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C1q/tumor necrosis factor-related protein-3 acts as a target treating hepatic fibrosis

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Abstract. – OBJECTIVE: C1q/tumor necrosis factor-related protein-3 (CTRP3) is demonstrated as a crucial factor that participated in various fibrotic diseases. Activation of hepatic stellate cell in liver takes a critical effect on the pathogenesis of hepatic fibrosis. However, the role of CTRP3 in hepatic fibrosis remains elusive. Our present study aimed to explore the molecular mechanism of CTRP3 in fibroblast activation and the development of hepatic fibrosis.

MATERIALS AND METHODS: We carried out overexpression (OE) of CTRP3 or knockout (KO) of CTRP3 in hepatic stellate cells (HSCs), respectively. Then, transforming growth factor-beta (TGF-β) was used to stimulate HSCs activation. Adult male C57BL/6J mice were treated tetrachloromethane by intraperitoneal injection and mice injected saline were served as control. Recombinant CTRP3 (RC-CTRP3) was employed to treat CCl4-induced liver fibrosis. Then, the expression of fibrotic biomarkers, Notch signaling pathway-associated factors, liver histology and liver function were investigated in vivo, respectively.

RESULTS: Our results showed that CTRP3 decreased in fibrotic liver and TGF-β treated HSCs. In vitro, CTRP3 inhibited the activation of HSCs and impeded extracellular matrix (ECM) including collagen I and fibronectin via inhibiting Notch-1/Jagged-1 signaling pathway. In vivo, the indexes of fibrogenesis in liver fibrotic mice received RC-CTRP3 were mitigated via regulation of Notch-1/Jagged-1 signaling pathway. Moreover, liver histology and liver function were improved through the increase of CTRP3 level.

CONCLUSIONS: The results proved that CTRP3 as a distinguished anti-fibrotic target inhibited HSCs activation by TGF-β inducement and protected the liver tissue in the process of liver fibrosis.

Key Words:
CTRP3, Hepatic fibrosis, HSCs, Notch/Jagged signaling pathway.

Introduction

Hepatic fibrosis is a chronic liver disease causing high morbidity and mortality[1]. Due to the wound-healing response to repeated injury, chronic injury in liver, such as inflammation, viral insult and excessive alcohol, eventually develops to hepatic cirrhosis characterized by the accumulation of fibrotic cells and the deposition of massive extracellular matrix (ECM) proteins[2,3]. Hepatic stellate cells (HSCs), located in the Disse space between the hepatic cords and the hepatic sinus wall, are the main interstitial cell group in the hepatic tissue[4]. Under normal conditions, HSC metabolizes and stores vitamin A and fat[5]. Importantly, it synthesizes collagen and proteoglycans, therefore, HSC is considered to be the main synthetic cell of ECM in liver. When liver is stimulated by biochemical factors, HSC storing vitamin A transforms into myofibroblast through phenotypic transformation, characterized by the expression of a variety of cytokines and massive proliferation, the synthesis of ECM in large quantities, the high expression of α-smooth muscle actin (α-SMA), and the contraction function[6,7]. The level of transforming growth factor-beta (TGF-β) is found to increase in serum and tissue during the fibrogenic process[8,9]. The overexpression of TGF-β can bind to TGF-β receptor on the surface

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of HSC, upregulate ECM expression, and induce fiber formation[10]. At the same time, activated HSC and myofibroblasts can continue to stimulate their own secretion of TGF, further activating HSC[11]. HSC trans-differentiation is mediated through the classic TGF/Smad signaling pathway[12]. However, previous studies[13-15] have reported that Notch/Jadded signaling pathway participates in various biological events, including proliferation, migration and differentiation. In fibrogenesis, the level of Notch/Jadded pathway was reported to increase after TGF-β stimuli in various cells. Inhibition of Notch/Jadded signaling pathway *via* small interfering RNA (siRNA) or pathway inhibitor to reduce the fibrotic markers after TGF-β-induced fibrogenesis[16]. Conversely, increased expression of Notch/Jadded pathway after TGF-β treatment promotes tissue fibrosis. C1q/tumor necrosis factor-related proteins (CTRPs), as a novel adipokine in CTRPs family, have been verified to be expressed in multiple tissue and organs[17,18]. It has been demonstrated that CTRP3 plays a vital role in cellular metabolism. Interestingly, CTRP3 was also showed an anti-fibrotic factor in fibrogenesis. The overexpression of CTRP3 attenuates post-infarct cardiac fibrosis by targeting Smad3 activation and inhibiting myofibroblast differentiation[19]. Therefore, we speculate that CTRP3 may reduce HSC differentiation to myofibroblast after TGF-β stimuli and play a protective role in hepatic fibrosis process. We here demonstrated that CTRP3 acts as a regulator in HSC differentiation *in vitro* and *in vivo*; further, increased CTRP3 expression in tetrachloromethane injected mice mitigates the severity of hepatic fibrosis, providing a novel insight into treatments of liver cirrhosis.

Materials and Methods

Cell Culture and Treatment

Hepatic stellate cells (HSCs) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and seeded in flasks. HSCs were seeded in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 1% penicillin, and 1% streptomycin. When cells grew up to suitable confluence, we conducted gene editing using transfection and further induced myofibroblast differentiation *via* transforming growth factor-beta (TGF-β, 20 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) for 24 h.

Cell Transfection

CTRP3 full length complementary deoxyribose nucleic acid (cDNA) and small interfering RNA (siRNA) of CTRP3 was loaded into pcDNA3.1 vector (Genechem, Nanjing, China). HSCs were transfected with them or vectors using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) directed by the manufacturer's protocols. The transfection efficiency was measured with Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

Hepatic Fibrosis Model

C57BL/6J mice (male, 6-8 week, 20-22 g) were selected for hepatic fibrosis model. All animals were obtained from Xi'an Jiaotong University Animal Center. This study was approved by the Animal Ethics Committee of Xi'an Jiaotong University Animal Center. Mice were randomly divided into three groups. Saline group was intraperitoneally injected with equal volume saline same to tetrachloromethane (CCl_4) group; CCl_4 group was intraperitoneally injected with CCl_4 dissolved in olive oil (0.15 mL/kg, 20%) twice a week. In CCl_4+RC-CTRP3 group, mice were received RC-CTRP3 *via* tail vein injection per day.

RT-PCR

Cell and tissue were harvested using TRIzol solution in a Total RNA Extraction Kit (YiFeiXue, Shanghai, China) followed the manufacturer's protocols. Reverse transcription was conducted using a qScript Flex cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA, USA). RNA measurement was performed using a SYBR1 Taq™ Kit (TaKaRa, Tokyo, Japan) in an ABI PRISM 7000 system (Applied Biosystems, Foster City, CA, USA). The level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalization. The primers were recorded at Table I.

Immunoblot

Protein of cell and tissue was isolated using a Total Protein Isolation Kit (YiFeiXue, Shanghai, China) according to the manufacturer's instructions. Protein concentration was detected using bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Electrophoresis and transferring were performed to separate protein. Primary antibodies and secondary antibodies were as follows: CTRP3 (1:1000, Abcam, Cambridge, MA, USA), Notch-1 (1:1000, Abcam, Cambridge, MA, USA), Jadded-1 (1:1000, Abcam, Cambridge, MA, USA), GAPDH (1:2000, Cell signaling technique, Danvers, MA, USA), HRP-Anti-Rabbit

Table I. Primer sequences of quantitative reverse transcription-polymerase chain reaction.

Oligo Name		Sequence (5′ --------> 3′)
CTRP3	Forward	GCCTTTGCTTTTCCTCCCATT
	Reverse	CCTTGGTAACCACGAAATCCA
collagen I	Forward	TGCTCGTCGCCGCTGTCCTT
	Reverse	TTGGGTCCTACAATATCCTTGATGTCTCC
fibronectin	Forward	TTCAAGTGTGATCCCCATGAAG
	Reverse	CAGGTCTACGGCAGTTGTCA
α-SMA	Forward	TCCGGGACATCAAGGAGAAAC
	Reverse	GCCCATCAGGCAACTCGTAA
GAPDH	Forward	TGGCCTTCCGTGTTCCTAC
	Reverse	GAGTTGCTGTTGAAGTCGCA

antibody (1:10000, YiFeiXue, Shanghai, China). Then protein was measured using enhanced chemiluminescence method.

Immunohistochemical Staining

Tissue was collected in 4% paraformaldehyde and turned out to 5 μm paraffin sections. Sections were incubated with primary antibodies (Beyotime, Shanghai, China) at 4°C overnight. Washed by PBS, sections were incubated with corresponding secondary antibodies, and then, performed coloring treatment using diaminobenzidine (DAB) method (Solarbio, Beijing, China). Nucleus was performed counterstaining with hematoxylin for 5 s. Then, the images were captured using a microscope.

IF Staining

Paraffin sections were conducted antigen blocking for 1 h at room temperature. Then, sections were treated with primary antibodies for incubation overnight at 4°C. Washed, sections further carried out fluorescence secondary antibodies treatment for 1 h in dark at room temperature. Fluorescence images were collected using a fluorescence microscope system.

Histology Assessment

Hematoxylin-eosin (HE) staining and Masson's trichrome staining were used to visualize the degree of damage and fibrosis in liver tissue. Staining was performed using HE Staining Kit and Masson's Trichrome Staining Kit (Beyotime, Shanghai, China) according to manufacturer's protocols, respectively. Photos were gathered with a microscope.

Liver Function

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bile acid (TBA) were examined using Dirui CS-T300 Chemistry Analyzer and corresponding kits (Dirui Medical Technology, Changchun, China). Hydroxyproline content was measured using an assay kits according to the manufacturer's instructions.

Statistical Analysis

Data were described as the means ± standard deviations (SD). The differences between the two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Data were collected and analyzed using Statistical Product and Service Solutions (SPSS) 21.0 software (IBM Corp., Armonk, NY, USA). $p<0.05$ is considered as statistical significance.

Results

Loss of CTRP3 Is a Crucial Marker of Fibrogenesis in Liver

Firstly, we detected the RNA levels of CTRP3 and collagen I, a representative marker of fibrosis in liver tissue injected with CCl_4 or not at 2 weeks, 4 weeks and 8 weeks post fibrosis induction (Figure 1A and 1B). It was found that CCl_4 injection remarkably reduced the expression of CTRP3 in liver compared with that in NC liver. Moreover, the liver tissue was used to evaluate the expressions of collagen I and CTRP3 in liver at 2, 4, and 8 weeks post CCl_4 treatment, displaying that CCl_4 injection significantly increased the positive area of collagen I in liver, whereas the positive area of CTRP3 was reduced (Figure 1C and 1D). The results indicate that CTRP3 decrease may play a vital role in the development of CCl_4-induced liver fibrosis.

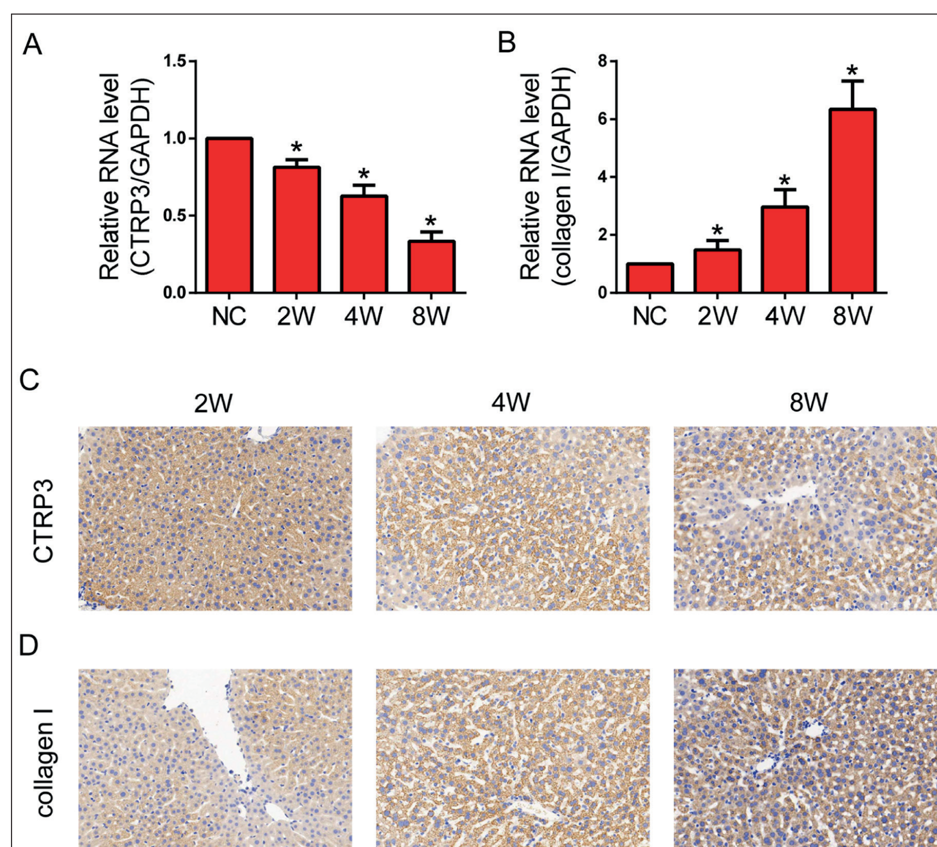

Figure 1. Loss of CTRP3 is a crucial marker of fibrogenesis in liver. **A,** The relative RNA expression of CTRP3 in liver at 2 weeks (W), 4 W, and 8 W post CCl_4 treatment. **B,** The relative RNA expression of collagen I in liver at 2 W, 4W, and 8 W post CCl4 treatment. **C,** The images of CTRP3 IHC in liver at 2 W, 4W, and 8 W post CCl4 treatment, (magnification: 200×). **D,** The images of collagen I IHC in liver at 2 W, 4 W, and 8 W post CCl_4 treatment, (magnification: 200×). "*" means *vs.* NC group with statistical significance.

CTRP3 Regulates HSCs Differentiation to Myofibroblast Via Notch/Jadded Pathway

To clarify the molecular mechanism of CTRP3 in liver fibrosis, we selected HSCs, a major interstitial cell in fibrogenesis of liver cirrhosis, to investigate OE or KO of CTRP3 in TGF-β induced HSC-to-myofibroblast. The RNA expression of CTRP3 was firstly examined using RT-PCR, showing OE or KO treatment indeed increased or decreased CTRP3 expression in HSCs (Figure 2A). Furthermore, the markers of myofibroblast including collagen I, fibronectin and α-smooth muscle actin (α-SMA) were measured in transcription level, finding that TGF-β markedly increased these factors in HSC-to-myofibroblast process. However, OE of CTRP3 reduced TGF-β induced increase of fibrotic markers in HSC-to-myofibroblast, and KO of CTRP3 elevated the expressions of collagen I, fibronectin and α-SMA after TGF-β treatment (Figure 2B-2D). Consistently, we found that the protein expression of CTRP3 decreased with the α-SMA, collagen I and fibronectin increase in HSCs after TGF-β induction (Figure 2E and 2F). Mechanically, we detected Notch-1 and Jadded-1 protein using Western blotting, it was found that TGF-β induction markedly increased Notch-1 and Jadded-1 expressions, KO of CTRP3 enhanced Notch-1 and Jadded-1 increase after TGF-β treatment but OE of CTRP3 significantly reduced Notch-1 and Jadded-1 level in TGF-β-induced HSCs (Figure 2G). The results show that CTRP3 regulates HSC-to-myofibroblast *via* Notch/Jadded signaling pathway.

Increased CTRP3 Mitigates Fibrogenesis in CCl4-Induced Liver Fibrosis

To evaluate the effect of CTRP3 on liver fibrosis model, we used RC-CTRP3 in mice with CCl_4 injection. The CTRP3 protein in mice was measured using Western blot at 8 weeks post CCl_4 injection, showing that the expression of CTRP3 decreased in CCl_4 group and the high level of

CTRP3 was in CCl_4+RC-CTRP3 group (Figure 3A). Moreover, the IHC staining of CTRP3 in sections exhibited that CTRP3 positive area decreased in liver but RC-CTRP3 rescued the level of positive CTRP3 area (Figure 3B). The collagen I and α-SMA in RNA level were measured by RT-PCR, it was exhibited that increased collagen I and α-SMA was in CCl_4-induced liver, whereas increased CTRP3 reduced collagen I and α-SMA expression (Figure 3C and 3D). Consistently, IHC staining showed that collagen I and α-SMA positive area in CCl_4-induced liver were reduced after

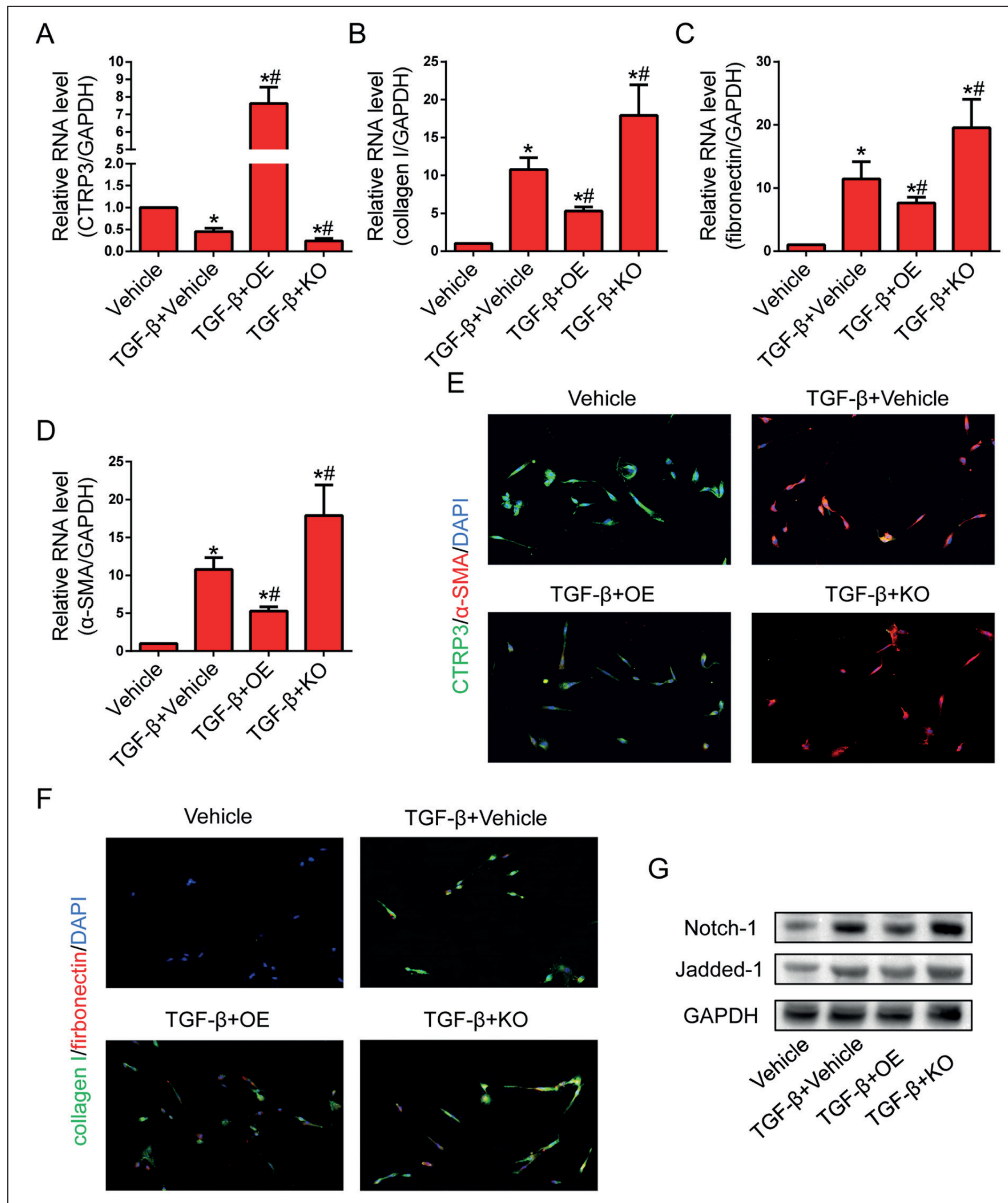

Figure 2. CTRP3 regulates HSCs differentiation to myofibroblast *via* Notch/Jadded pathway. The relative RNA expression of CTRP3 (**A**), collagen I (**B**), fibronectin (**C**), and α-SMA (**D**) in HSC in Vehicle, TGF-β+Vehicle, TGF-β+OE, and TGF-β+KO group. **E,** The IF photos of CTRP3 and α-SMA in HSC in Vehicle, TGF-β+Vehicle, TGF-β+OE, and TGF-β+KO group, (magnification: 200×). **F,** The IF photos of collagen I and fibronectin in HSC in Vehicle, TGF-β+Vehicle, TGF-β+OE, and TGF-β+KO group, (magnification: 200×). **G,** The protein bands of Notch-1 and Jadded-1 in Vehicle, TGF-β+Vehicle, TGF-β+OE, and TGF-β+KO group. "*" means *vs.* Vehicle group and "#" means *vs.* TGF-β+Vehicle group with statistical significance.

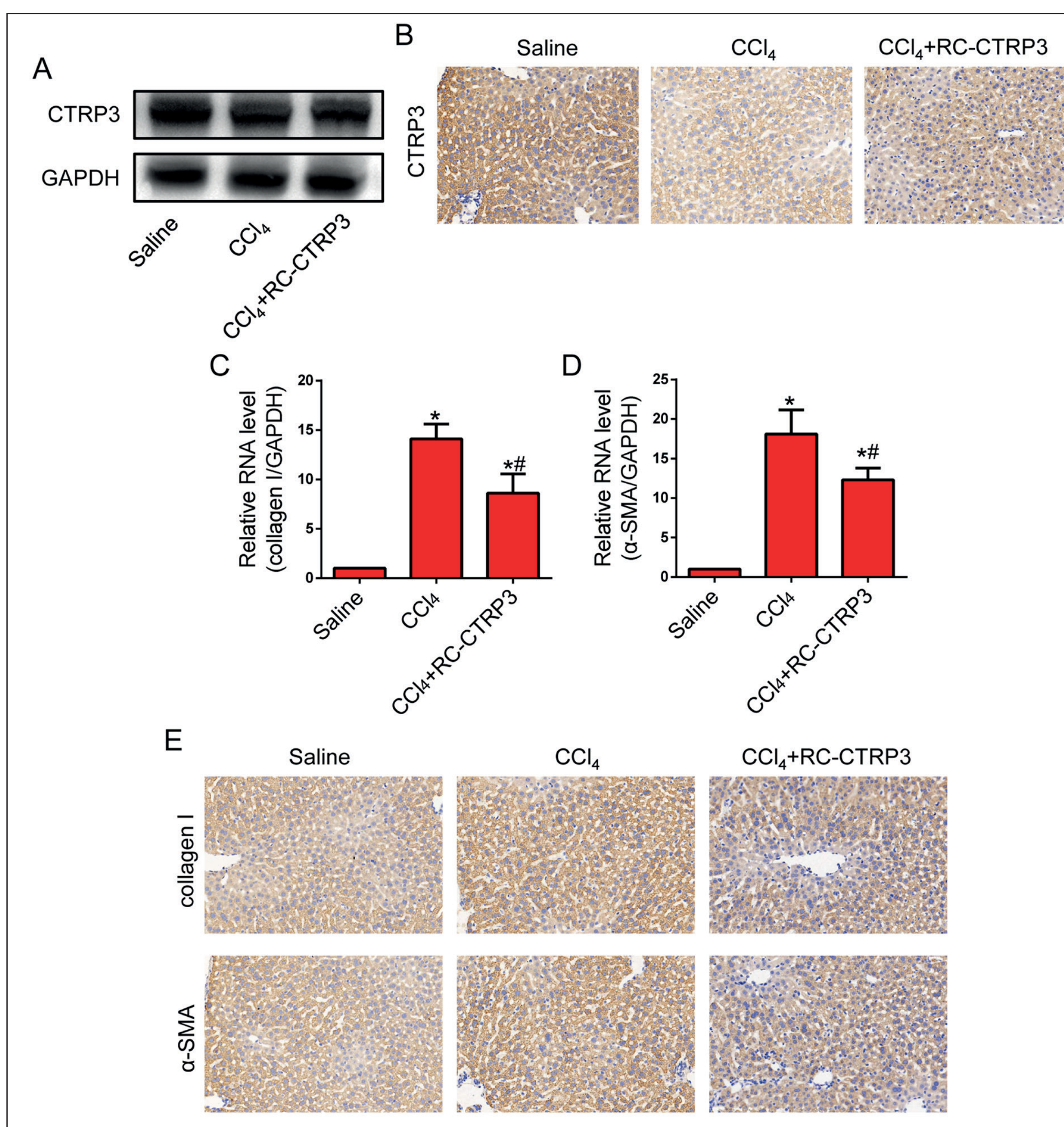

Figure 3. Increased CTRP3 mitigates fibrogenesis in CCl4-induced liver fibrosis. **A,** The protein bands of CTRP3 in Saline, CCl_4, and CCl_4+RC-CTRP3 group at 8 W post CCl_4 treatment. **B,** The IHC images of CTRP3 in liver in Saline, CCl_4, and CCl_4+RC-CTRP3 group at 8 W post CCl_4 treatment, (magnification: 200×). The relative RNA expression of collagen I (**C**) and α-SMA (**D**) in Saline, CCl_4, and CCl_4+RC-CTRP3 group at 8 W post CCl_4 treatment. **E,** The IHC images of collagen I and α-SMA in liver in Saline, CCl_4, and CCl_4+RC-CTRP3 group at 8 W post CCl_4 treatment, (magnification: 200×). "*" means *vs.* Saline group and "#" means *vs.* CCl_4 group with statistical significance.

RC-CTRP3 administration (Figure 3E). Taken together, increased CTRP3 attenuates fibrogenesis in liver fibrosis model.

Rescue of CTRP3 Improves Liver Histology and Function

Lastly, we evaluated the therapeutic action of increased CTRP3 on liver fibrosis mice at 8 weeks post treatment. The histologic assessment was recurred to HE and Masson staining. HE staining showed that severe tissue disorder was in CCl_4 group, however, CTRP3 rescue mitigated the histologic disorder of liver tissue (Figure 4A). Moreover, Masson staining exhibited more fibers in CCl_4-induced liver than in CTRP3 utilization (Figure 4B). Consistently, hydroxyproline content assay showed more hydroxyproline in CCl_4 compared with that in CCl4+RC-CTRP3

group (Figure 4C), indicating that reinforce of CTRP3 reduced deposition of collagenous fiber in liver. ALT, AST and TBA were detected for liver function assessment, showing that ALT, AST and TBA levels in serum at 8 weeks post injury were reduced after CTRP3 increase treatment (Figure

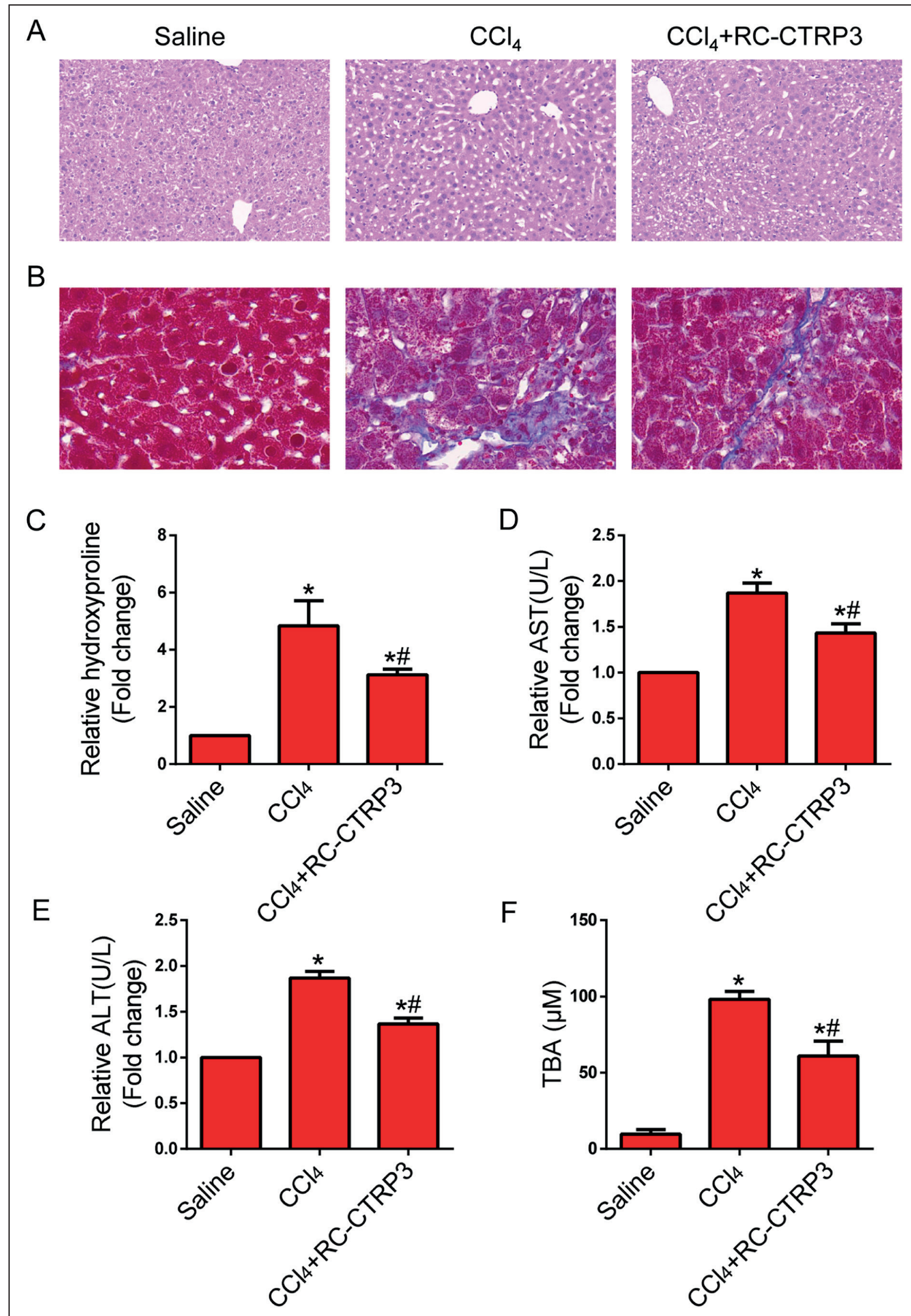

Figure 4. Rescue of CTRP3 improves liver histology and function. **A,** The HE images of liver in Saline, CCl_4, and CCl_4+RC-CTRP3 group at 8 W post CCl_4 treatment, (magnification: 200×). **B,** The Masson images of liver in Saline, CCl_4, and CCl_4+RC-CTRP3 group at 8 W post CCl_4 treatment, (magnification: 200×). The content of hydroxyproline (**C**), AST (**D**), ALT (**E**) and TBA (**F**) in Saline, CCl_4, and CCl_4+RC-CTRP3 group at 8 W post CCl_4 treatment. "*" means *vs.* Saline group and "#" means *vs.* CCl_4 group with statistical significance.

4D-4F). Hence, the results suggest that increased CTRP3 ameliorates liver histology and function in liver fibrosis.

Discussion

Several studies have shown that CTRP3 plays an antagonistic role in the fibrosis of various organs and tissues. CTRP3 can reduce the transformation of fibroblasts to myofibroblasts in the area of myocardial injury after myocardial infarction[20]. In addition, CTRP3 has been reported to reduce the differentiation, migration, and proliferation of vascular adventitia fibroblasts and inhibit the deposition of ECM and the expression of CTGF[21]. In Crohn's disease, Hofmann et al[21] found that CTRP3 plays an inhibitory role in fibroblasts in the lamina propria of the colon. Herein, CTRP3, which is highly expressed in HSCs, was significantly reduced in the fiber repair of liver injury induced by CCl_4 but the overexpression of CTRP3 plays an antagonistic role in the process of liver fibrosis. Increased TGF-β-induced liver fibrosis characterized by extensive extracellular matrix synthesis and secretion and inhibition of extracellular matrix degradation, promotes massive myofibroblast activation and proliferation. TGF-β-treated HSCs significantly lead to fibrosis progression. OE or KO of CTRP3 was treated with HSCs after TGF-β treatment, showing that CTRP3 increase reduces α-SMA and collagen-I expression. Besides, KO of CTRP3 treatment promotes TGF-β-induced HSC differentiation and increased ECM. The expression of Notch/Jadded signaling pathway has been found to increase in liver fibrosis[22]. Moreover, the activation of Notch signaling pathway in HSCs results in the progression of interstitial fibrosis, and the inhibition of Jadded-1 reduces liver fibrosis. A report[23] showed that Kruppel-like factor 4 can also mediate fibrotic transition of HSCs through Notch signaling pathway. These findings suggest that Notch signaling pathway plays an important role in liver fibrosis. In the present study, we found that the expressions of Notch-1 and Jadded-1 are upregulated in TGF-β-induced HSCs and in CCl_4 mediated hepatic fibrosis models. However, CTRP3 inhibits TGF-β-mediated fibrotic activation *via* reducing the protein expressions of Notch-1 and Jagged-1. Additionally, KO of CTRP3 increases the Notch/Jadded signaling pathway and promotes the synthesis of fibrogenic factors in HSCs after TGF-β treatment. These results imply that CTRP3 plays an inhibitory role in TGF-β-induced liver fibrosis by blocking Notch/Jadded signaling pathway. CTRP3 has been reported to alleviate myofibroblast differentiation in human HSCs with TGF-β utilization *via* inhibiting the activation of the Smad family[24]. We firstly reported that CTRP3 inhibited liver fibrosis by regulating Notch/Jadded signaling pathway and Smad signaling pathway. The current study complemented the previous findings in the fibrogenesis process of HSCs following TGF-β inducement. Importantly, previous studies have shown that inflammation and apoptosis are closely related to the progression of organ fibrosis, while CTRP3 has been shown to play an important role in the regulation of inflammation and apoptosis. More notably, the Notch signaling pathway also involves in inflammation and apoptosis. Therefore, CTRP3 may plays an anti-inflammatory and anti-apoptotic role by inhibiting Notch signaling pathway to result in fibrosis inhibition, which needs to be explored in further studies.

Conclusions

Taken together, CTRP3 mitigates the development of liver fibrosis, improves histological and functional protection of liver, and inhibits TGF-β-mediated fibrosis in HSCs by inhibiting Notch/Jadded signaling pathway. The results confirm that CTRP3 is an effective anti-hepatic fibrosis target of drug therapy in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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