracellular matrix (ECM) in the liver, is a key part of the course of cirrhosis or primary hepatic carcinoma. Hepatic stellate cells (HSC) are sensitive to hepatic injury and, thus, are the most important effector of liver fibrosis [1]. Studies have demonstrated that activated HSCs aggravate liver fibrosis through characteristic

Z. Li, J. Wang and Q. Zeng contributed equally to this work.

Zujiang Yu
and Zhiqin Li

Department of Infectious Diseases, The First Affiliated Hospital of Zhengzhou University
No. 1 Jianshe East road, Zhengzhou 450052, Henan Province (China)
Tel. 18603710022, E-Mail johnyuem@zzu.edu.cn; fcclizq@zzu.edu.cn

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expression of α-smooth muscle actin (α-SMA) and synthesis and secretion of type 1 collagen (Col-1)-rich ECM [2]. Further, the TGF-β/Smad signaling pathway, as the most important for HSC activation, has been shown to be regulated by a variety of key molecules, including long non-coding RNAs (lncRNA) and microRNAs (miRNA), in the progression of liver fibrosis [3, 4].

The aberrant expression of miRNAs was confirmed in some studies to be involved in liver diseases such as viral hepatitis, alcoholic or non-alcoholic steatohepatitis, autoimmune liver diseases and ischemia-reperfusion liver injury [5]. Cumulative studies have provided the specific miRNA expression pattern for different causes of injury to the liver. In some studies, miR-542, miR-652 and miR-181b levels were significantly decreased in the serum of patients with cirrhosis [6, 7]. Moreover, both miR-19b and miR-29 were identified as anti-fibrotic miRNA [8, 9]. Notably, the application of miR122 inhibitor to alleviate hepatic fibrosis has shown positive results [10]. MiRNA-based therapy provides a new direction for the prevention and treatment of liver fibrosis. In one study, miR-148a mimetic treatment was found to significantly reduce liver fibrosis of developed tumors in mice [11]. Additionally, it has been reported that miR-148a induced autophagy and inhibited proliferation of HSCs, suggesting the potential to control miR-148a in hepatic fibrosis [12].

The important regulatory function of lncRNA in growth, development and disease progression has been generally recognized, and their functions and mechanisms in hepatic fibrosis have received considerable attention as well. For instance, maternally expressed gene 3 (MEG3), an important tumor-associated lncRNA, was dramatically decreased in human and mouse fibrotic livers and was further confirmed to inhibit TGF-β1-induced HSC activation in one study [13]. Additionally, lncRNA homeobox transcript antisense RNA (HOTAIR) and *Alu*-mediated p21 transcriptional regulator (APTR) promoted HSC proliferation and activation, contributing to the progression of liver fibrosis. HOXA distal transcript antisense RNA (HOTTIP), located on 7p15.2, plays a pivotal role in multiple human cancers, including gastric cancer, hepatocellular carcinoma and colorectal cancer [14-16]. However, its function in liver fibrosis is still unclear. Given the possible interactions between HOTTIP and miR-148a, and based on bioinformatics analysis, we inferred that there may be functional correlations between HOTTIP and miR-148a. Thus, the aim of the current study was to determine the effect and the underlying mechanisms of HOTTIP and miR-148a in liver fibrosis.

Materials and Methods

Human specimens

Liver samples were collected from patients with liver fibrosis in chronic hepatitis B (n=30), patients with liver cirrhosis in chronic hepatitis B (n=30) and healthy controls (n=30) to be used for analysis of HOTTIP. The healthy controls recruited for this study did not have any liver diseases or viral hepatitis. The participants with liver cirrhosis or liver fibrosis in chronic hepatitis B were diagnosed at The First Affiliated Hospital of Zhengzhou University by liver biopsy and/or imaging diagnostic methods and underwent either a partial hepatectomy or liver biopsy. Exclusion criteria were being less than 18 years old, having a co-infection of hepatitis C virus (HCV) or human immunodeficiency virus (HIV), having liver cancer, being pregnant or currently lactating, having a history of drug or alcohol abuse, or having an autoimmune disease. Written informed consent was obtained from each patient enrolled in the study, which was approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University.

Mouse studies

C57BL/6J mice (8 weeks old) were purchased from Zhengzhou University. Liver injury was induced in the mice by intraperitoneal injection of CCl_4, a typical method for inducing liver fibrosis. For liver fibrosis, one group of mice (n=12) received 10% CCl_4 (Sigma-Aldrich, St. Louis, MO, USA) dissolved in olive oil at a dose of 7μL/g of body weight two times per week for 6 consecutive weeks (12 times). Another group of mice (n=12) used as the controls received only olive oil. After 6 weeks, the mice were killed by breaking cervical

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vertebra, and liver tissues were removed for use in the study.

For delivery of the expressing sh-HOTTIP adenoviral vector, Ad-shHOTTIP, the CCl_4-treated mice received Ad-shHOTTIP (1×10^9 pfu/100μL) or its control (1×10^9 pfu/100μL) by intravenous injection once every 2 weeks for 6 weeks (three times). After 6 weeks, the mice were killed by breaking cervical vertebra, and the liver tissues were used for analysis. In this part of the study, C57BL/6J mice were randomly divided into four groups (n=12 per group): control, CCl_4 group, CCl_4+Ad-shCtrl group, and CCl_4+Ad-shHOTTIP group. The mouse experimental protocols were performed following "The Guide for the Care and Use of Laboratory Animals" and approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University.

Culture of primary HSCs and hepatocytes

The primary HSCs were isolated according to the detailed protocol in [17-19] and then cultured in DMEM (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS, Gibco, Waltham, MA, USA), 1% penicillin-streptomycin (HyClone, South Logan, UT, USA) at 37°C with 5% CO_2. The primary hepatocytes were isolated as described in [20] and were cultured in Williams' medium E (Biochrom, Cambridge, UK) supplemented with 10% FBS (Gibco, Waltham, MA, USA), 1% penicillin-streptomycin (HyClone, South Logan, UT, USA), at 37°C with 5% CO_2.

Quantitative real-time PCR (qRT-PCR) analysis

In this study, the expressions of HOTTIP, miR-148a, α-SMA, Col-1, TGFBR1, TGFBR2, Smad2, and Smad3 mRNA were determined in liver tissues or cells through the use of qRT-PCR analysis. Total RNA was extracted from liver tissues or cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of total RNA was quantified on a NanoDrop 2000C spectrophotometer (Thermo Scientific, Waltham, MA, USA). The total RNA (equal amounts of each sample) was reverse transcribed into cDNA using PrimeScript cDNA Synthesis Kit (TaKaRa Biotechnology, Kusatsu, Japan). The qRT-PCRs of HOTTIP, α-SMA, Col-1, TGFBR1, TGFBR2, Smad2 and Smad3 were performed using SYBR Green Real-Time PCR Master Mixes (Applied Biosystems, Foster City, CA, USA) with specific primers on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primers used were as follows: HOTTIP, (forward) 5'-CACACTCACATTCGCACACT-3' and (reverse) 5'-TCCAGAACTAAGCCAGCCATA-3'; α-SMA, (forward) 5'-CTGACAGAGGCACCACTGAA-3' and (reverse) 5'-CATCTCCAGAGTCCAGCACA-3'; Col-1, (forward) 5'-GATTGAGAACATCCGCAGC-3' and (reverse) 5'-CATCTTGAGGTCACGGCAT-3'; TGFBR1, (forward) 5'-AAACTTGCTCTGTCCACGG-3' and (reverse) 5'-AATGGCTGGCTTTCCTTG-3'; TGFBR2, (forward) 5'-GTAATAGGACTGCCCATCCAC-3' and (reverse) 5'-GATTTCTGGTTGTCACAGGTG-3'; Smad2, (forward) 5'-AGCAGAATACCGAAGGCAG-3' and (reverse) 5'-TTTGTCCAACCACTGTAGAGGT-3'; Smad3, (forward) 5'-GCTGCTCTCCAATGTCAACAG-3' and (reverse) 5'-TCTTCCGATGTGTCTCCGT-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. For miR-148a expression analysis, total RNA extracted from liver tissues and cells was reverse transcribed into cDNA using the Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA) and the qRT-PCR was performed using SYBR Green master mix (CoWin Biotech, Beijing, China) according to the manufacturer's instructions. U6 was used as the internal control for miR-148a.

Western blot analysis

The protein expressions of α-SMA, Col-1, TGFBR1, TGFBR2, Smad2 and Smad3 were determined using Western blot analysis. Total protein was extracted from liver tissues or cells using lysis buffer (Beyotime Biotechnology, Haimen, China) according to the manufacturer's instructions. The concentration of total protein was quantified using a BCA kit (Beyotime Biotechnology, Haimen, China) according to the manufacturer's instructions. The total protein (20μg per sample) was separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Burlington, MA, USA), and the PVDF membranes were blocked with 5% skimmed milk for 1h. The proteins on PVDF membranes were then incubated with the primary antibodies of anti-α-SMA (1:2000, Abcam, Cambridge, UK), anti-Col-1 (1:1000, Novus Biologicals), anti-TGFBR1 (1:2000, Abcam, Cambridge, UK), anti-TGFBR2 (1:2000, Abcam, Cambridge, UK), anti-Smad2 (1:1000, Novus Biologicals, Littleton, CO, USA), anti-Smad3 (1:1000, Novus Biologicals, Littleton, CO, USA) and β-actin (1:2000, ProteinTech Group, Inc., Chicago, IL, USA) overnight at 4°C. The proteins on were then incubated with the appropriate HRP-conjugated secondary antibodies. The bands were visualized using ECL Substrate (Thermo Scientific, Waltham, MA, USA) and quantified using Quantity One imaging software

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(Bio-Rad, Hercules, CA, USA).

Analysis of hydroxyproline (HYP) content

The hydroxyproline (HYP) content of mouse liver tissues was measured using the commercial HYP colorimetric assay kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions. Briefly, the liver tissue homogenates were hydrolyzed overnight and then centrifugated to collect the supernatant. After being dried under vacuum, the extract was used for analysis of HYP content.

Cell transfection

In this study, the transfections of si-HOTTIP (Shanghai GenePharma Co., Ltd., Shanghai, China), pcDNA-HOTTIP (Shanghai GenePharma Co., Ltd., Shanghai, China), miR-148a mimic and inhibitor (RiboBio, Guangzhou, China) were used for modulating the expression of HOTTIP and miR-148a in HSCs *in vitro*. The HSCs were cultured at a concentration of 2.0×10^5 cells/ml overnight and then transfected with si-HOTTIP (30nM), pcDNA-HOTTIP (30nM), miR-148a mimic (20nM), miR-148a inhibitor (20nM), or their control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

MTT assays

Cell viability was determined using MTT assays. The HSCs (2.0×10^3 cells/well) were cultured in 96-well plates overnight, and then transfected with si-HOTTIP (30nM) or its control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 24h of transfection, the cells in each well were incubated with MTT solution (20μL, 5mg/mL) for 4h at 37°C. After removal of the medium, DMSO (150μL) was added into each well for 10min. The absorbance of each well was detected at 490 nm on a microplate spectrophotometer (Molecular Devices, San Jose, CA, USA).

Luciferase activity assays

Dual-Luciferase pmirGLO plasmid was purchased from Promega (Madison, WI, USA). The HOTTIP luciferase reporter vectors containing miR-148a binding site (pmirGLO-HOTTIP) or mutated site (pmirGLO-HOTTIP-MU) were constructed by Shanghai GenePharma Co., Ltd. HEK293T cells were cultured in 24-well plates and transfected with miR-148a mimic/inhibitor and pmirGLO plasmid using Lipofectamine 2000 and X-tremeGENE HP (Roche Applied Science, Penzberg, Germany), respectively. After 48h of transfection, the luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Dual-Luciferase Reporter Assay was also performed as described above to validate the direct binding between miR-148a and TGFBR1/TGFBR2. Three independent experiments were carried out.
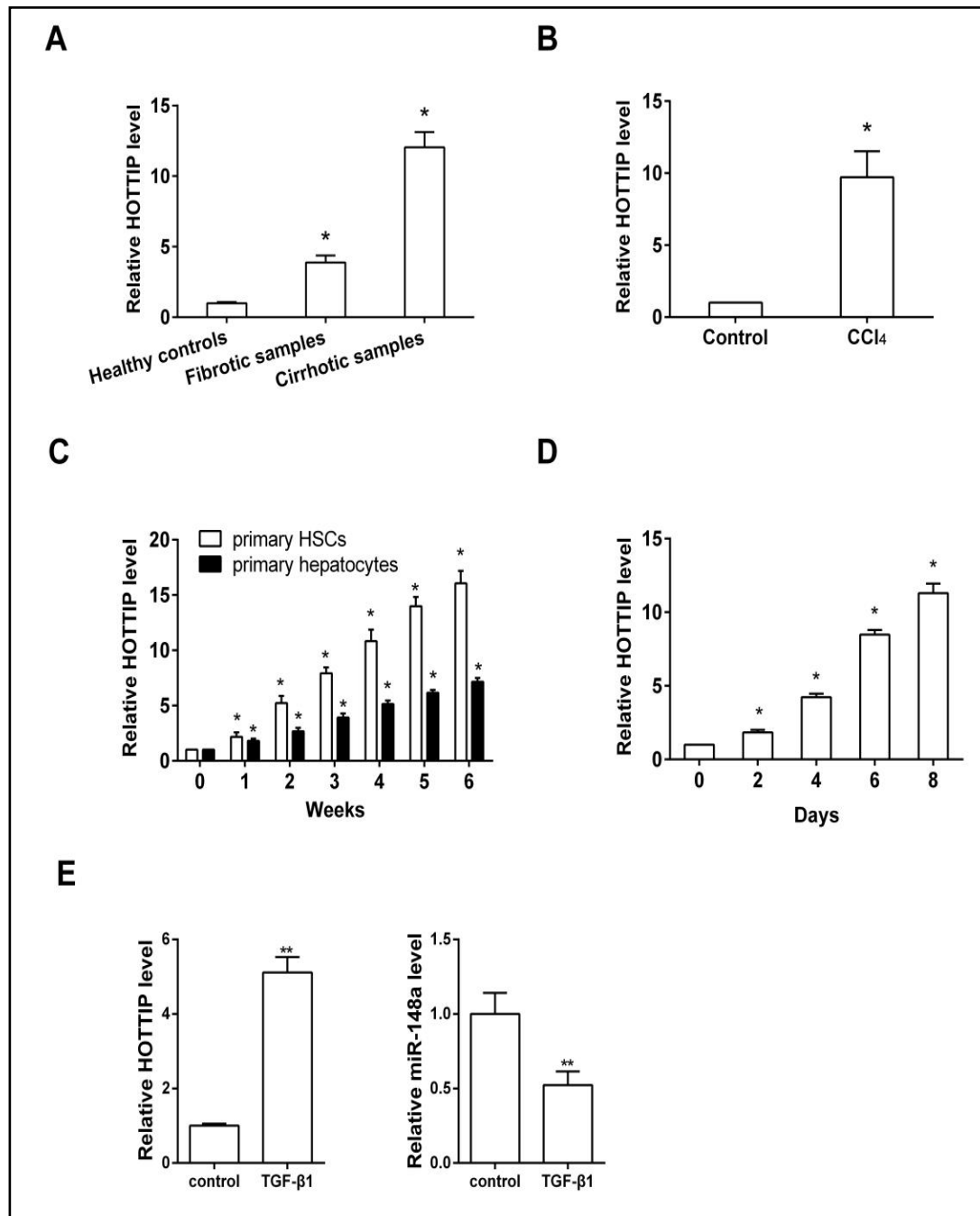
Statistical analysis

All data from three independent experiments were expressed as mean values±standard deviation (SD). The comparison between two groups was conducted using Student's test on GraphPad 6.0 software (Prism, La Jolla, CA, USA), and $P < 0.05$ was considered statistically significant.
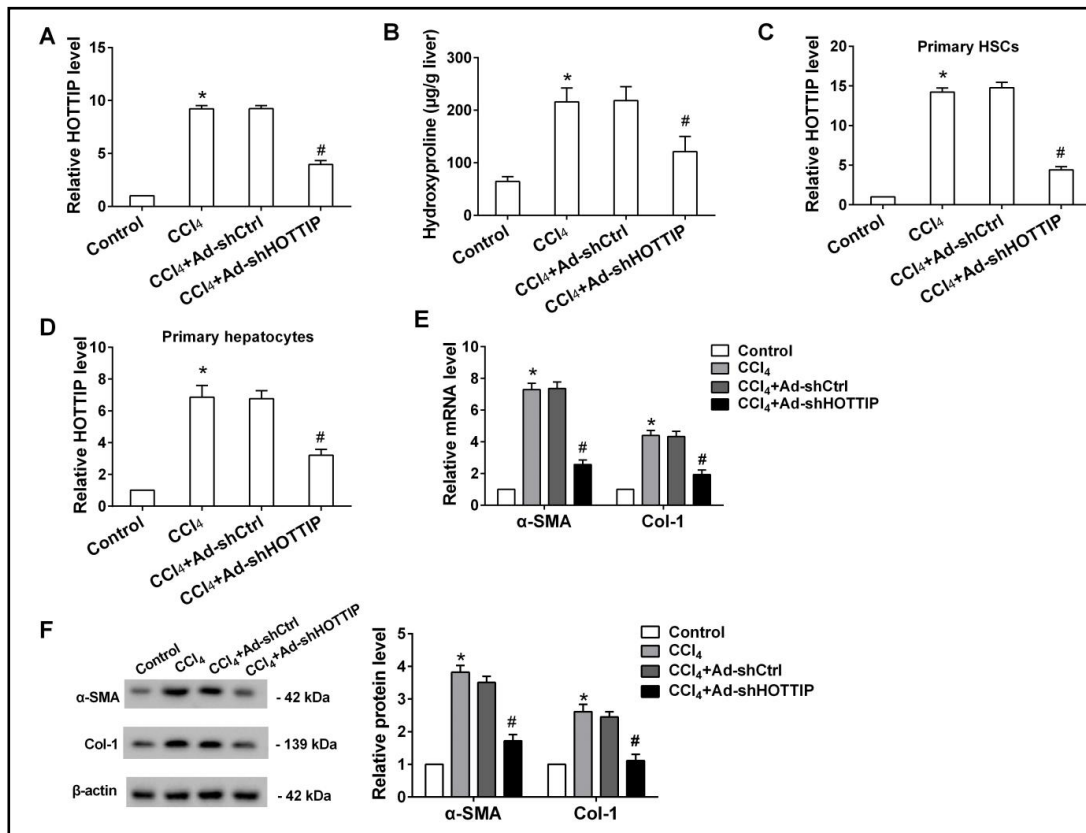
Results

HOTTIP was upregulated during the progression of liver fibrosis

The liver samples from patients with liver fibrosis in chronic hepatitis B (Fibrotic samples, n=30), patients with liver cirrhosis (Cirrhotic samples, n=30), and healthy controls (n=30) were collected and used for analysis of the relative expression of HOTTIP during the progression of liver fibrosis. HOTTIP expressions in fibrotic liver samples and cirrhotic liver samples were significantly upregulated in comparison to the healthy liver controls, and cirrhotic samples exhibited the highest level of HOTTIP (Fig. 1A). Moreover, HOTTIP expression was substantially induced in the mouse liver tissues (n=12) by intraperitoneal injection of CCl_4 (Fig. 1B), a typical method for inducing liver fibrosis. We further analyzed the expressions of HOTTIP in the primary HSCs and primary hepatocytes isolated from the CCl_4-treated mice and revealed that HOTTIP increased significantly in both primary HSCs and hepatocytes with the extension of CCl_4 inducing time (Fig. 1C). Notably, it increased

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Fig. 1. HOTTIP was upregulated during the progression of liver fibrosis. (A) The relative expression of HOTTIP in liver samples from patients with liver fibrosis in chronic hepatitis B (Fibrotic samples, n=30), patients with liver cirrhosis in chronic hepatitis B (Cirrhotic samples, n=30) and healthy controls (n=30). *P<0.05 vs. Healthy controls. The liver fibrosis in C57BL/6J mice was induced by intraperitoneal injection of CCl_4. The mice (n=12) that received olive oil were used as the control for the CCl_4-treated mice. (B) The relative expression of HOTTIP in liver tissues taken from the CCl_4-treated mice (n=12) and the control mice (n=12). *P<0.05 vs. Control. (C) The relative expression of HOTTIP in the primary HSCs and primary hepatocytes isolated from the CCl_4-treated mice at 0, 1, 2, 3, 4, 5, and 6 weeks. *P<0.05 vs. 0 week. (D) The relative expression of HOTTIP in the cultured primary HSCs isolated from healthy mice. *P<0.05 vs. 0 day. (E) Human LX-2 HSCs were treated with transforming growth factor (TGF)-β1 (2.0 ng/mL) for 24 h. The relative expression of HOTTIP and miR-148a were analyzed. **P<0.01 vs. control.

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Fig. 2. Down-regulation of HOTTIP alleviated the CCl$_4$-induced liver fibrosis in mice. C57BL/6J mice were randomly divided into four groups (n=6 per group): control, CCl$_4$ group, CCl$_4$+Ad-shCtrl group, and CCl$_4$+Ad-shHOTTIP. Ad-shHOTTIP, expressed sh-HOTTIP adenoviral vector; Ad-shCtrl, the adenoviral vector expressing scrambled shRNA for sh-HOTTIP. (A) The relative expression of HOTTIP in the liver tissues from all mice. (B) The hydroxyproline (HYP) content detected in liver tissues from the mice. (C) The relative expression of HOTTIP in the primary HSCs and (D) in the primary hepatocytes isolated from the mice. (E-F) The mRNA and protein expression of α-SMA and type I collagen (Col-1) in liver tissues of the mice. *P<0.05 vs. control; #P<0.05 vs. CCl$_4$+Ad-shCtrl.

more significantly in the primary HSCs (Fig. 1C). Therefore, the relative expressions of HOTTIP in the cultured primary HSCs isolated from healthy mice were also determined up to day 8, and results showed that HOTTIP expressions were significantly elevated in a time-dependent manners (Fig. 1D). Moreover, treatment with TGF-β1, a widely used stimulus for HSC activation, significantly upregulated the expression of HOTTIP and miR-148a in human LX-2 HSCs (Fig. 1E). These results suggest that greater expression of HOTTIP was linked to the progression of liver fibrosis.

Downregulation of HOTTIP alleviated the CCl$_4$-induced liver fibrosis in mice

To determine the role of the abnormal expression of HOTTIP in liver fibrosis, the CCl$_4$-treated mice received the expressing sh-HOTTIP adenoviral vector Ad-shHOTTIP to reduce HOTTIP levels *in vivo*, and it effectively downregulated HOTTIP expression in the liver tissues of the CCl$_4$-treated mice (Fig. 2A). Moreover, the high HYP content induced in liver tissues by CCl$_4$ was significantly decreased by Ad-shHOTTIP (Fig. 2B). We also observed that the relative expression of HOTTIP was markedly reduced by Ad-shHOTTIP delivery in both the primary HSCs and primary hepatocytes of the CCl$_4$-treated mice (Figures 2C and 2D). The mouse liver tissue samples were analyzed for mRNA and protein expressions of α-SMA and Col-1, which were the important factors in liver fibrosis, and the results showed that Ad-
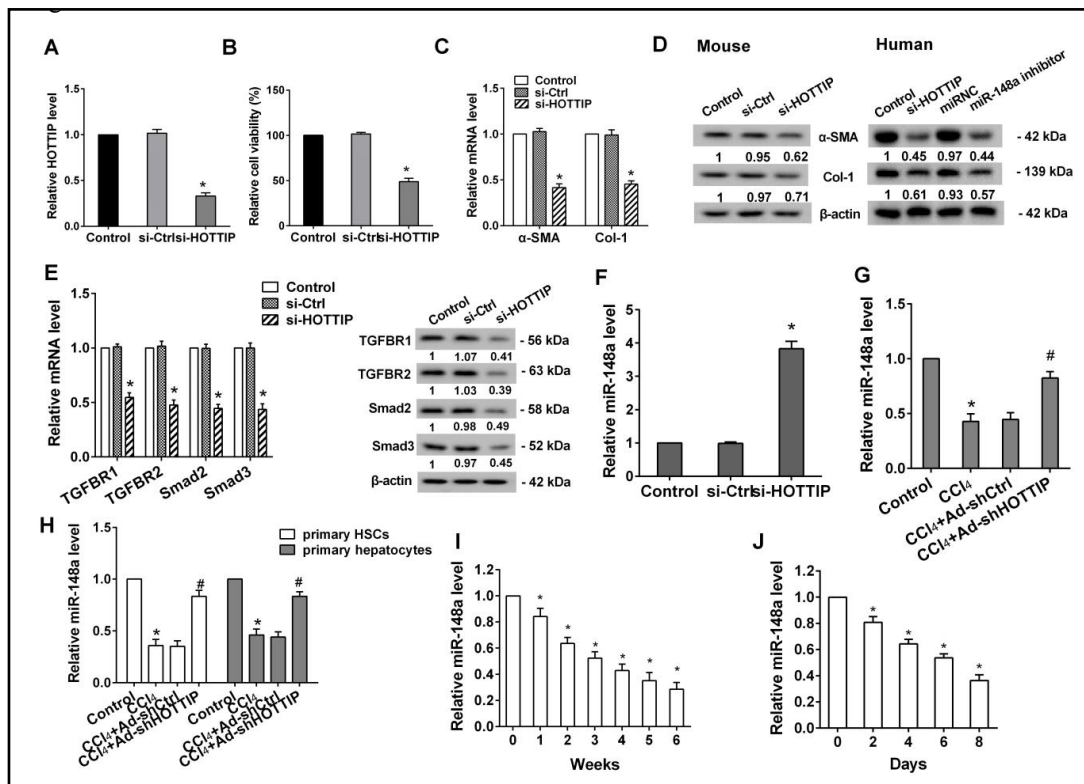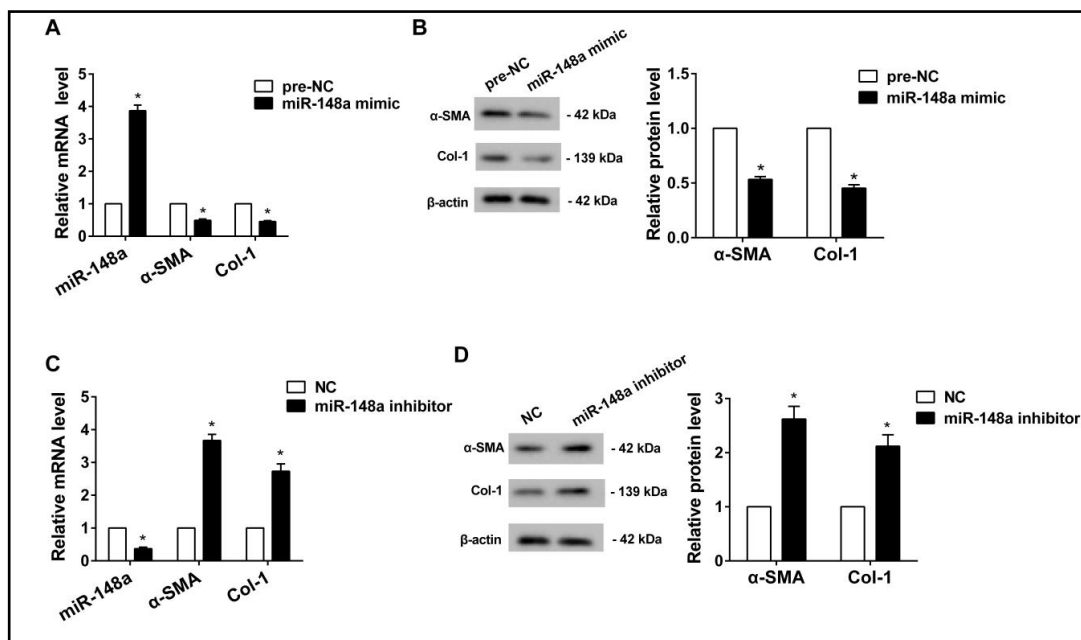
Fig. 3. Downregulation of HOTTIP inhibited the viability and activation of HSCs in vitro. The primary HSCs were cultured and transfected with si-HOTTIP and its control, si-control (si-Ctrl). The cells with routine cultivation were used as the control. (A) The transfection efficiency of si-HOTTIP in vitro was determined using qRT-PCR. (B) The relative viability was detected using MTT assays. (C-D) The mRNA and protein expressions of α-SMA and Col-1 were detected in the primary HSCs transfected with si-HOTTIP or si-control or in the human LX-2 cells transfected with Si-NC, Si-HOTTIP, miRNC or miR-148a mimic and treated with TGF-β1. Si-NC, the negative control for Si-HOTTIP; miRNC, the negative control for miR-148a mimic. (E) The mRNA and protein expressions of TGFBR1/2 and Smad2/3 were detected. (F) The relative expression of miR-148a in the primary HSCs transfected with si-HOTTIP or si-control. (G) The relative expression of miR-148a was analyzed in liver tissues of the CCl$_4$-treated mice with or without Ad-shHOTTIP administration. (H) The relative expression of miR-148a was analyzed in the primary HSCs and primary hepatocytes isolated from the CCl$_4$-treated mice with or without Ad-shHOTTIP administration. (I) The relative expression of miR-148a was analyzed in the primary HSCs isolated from the CCl$_4$-treated mice at 0, 1, 2, 3, 4, 5, and 6 weeks. (J) The relative expression of miR-148a was analyzed in the cultured primary HSCs isolated from healthy mice during culture days. For (A)-(F), *P<0.05 vs. si-Ctrl; For (G)-(J), *P<0.05 vs. control; #P<0.05 vs. CCl$_4$+Ad-shCtrl.

shHOTTIP effectively decreased α-SMA and Col-1 expression at both the mRNA and protein levels (Figures 2E and 2F). Taken together, this indicates that suppression of HOTTIP could alleviate CCl$_4$-induced liver fibrosis in mice.

Downregulation of HOTTIP inhibited the viability and activation of HSCs in vitro

We further examined the effect of HOTTIP on the viability and activation of HSCs *in vitro*. As indicated in Fig. 3A, HOTTIP siRNA (si-HOTTIP) transfection significantly inhibited HOTTIP expression in the cultured HSCs. In addition, the relative viability of HSCs were significantly reduced after transfection of si-HOTTIP (Fig. 3B). The decrease of HOTTIP expression downregulated both the mRNA and protein levels of α-SMA and Col-1 (Figures 3C and 3D left). In LX-2 cells, Western blotting demonstrated that decreased HOTTIP or decreased miR-148a reduced the protein levels of α-SMA and Col I that had been induced

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Fig. 4. MiR-148a inhibited the activation of HSCs in vitro. (A) The primary HSCs were cultured and transfected with miR-148a mimic or its control, pre-NC. The relative expressions of miR-148a, α-SMA and Col-1 mRNA were analyzed in the HSCs transfected with miR-148a mimic or pre-NC. (B) The protein expressions of α-SMA and Col-1 were also analyzed. (C) The primary HSCs were transfected with miR-148a inhibitor or its control, NC. The relative expressions of miR-148a, α-SMA and Col-1 mRNA were analyzed in the HSCs transfected with miR-148a inhibitor or NC. (D) The protein expressions of α-SMA and Col-1 were analyzed. For (A)-(B), *P<0.05 vs. pre-NC; For (C)-(D), *P<0.05 vs. NC.
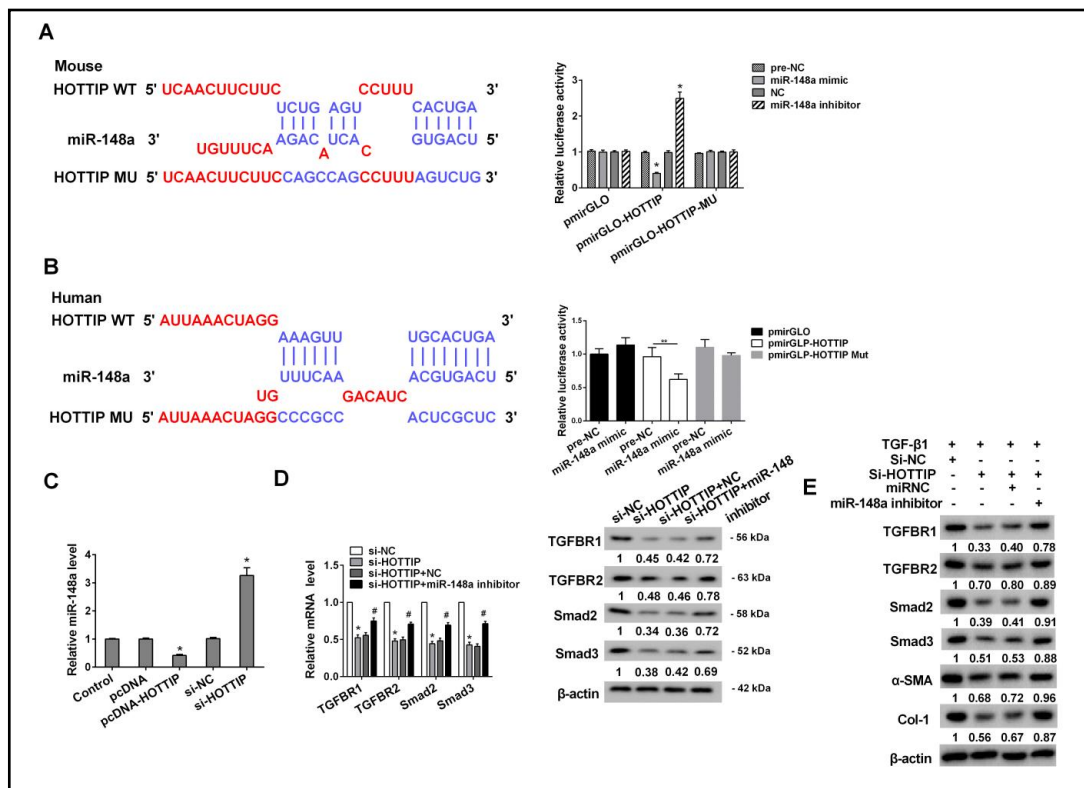
by TGF-β1 (Fig. 3D right). The TGF-β/Smad signaling pathway was confirmed to be closely related to HSCs activation of liver fibrosis *in vivo* and *in vitro*. Thus we next explored the effect of HOTTIP silencing on the TGF-β/Smad signaling pathway in HSCs and found that HOTTIP silencing induced a significant decrease in the mRNA and protein expression of TGFBR1, TGFBR2, Smad2 and Smad3 in HSCs *in vitro* (Fig. 3E). Taken together, these results suggest that HOTTIP silencing inhibits the activation of HSCs through the TGF-β/Smad signaling pathway.

Given the possible interactions between HOTTIP and miR-148a, and based on bioinformatics analysis, we profiled miR-148a expression in HSCs after HOTTIP silencing. As expected, downregulation of HOTTIP promoted miR-148a expression in HSCs (Fig. 3F). Moreover, miR-148a expression was significantly downregulated in the liver tissues of the CCl₄-treated mice, while HOTTIP silencing by Ad-shHOTTIP significantly enhanced miR-148a in the CCl₄-treated mice (Fig. 3G). It was also observed that miR-148a expression was inhibited by CCl₄ in the primary HSCs and hepatocytes, and HOTTIP silencing elevated miR-148a expression (Fig. 3H). The relative expressions of miR-148a in the primary HSCs isolated from the CCl₄-treated mice were found to decrease in a time-dependent manner (Fig. 3I). Similarly, miR-148a expressions were also gradually decreased in the cultured primary HSCs during culture days (Fig. 3J). These data indicate that miR-148a was involved in liver fibrosis by affecting HSC activation.

miR-148a inhibited the activation of HSCs in vitro
Based on the above findings, we sought to investigate the precise role of miR-148a in HSC activation. The induction of miR-148a in HSCs significantly reduced the mRNA levels of α-SMA and Col-1 when compared with the untransfected HSCs (Fig. 4A). Additionally, the protein expressions of α-SMA and Col-1 were also significantly decreased by miR-148a overexpression in both mouse and human HSCs (Fig. 3D and 4B). On the other hand, the

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Fig. 5. The downregulated HOTTIP reduced TGFBR1/2 and Smad2/3 in HSCs through negative regulation of miR-148a. (A) The predicted HOTTIP binding site in miR-148a based on DIANA Tools online systems. Dual-Luciferase Reporter Assay was performed to validate the direct binding between mouse HOTTIP and miR-148a. The reporter vectors pmirGLO-HOTTIP or pmiRGLO-HOTTIP-mutated-type (pmirGLO-HOTTIP-MU), together with miR-148a mimic/inhibitor were transfected into HEK293T cells, and the relative luciferase activity was detected after 48h transfection. *P<0.05 vs. pre-NC or NC. (B) The diagram of human HOTTIP putative binding sites in has-miR-148a, and the relative luciferase activity was detected after 48h transfection. pre-NC, the negative control for miR-148a mimic. **P<0.01 vs. pre-NC. (C) The relative expressions of miR-148a were analyzed in the primary HSCs transfected with pcDNA-HOTTIP, si-HOTTIP, or their control, pcDNA, and si-NC. *P<0.05 vs. pcDNA or si-NC. (D) The mRNA and protein expressions of TGFBR1/2 and Smad2/3 were analyzed in the primary HSCs transfected with si-HOTTIP and miR-148a inhibitor or si-NC. *P<0.05 vs. si-NC; #P<0.05 vs. si-HOTTIP+NC. (E) LX-2 cells were divided into four groups: transfected with Si-NC and treated with TGF-β1; transfected with Si-NC and treated with TGF-β1; co-transfected with Si-HOTTIP and miRNC and treated with TGF-β1; and co-transfected with Si-HOTTIP and miR-148a inhibitor and treated with TGF-β1. The protein expression of TGFBR1, TGFBR2, Smad2, Smad3, α-SMA and Col I were analyzed. Si-NC, the negative control for Si-HOTTIP; miRNC, the negative control for miR-148a inhibitor.

mRNA and protein expressions of α-SMA and Col-1 were significantly increased in miR-148a knockdown HSCs (Figures 4C and 4D). Overall, our results showed that, in contrast to HOTTIP, miR-148a had negative effects on HSC activation.

The down-regulated HOTTIP reduced TGFBR1/2 and Smad2/3 in HSCs through negative regulation of miR-148a

To test whether miR-148a mediated the effect of HOTTIP on HSCs activation, we first performed the Dual-Luciferase Reporter Assay to validate the direct binding between mouse HOTTIP and miR-148a based on the predicted HOTTIP binding site in miR-148a (Fig. 5A). The HOTTIP luciferase reporter vectors containing the miR-148a binding site (pmirGLO-HOTTIP) or mutated site (pmirGLO-HOTTIP-MU) were constructed. The miR-148a levels
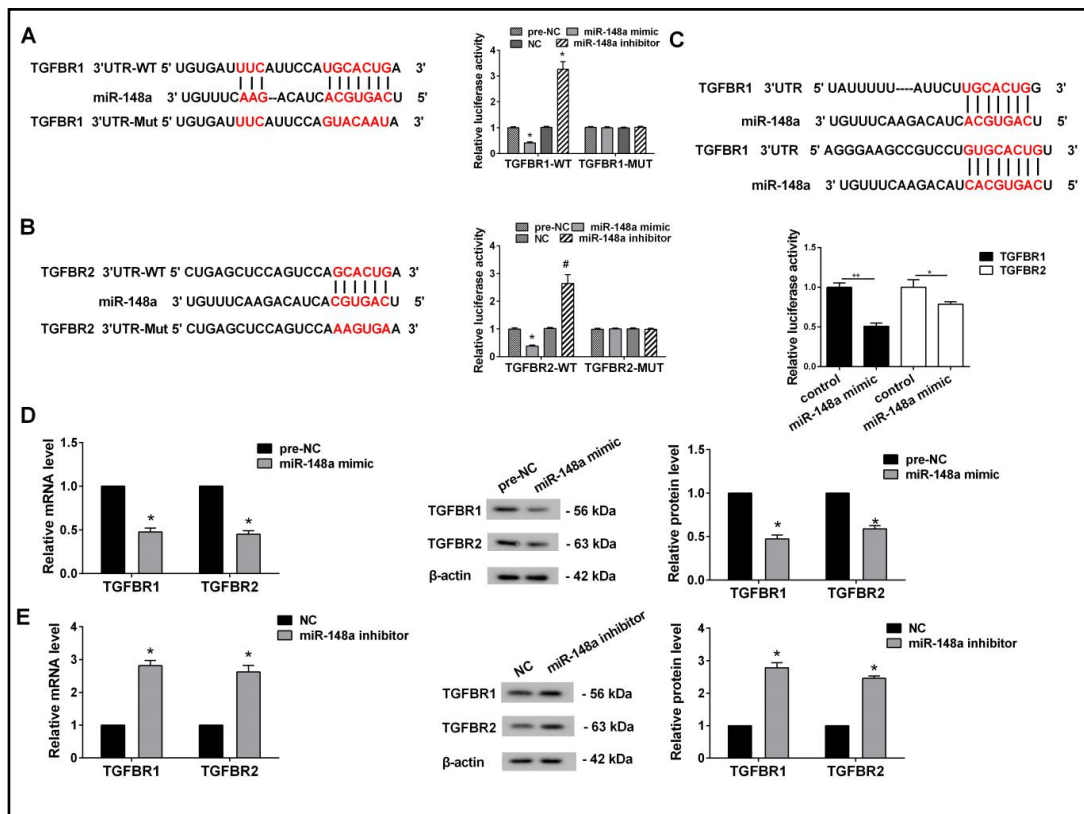
Fig. 6. MiR-148a targeted TGFBR1 and TGFBR2 and, thus, downregulated their expression in HSCs. (A) The putative miR-148a binding site presented in TGFBR1 3-UTR based on microRNA.org software. Dual-Luciferase Reporter Assay was performed in HEK293T cells to validate the direct binding between TGFBR1 3-UTR and miR-148a. *P<0.05 vs. pre-NC or NC. (B) The putative miR-148a binding site presented in TGFBR2 3-UTR based on microRNA.org software. Dual-Luciferase Reporter Assay was performed to validate the direct binding between TGFBR2 3-UTR and miR-148a in HEK293T cells. *P<0.05 vs. pre-NC or NC. (C) The putative has-miR-148a binding sites presented in the 3-UTR of TGFBR1 and TGFBR2. Luciferase assay was performed with the wild-type TGFBR1 and TGFBR2 constructs. control, the negative control for miR-148a mimic. *P<0.05, **P<0.01 vs. control. (D) The effect of miR-148a overexpression on TGFBR1 and TGFBR2 expression in HSCs. The mRNA and protein expressions of TGFBR1 and TGFBR2 were analyzed in the primary HSCs transfected with miR-148a mimic or pre-NC. *P<0.05 vs. pre-NC. (E) The effect of miR-148a knockdown on TGFBR1 and TGFBR2 expression in HSCs. The mRNA and protein expressions of TGFBR1 and TGFBR2 were analyzed in the primary HSCs transfected with miR-148a inhibitor or NC. *P<0.05 vs. NC.

negatively regulated the relative luciferase activity of pmirGLO-HOTTIP but had no obvious effect on pmirGLO-HOTTIP-MU luciferase activity in HEK293T cells, suggesting the interaction between HOTTIP and miR-148a in a sequence-specific manner (Fig. 5A). Bioinformatics analysis and luciferase assays also supported the interaction between human HOTTIP and has-miR-148a (Fig. 5B). Indeed, the relative expression of miR-148a was inhibited by HOTTIP, but it was elevated by HOTTIP knockdown in the primary HSCs (Fig. 5C). To determine whether miR-148a was involved in regulation of the TGF-β/Smad signaling pathway due to effects from HOTTIP, we examined the effect of simultaneous downregulation of HOTTIP and miR-148a on the TGF-β/Smad signaling pathway. Notably, downregulated miR-148a markedly restored the mRNA and protein expression of TGFBR1, TGFBR2, Smad2, and Smad3 that had been decreased by HOTTIP silencing in both mouse and human HSCs (Fig. 5D and 5E). miR-148a knockdown also markedly restored the decreased mRNA and protein expression of α-SMA and Col-1 that had been caused by HOTTIP silencing in both mouse and human HSCs (Fig. 5E). These data suggest that miR-148a mediated the inhibition of HOTTIP

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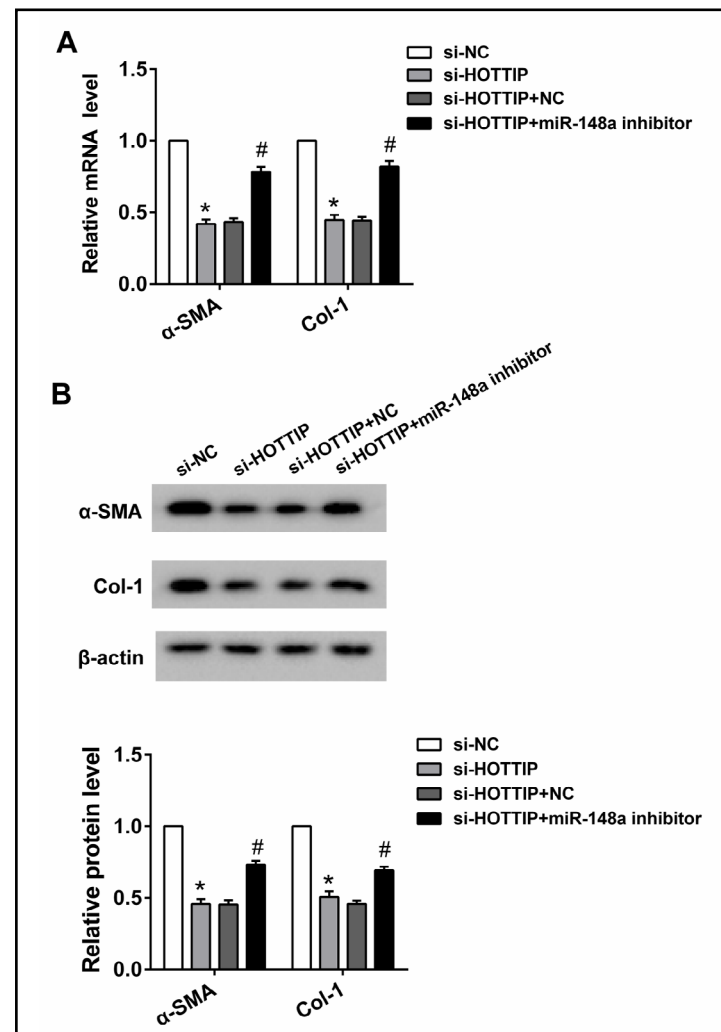Li et al.: The Role of HOTTIP in Liver Fibrosis

silencing on TGFBR1/2 and Smad2/3 in HSCs.

miR-148a targeted TGFBR1 and TGFBR2, and thus downregulated their expression in HSCs
Based on microRNA.org software, both TGFBR1 and TGFBR2 were projected to be the targets of miR-148a (Figures 6A and 6B). Results from the Dual-Luciferase Reporter Assay supported our hypothesis, revealing that miR-148a expression negatively regulated the luciferase activity of the TGFBR1/TGFBR2 luciferase reporter vectors containing the miR-148a binding site but had no obvious effect on the luciferase activity of luciferase reporter vectors containing a mutated site in HEK293T cells (Figures 6A and 6B). Moreover, the human TGFBR1 and TGFBR2 3'UTR sequences were found to contain target sites for miR-148a (Fig. 6C). Luciferase assays validated that TGFBR1 and TGFBR2 were the targets of hsa-miR-148a (Fig. 6C). Further studies showed that the forced miR-148a significantly inhibited the mRNA and protein expressions of TGFBR1 and TGFBR2 in primary HSCs

Fig. 7. Downregulation of HOTTIP inhibited the activation of HSCs in vitro via negative regulation of miR-148a. The relative expressions of (A) α-SMA and Col-1 mRNA and (B) protein were analyzed in the primary HSCs transfected with si-HOTTIP or si-HOTTIP and miR-148a inhibitor or si-HOTTIP and NC. *P<0.05 vs. si-NC; #P<0.05 vs. si-HOTTIP+NC.

as compared with the control (Fig. 6D), and downregulation of miR-148a in HSCs had the opposite effect on TGFBR1 and TGFBR2 expression (Fig. 6E). Thus, we confirmed that both TGFBR1 and TGFBR2 were the targets of miR-148a.

Down-regulation of HOTTIP inhibited the activation of HSCs in vitro via negative regulation of miR-148a
We next focused on whether HOTTIP inhibited the activation of HSCs by regulating miR-148a. We observed that the miR-148a knockdown markedly restored the decreased mRNA and protein expression of α-SMA and Col-1 that had been caused by HOTTIP silencing in both mouse and human HSCs (Figures 5E, 7A and 7B). These results corroborated that HOTTIP contributed to HSC activation, at least in part, through negative regulation of miR-148a.

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Li et al.: The Role of HOTTIP in Liver Fibrosis

Discussion

Liver fibrosis is a pathological change caused by chronic liver damage and is characterized by impaired liver function and an excessive hepatic deposition of ECM in the Disse space of the sinusoidal cell layer [21]. Without an effective control, liver fibrosis further develops into irreversible end-stage liver cirrhosis or even liver cancer, with high mortality. HSC activation is the fundamental process in liver fibrosis resulting from different causes and impact factors [22]. In one study, in response to acute or chronic liver injury, normal resting HSCs were transformed into activated HSCs that synthesized and secreted collagen-rich ECM [23]. Targeting the regulatory mechanism of HSC activation may serve as an effective therapeutic strategy for liver fibrosis.

HOTTIP, located on 7p15.2, plays a pivotal role in a number of human cancers, including gastric cancer, hepatocellular carcinoma, and colorectal cancer [14-16]. High HOTTIP expression has been detected in human HCC specimens and associated with the clinical progression and disease outcome of HCC patients [15]. More importantly, HOTTIP has been demonstrated to dysregulate in early stage of human HCC formation [24]. In the current study, we showed that HOTTIP was up-regulated during the progression of liver fibrosis and that downregulation of HOTTIP significantly alleviated the CCl_4-induced liver fibrosis in mice, a well-established model for liver fibrosis. Moreover, we also identified HOTTIP as a potent inducer of HSC activation. Studies on the role of HOTTIP in human diseases have mainly focused on its oncogenic activity, which fuels tumor cell growth, metabolism, and metastasis. HOTTIP expression has been observed to elevate in developing hind limbs, and it is implicated in bat wing development [25]. HOTTIP has also been shown to be significantly upregulated in the chondrocytes of osteoarthritis, and it has been demonstrated to regulate cartilage integrity [26]. The present study is the first to evaluate the role of HOTTIP in liver fibrosis and confirmed that HOTTIP dysregulation is associates with *in vivo* HSC activation.

MiR-148a, as a liver-related miRNA, has been implicated in HCC initiation, progression and liver fibrosis. MiR-148a was previously reported to induce hepatocytic differentiation, thereby inhibiting the formation and development of HCC; remarkably, miR-148a mimetic treatment may significantly reduce liver fibrosis of developed tumors in mice [11]. Recombinant human TGFβ1, a widely used stimulus for HSC activation, has significantly increased the expression level of miR-148a in human HSC, while transfection of miR-148a inhibitor evidently reduced COL6A1 levels associated with HSC activation [27]. A recent study established a link between miR-148a and the Wnt/β-catenin pathway [28]. The Wnt/β-catenin pathway has been shown to play essential roles in HSC activation and to eventually promote the progression of liver fibrosis [29]. Obviously, these prior studies indicate that miR-148a could be an important determinant in liver fibrosis. In the present study, we showed that miR-148a overexpression inhibited the activation of HSCs *in vitro*, and we further showed the functional involvement of miR-148a in the development of liver fibrosis induced by HOTTIP. We identified that HOTTIP negatively regulated miR-148a in HSCs, and the new targets of miR-148a, TGFBR1, and TGFBR2 were significantly increased, thereby enhancing HSC activation. TGF-β/Smad signaling, tightly regulated by TGFBR1/TGFBR2 complexes, was the essential inducer for HSC activation and survival in liver fibrosis [30, 31].

HOTTIP was located at chr. 7p15.2 (27200421..27207259) and chr. 6B3 (52262775..52267603) in the human and mouse genomes, respectively, while miR-148a was located in human chr. 7p15.2 (25949919..25949986) and mouse chr. 6B3 (51269812..51269910), respectively. The interaction of HOTTIP/WDR5 (MLL) generates a transcription loop involving the activation of nearby genes at the 5' end of the locus HOXA (HOXA13-HOXA9). Importantly, the transcription loop is located about 300bp upstream of the HOXA (HOXA13-HOXA9) 5' end and the miR-148a gene is not in this transcription loop. The chromosome location and sequence similarity suggest that some lncRNAs might serve as the host genes of miRNA and act in close association with miRNAs expression and function. However, in the present study, we observed that HOTTIP negatively regulated miR-148a in a sequence-specific manner.

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Conclusion

Our study confirmed that HOTTIP is involved in the progression of liver fibrosis by promoting HSC activation and miR-148a downregulated by HOTTIP played a central role in this pathogenic process. Notably, TGFBR1 and TGFBR2 were identified as the new targets of miR-148a in HSCs. The high level of HOTTIP downregulated miR-148a, increased the levels of TGFBR1 and TGFBR2 and thus contributed to liver fibrosis (Fig. 8). Finally, our study further illustrated the underlying mechanisms for liver fibrosis and is expected to provide new insights into therapeutic strategy.

Fig. 8. Schema depicting the mechanisms of the involvement of the HOTTIP/miR-148a axis in liver fibrosis.
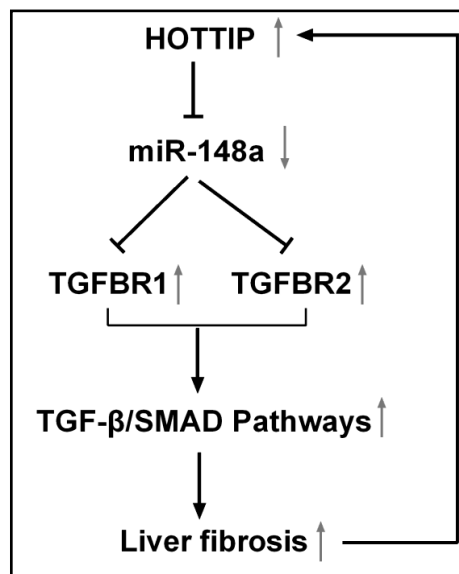
Acknowledgements

Disclosure Statement

The authors declare no conflicts of interest.

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