

Original Article

Zinc mediated hepatic stellate cell collagen synthesis reduction through TGF- β signaling pathway inhibition

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Abstract: This study is to investigate the effect and underlying mechanism of Zinc (Zn) on hepatic stellate cell collagen synthesis. The proliferation and collagen synthesis ability of LX-2 cells were detected after adding Zn. The collagen synthesis related proteins of MMP-13 and TIMP1 along with TGF- β signaling pathway related proteins were detected by Western blot. The role of TGF- β signaling pathway in collagen synthesis inhibition was identified by TGF- β RI siRNA silencing. Compared with control group, LX-2 cell proliferation ability was significantly inhibited at all Zn concentrations (50 μ M, 100 μ M and 200 μ M). Zn at 50 μ M did not affect the protein content of α SMA and type I collagen while 100 μ M and 200 μ M Zn could significantly inhibit α SMA expression. Compared with control group, gene expression and protein content of MMP-13 in 200 μ M Zn group was significantly increased while no difference in gene expression and protein content of TIMP1 was found. TGF- β RI content in 200 μ M Zn group was significantly decreased and the protein content of TGF- β RII was not affected. MMP-13 expression was significantly increased after TGF- β RI siRNA silencing. Further results showed that in LX-2 cells those TGF- β RI expression was inhibited, LX-2 cell proliferation ability and the expression of synthesis collagen related proteins of α SMA and type I collagen were greatly decreased. Zn could significantly inhibit the expression of α SMA and type I collagen by inhibiting TGF- β RI expression and promoting MMP-13 expression.

Keywords: Collagen synthesis, Zn, hepatic fibrosis, hepatic stellate cells

Introduction

Hepatic fibrosis is a common result of a variety of chronic liver injuries. During the pathological process, liver cell necrosis occurs after stimulated by inflammation factors, excessive extracellular matrix proteins deposition appears and abnormal hyperplasia occurs in fibrous connective tissue in the liver, eventually leading to the formation of liver cirrhosis [1, 2].

Hepatic stellate cells (HSCs) are the main source of excessive collagen during liver fibrosis [3, 4] and once they are activated, collagen expression ability of HSCs is significantly enhanced [5, 6]. Type I collagen is the main fibrin in extracellular matrix, its synthesis increases and its degradation reduces in the process of liver fibrosis [7-9]. The degradation of extracellular matrix depends on the activity of matrix

metalloproteinase (MMPs) [10]. According to the substrate specificity, matrix MMPs can be divided into five categories, namely interstitial collagenase (MMP-1, -8, -13), gelatinase (MMP-2, -9), matrix degrading enzyme (MMP-3, -7, -10, -11), membrane type matrix metalloproteinase (MMP-14, -15, -16, -17, -24, -25) and metal elastase (MMP-12) [10]. These enzymes are regulated by tissue inhibitor of matrix metalloproteinases (TIMPs) [11-13]. In the progress of liver fibrosis, the imbalance between the activities of MMPs and TIMPs is a key reason that leads to collagen deposition [13, 14].

Transforming growth factor β (TGF- β) is a profibrotic cytokine [15], which, can stimulate cell synthesis and deposition of extracellular matrix components such as type I, III, IV collagen, elastin, tenascin, osteonectin, biglycan proteoglycan and core protein polysaccharide [16]. HSCs

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are the main origin of TGF- β , meanwhile, macrophages, liver cells and platelets also can secrete TGF- β [17]. TGF- β mediated intracellular signal delivery is transmitted by Smad protein [18]. After binding to activated type II receptor, TGF- β phosphorylates type I receptor. The signal is transmitted to Smad2 and Smad3 proteins from type I receptor and phosphorylation takes place in Smad2 and Smad3 proteins [19]. Next, Smad2, Smad3 and Smad4 proteins form an oligomeric complex and transfer to nucleus, then, recognize and regulate transcription on specific sites of target DNA. At the same time, along with the activation of TGF- β signaling pathway, HSCs proliferation ability can be significantly increased [20]. Smad7 protein is an inhibitor of Smad signaling pathway. It can interfere the interaction of type I TGF- β receptor and Smad2 and Smad3 through interaction with type I TGF- β receptor thus effectively inhibits Smad2 and Smad3 phosphorylation and a series of subsequent downstream signals [21].

Zinc (Zn) is an essential trace element for human body with a variety of biological functions such as antioxidant, anti-inflammatory and anti apoptosis [22]. Zn deficiency is generally found in cirrhotic patients and patients with chronic liver disease [23, 24]. Zn is the inhibitor of collagen synthase (proline hydroxylase) [25] because it can compete with the iron ion binding site of enzymatic activity center, thus inhibit the enzyme activity, and as a result, inhibit collagen synthesis. In addition, Zn is a co activator of MMPs [26] and is an essential metal ion for MMPs activity maintenance. Zn deficiency can induce decreased MMPs activity, inhibition of collagen degradation and aggregation of collagen in liver tissue, hence, leading to the formation of fibrotic lesions.

In this study, human hepatic stellate cell line LX-2 cells were used as the research model to investigate the molecular mechanism collagen synthesis inhibition mediated by Zn.

Materials and methods

Cell treatment

The cultured LX-2 cells were divided into 4 groups, namely control group, 50 μ M group, 100 μ M group and 200 μ M group. The control group was LX-2 cells cultured with normal medium while corresponding final concentrations of

Zn was added to the 50 μ M, 100 μ M and 200 μ M groups. The cells were cultured with H-DMED medium in a cell culture incubator with 5% CO₂ and saturated humidity at 37°C.

Atomic absorption spectrometry

After cultured for 24 hours, the LX-2 cells were digestion with 1%TNE and collected by centrifugation. The cells was resuspended with vortex oscillation after adding 100 μ l 1% SDS and was lysed with ultrasonic. Cellular debris was removed by 12000 g centrifugation at 4°C for 10 min and clear lysate was reserved. Protein concentration was detected by commercial DAC protein assay Kit (Bio-rad, Hercules, USA). The LX-2 cell protein extraction was digested by adding 5 times volume of concentrated nitric acid. Sample contained Zn contents were detected by using the atomic absorption spectrometry method and the results were standardized with total protein content of LX-2 cells.

Methyl thiazol tetrazolium (MTT) assay

LX-2 cells proliferation was detected after culturing for 1 day, 2 days, 3 days, 4 days, 5 days, 6 days and 7 days. And, 5 wells in each group were measured. Each well was added with 200 μ L MCDB131 after the medium was absorbed and another 20 μ L MTT was added subsequently. After incubated for 3.5 hours in an incubator, the culture medium was absorbed and 100 μ L DMSO was added into each well. Another incubation of 5-10 minutes was carried out and the solution was transferred to another clean 96 wells petri dish. The OD value was measured at wavelength of 490 nm with continuous spectrum microplate reading instrument (Bio-rad, Hercules, USA).

Western blotting

After subject to sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes and blocked with 50 g/L skimmed milk at room temperature for 1 h. The membranes were incubated respectively with rabbit primary antibodies of TGF- β RI, TGF- β RII, MMP13, TIMP-1, α SMA, collagen I (1:5000) and GAPDH(1:2000)(Santa Cruz Biotechnology, Dallas, USA) at 4°C overnight. After washed with phosphate-buffered saline with Tween 20 (PBS-T) for 3 times of 15 min, the membranes

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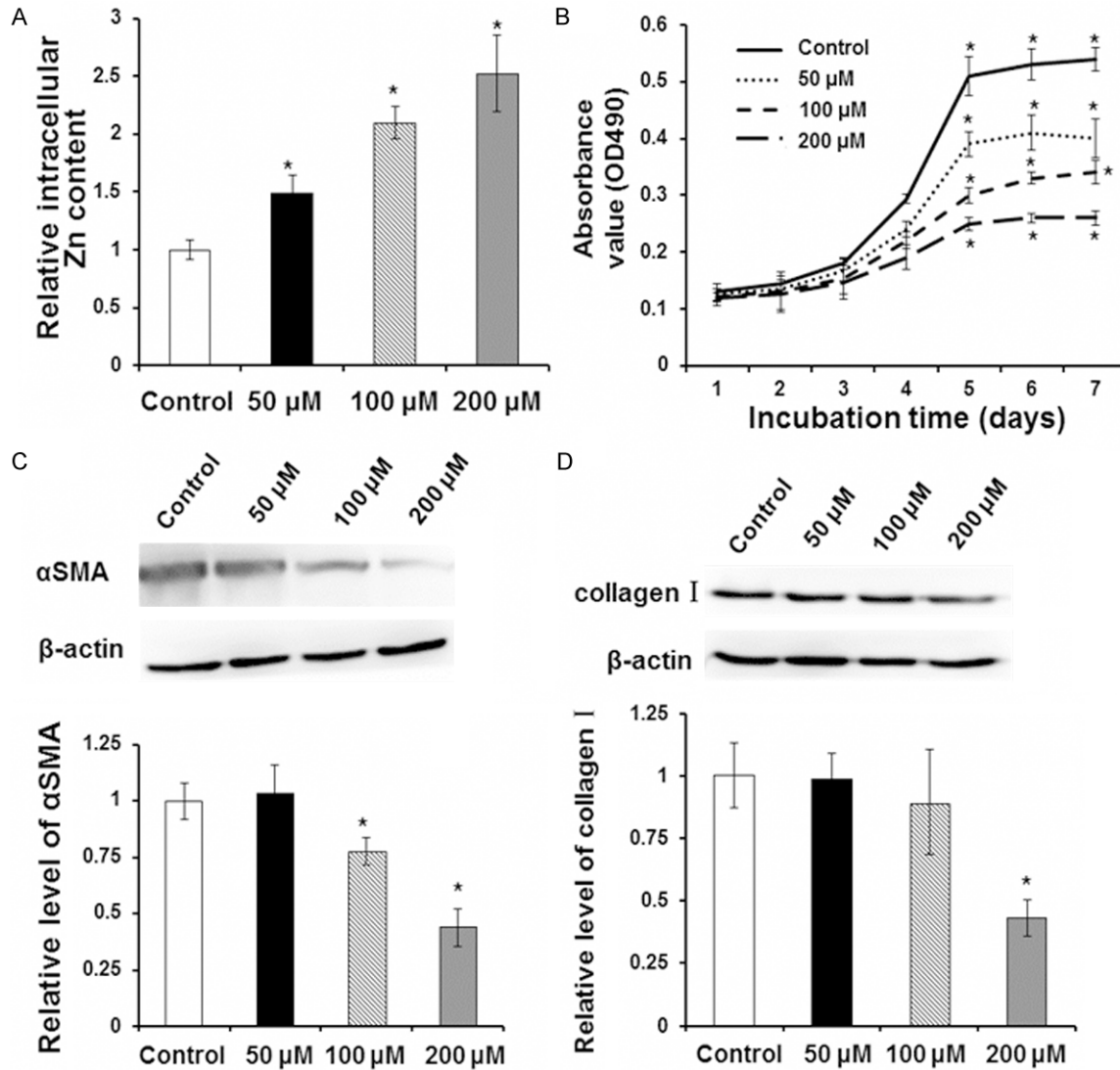


Figure 1. Effect of Zn on LX-2 cell proliferation and collagen synthesis. LX-2 cells were incubated with 0 μ M (control group), 50 μ M, 100 μ M and 200 μ M Zn for indicated times. A. After incubation for 24 h, intracellular Zn content was detected with atomic absorption spectrometry. B. After incubation for 1 day, 2 days, 3 days, 4 days, 5 days, 6 days and 7 days, cell proliferation was measured by MTT assay. C. After incubation for 48 h, level of α SMA was detected with Western blot analysis. Representative Western blot results were shown in the upper panel and quantitative Western blot results were shown in the lower panel. D. After incubation for 48 h, level of collagen I was detected with Western blot analysis. Representative Western blot results were shown in the upper panel and quantitative Western blot results were shown in the lower panel. Compared with control group, * $P < 0.05$.

were incubated with goat anti-rabbit-horseradish peroxidase conjugate antibody (1:1000; Santa Cruz Biotechnology, Dallas, USA) for 1 h at room temperature before washing with PBS-T for 3 times of 15 min. Then, the membranes were developed with enhanced chemiluminescence detection kit (Sigma-Aldrich, St. Louis, USA) for imaging. Image lab (Bio-Rad, Hercules, USA) software was used to acquire and analyze imaging signals. The relative content of protein was expressed as protein/GAPDH ratio.

siRNA silencing

All three targeting siRNAs of TGF- β RI were purchased from RiboBio (RiboBio Co., Ltd., Guangzhou, China). The siRNA sequences used were as follows: 5'-GAGGGCAUGUAGACGGUUA dTdT-3'; 3'-dTdT CUCCCGUACAUCUGCCAAU-5', (2) 5'-ACAAU AUGCUACCUCAAA dTdT-3'; 3'-dTdT UGUUUAUCGAUGGAGGUUU-5' and (3) 5'-GACUGGAUCCAUACAAUUAU dTdT-3'; 3'-dTdT CU-GACCUAGGUAUGUUUAU-5'. TGF- β RI was silen-

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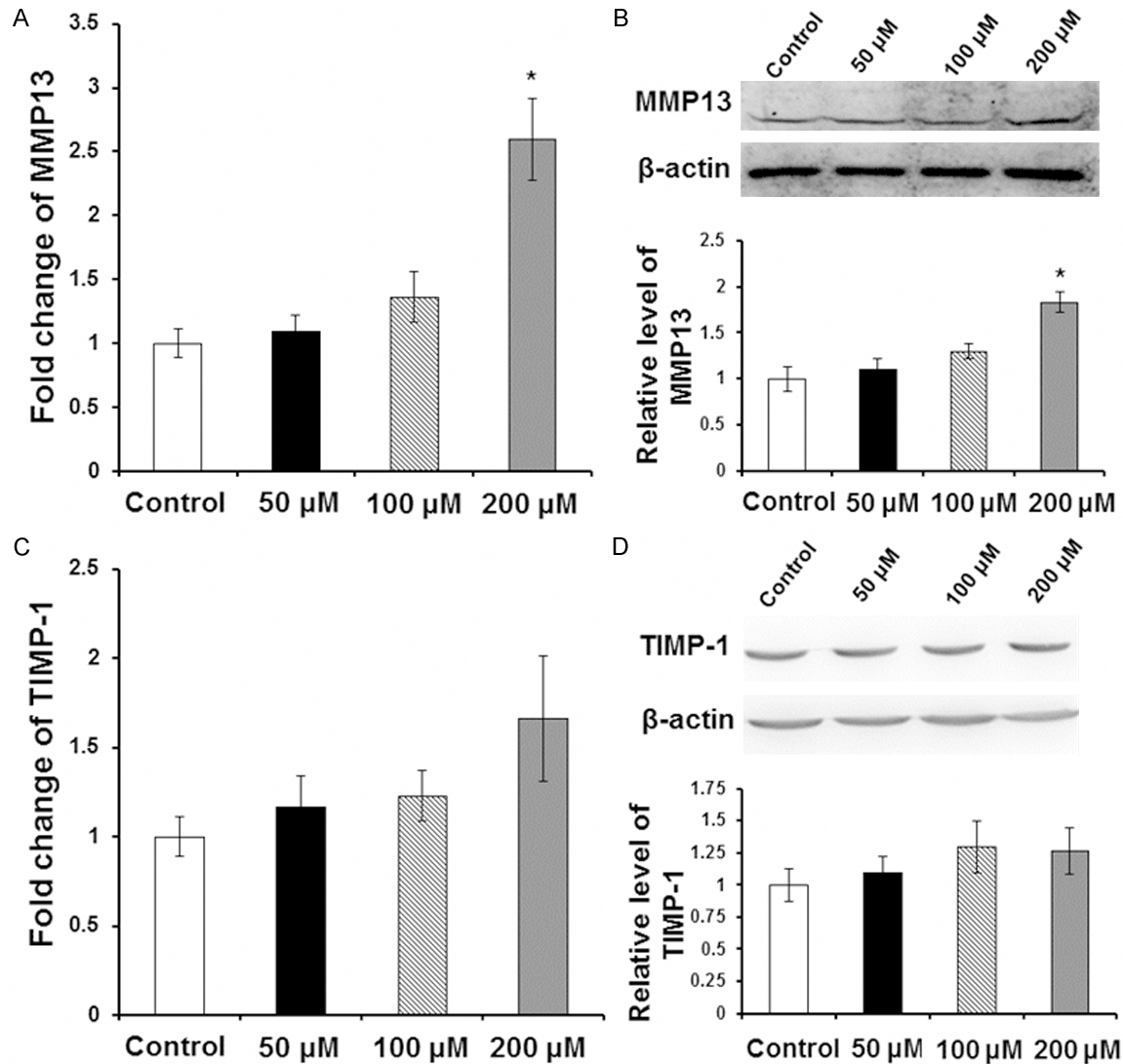


Figure 2. Effect of Zn on expression level of MMP13 and TIMP-1. LX-2 cells were incubated with 0 μ M (control group), 50 μ M, 100 μ M and 200 μ M Zn for 24 h. Expression levels of MMP13 and TIMP-1 were detected with qRT-PCR and Western blot analysis. A. Relative expression level of MMP13 by qRT-PCR. B. Representative and quantitative Western blot results of MMP13. C. Relative expression level of TIMP-1 by qRT-PCR. D. Representative and quantitative Western blot results of TIMP-1. Compared with control group, * $P < 0.05$.

ced with 30 nM siRNAs and mismatched siRNA was used as control. Lipofectamine 2000 (Invitrogen, New York, USA) was used as transfection reagent. After cultured for 48 hours, the transfected cells were collected using trypsin and analyzed by RT-PCR.

RT-PCR analysis

Total RNA was isolated by Trizol method and was quantified by XXX spectrophotometer. Samples those with OD260/OD280 ratio between 1.8-2.00 were taken as good quality RNA. The quality of RNA was further analyzed

by Experion Bioanalyzer (BioRad, Hercules, USA) and first-strand cDNA was prepared by using PrimeScript[®] RT reagent Kit (Takara, Japan) following manufacturer's instructions. The samples were electrophoresed on 1.5% agarose gel and the bands were analyzed by using band intensity software. Relative expression amounts of miRNA were calculated by $2^{-\Delta\Delta T} \pm$ SEM.

Statistical analysis

All the statistical analyses were performed using SPSS version 13.0 (SPSS Inc, Chicago, IL,

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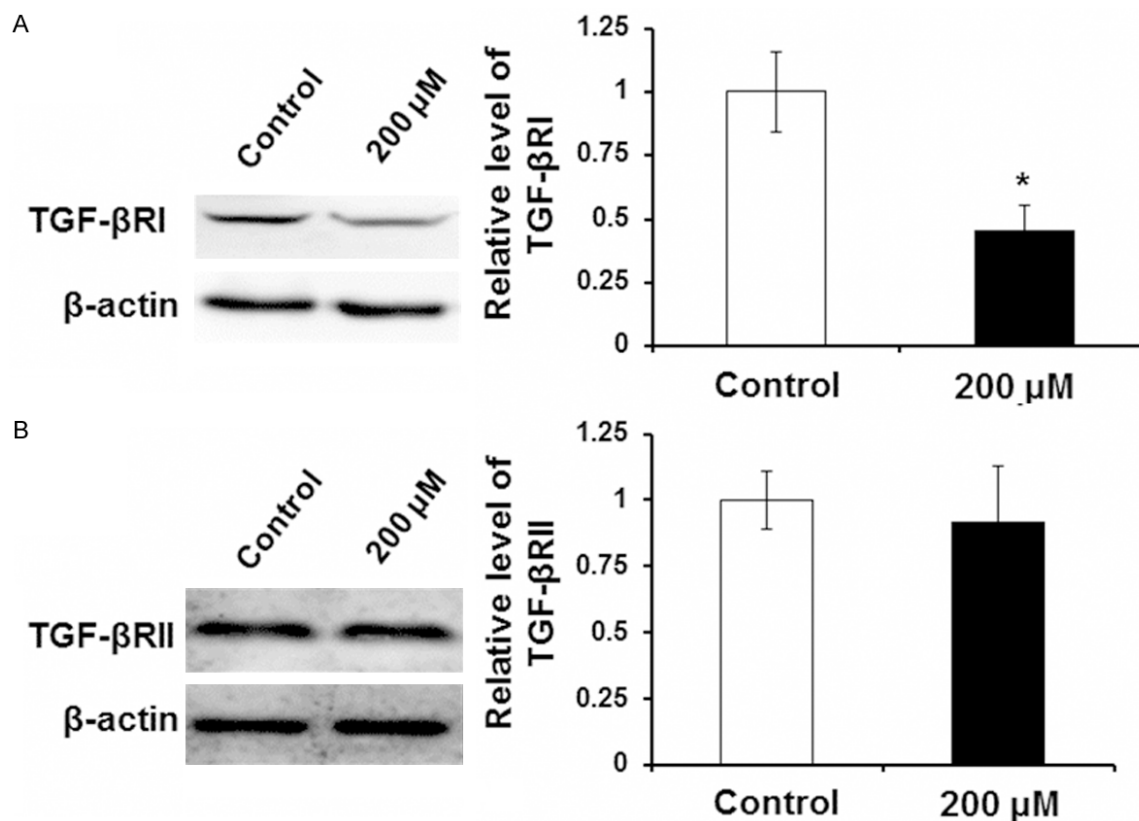


Figure 3. Effect of Zn on expression level of TGF- β RI and TGF- β RII. LX-2 cells were incubated with 0 μ M (control group) and 200 μ M Zn for 24 h. Expression levels of TGF- β RI and TGF- β RII were detected with Western blot analysis. A. Representative and quantitative Western blot results of TGF- β RI. B. Representative and quantitative Western blot results of TGF- β RII. Compared with control group, * $P < 0.05$.

USA) for Windows. The test was used to compare between different groups and P value less than 0.05 was considered as statistically significant.

Results

Zn inhibits LX-2 cell proliferation and collagen synthesis

To detect the inhibitory effect of Zn in cell proliferation and collagen synthesis, corresponding analysis were carried out using LX-2 cell model after cultured with Zn for 24 hours. Zn concentration was detected by atomic absorption spectrometry. LX-2 cell proliferation ability was measured using the MTT method. The expressions of α SMA and type I collagen were analyzed by Western blot. As shown in (Figure 1A), as extracellular Zn concentration increased, intracellular Zn concentration significantly increased. Compared with the control group, LX-2 cell proliferation ability was significantly inhibited at all Zn concentrations of 50 μ M,

100 μ M and 200 μ M (Figure 1B). Western blot results showed that Zn with a final concentration of 50 μ M did not affect the protein content of α SMA and type I collagen in LX-2 cells while 100 μ M and 200 μ M Zn could significantly inhibit α SMA expression ($P < 0.05$). Compared with the control group, 100 μ M Zn could inhibit type I collagen expression but the difference was not significant while 200 μ M Zn had a significant effect on the expression type I collagen ($P < 0.05$) (Figure 1C, 1D). As inhibition of 200 μ M Zn was apparent, the concentration of 200 μ M Zn was adopted in the subsequent experiments. Taken together, the results showed that high concentration of Zn significantly inhibited LX-2 cell proliferation ability and collagen synthesis ability and the effect of a final concentration of 200 μ M Zn was most obvious.

Zn inhibits LX-2 cell collagen synthesis by increasing MMP-13 expression

To find out how Zn inhibits LX-2 cell collagen synthesis, qRT-PCR and Western blot were ad-

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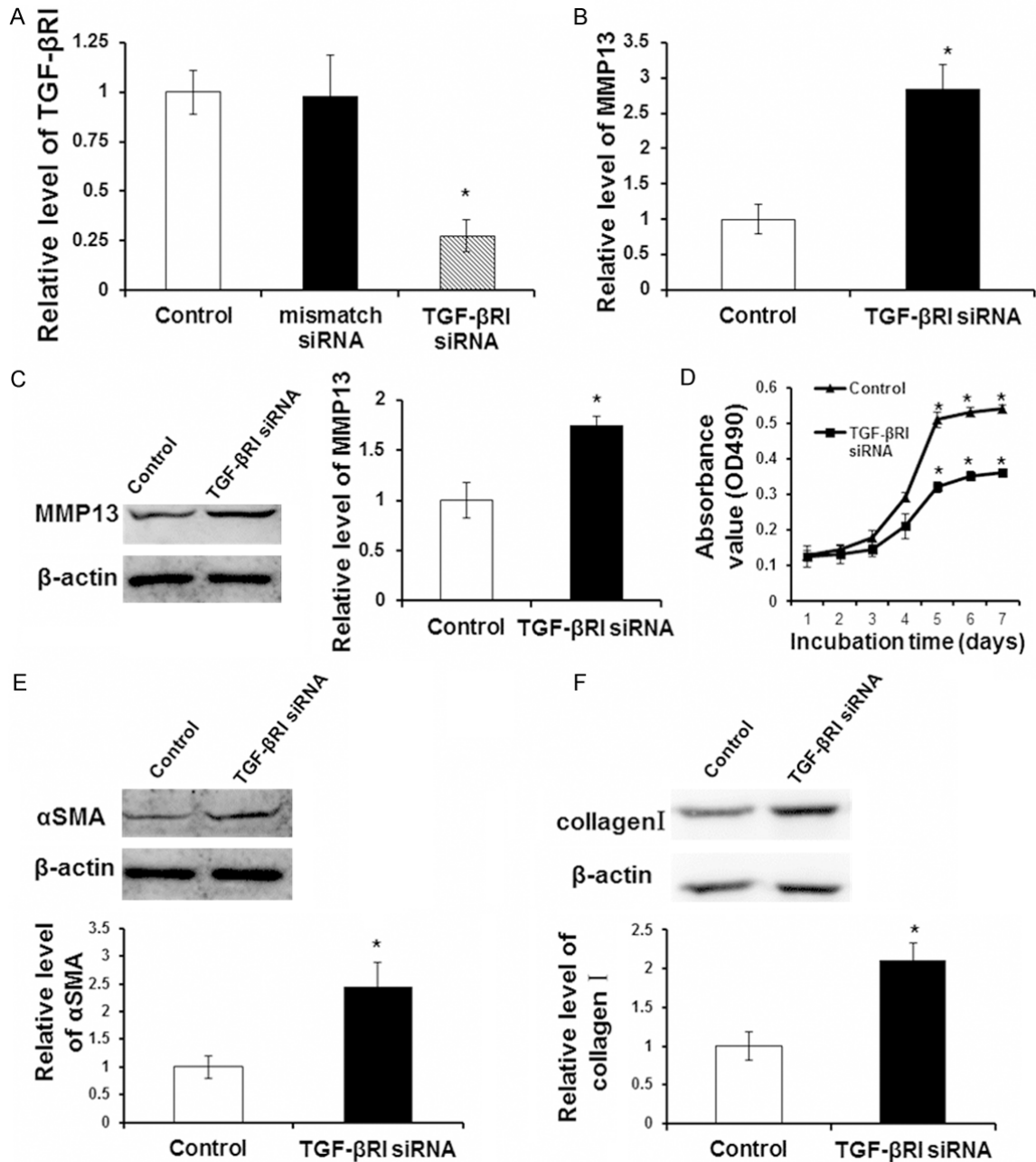


Figure 4. Inhibition of TGF- β RI by siRNA silencing. LX-2 cells were transfected with mismatch siRNA, TGF- β RI siRNA or un-transfected (control group) and then were cultured for 48 h. A. Transfection efficacy was detected by qRT-PCR. Relative level of TGF- β RI was shown. B. MMP13 expression at mRNA level after TGF- β RI siRNA was detected by qRT-PCR. C. MMP13 expression at protein level after TGF- β RI siRNA was detected by Western blot analysis. Representative and quantitative Western blot results were shown. D. Cell proliferation was detected by MTT assay. E. The α SMA expression at protein level after TGF- β RI siRNA was detected by Western blot analysis. Representative and quantitative Western blot results were shown. F. The collagen I expression at protein level after TGF- β RI siRNA was detected by Western blot analysis. Representative and quantitative Western blot results were shown. Compared with control group, * $P < 0.05$.

opted to detect levels of MMP-13 and TIMP-1 respectively. LX-2 cells were cultured for 24 h with 0 μ M (control group), 50 μ M, 100 μ M and 200 μ M of Zn. Compared with the control

group, MMP-13 expression at mRNA level (Figure 2A) and protein level (Figure 2B) at the concentration of 200 μ M was significantly increased ($P < 0.05$). However, there was no

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significant difference in TIMP-1 expression level (**Figure 2C, 2D**). To sum up, the results demonstrate that Zn inhibits collagen synthesis ability of LX-2 cells by increasing the expression of collagen degradation associated matrix metalloproteinase MMP-13.

Zn inhibits expression of TGF- β RI

To identify whether Zn can inhibit TGF- β RI and TGF- β RII expression, Western blot was performed. LX-2 cells were incubated with 0 μ M (control group) and 200 μ M Zn for 24 h. As shown in (**Figure 3A**), TGF- β RI protein level in 200 μ M Zn group was significantly decreased while compared with the control group ($P < 0.05$). However, Zn with a final concentration of 200 μ M did not affect the protein level of TGF- β RII (**Figure 3B**). In conclusion, the result argues that Zn could inhibit collagen synthesis ability of LX-2 cells by decreasing the content of TGF- β RI.

TGF- β RI targeted siRNA silencing results

To further identify whether the inhibitory effect of Zn on LX-2 cell proliferation and collagen synthesis was achieved by TGF- β RI inhibition, TGF- β RI targeted siRNA silencing, MTT assay, qRT-PCR and Western blot were carried out. LX-2 cells were divided into two groups. One was the control group, and the other was TGF- β RI siRNA group, of which LX-2 cells were transfected with siRNA targeting silence TGF- β RI. After culturing for 48 hours, proliferation of LX-2 cells was assayed using MTT assay. MMP-13 expression was detected by qRT-PCR and Western blot. Collagen synthesis related proteins of α SMA and type I collagen were analyzed by Western blot. As shown in (**Figure 4A**), compared with the control group, mismatch siRNA did not affect the expression of TGF- β RI in LX-2 cells while targeting silence siRNA significantly inhibited TGF- β RI expression ($P < 0.05$). Compared with the control group, once TGF- β RI expression was inhibited, MMP-13 expression was significantly increased ($P < 0.05$) (**Figure 4B, 4C**). Further results showed that in LX-2 cells with inhibited TGF- β RI expression, LX-2 cell proliferation ability (**Figure 4D**) and the expression of synthesis collagen related proteins α SMA (**Figure 4E**) and type I collagen (**Figure 4F**) were significantly decreased ($P < 0.05$). In summary, these results showed that TGF- β RI inhibition had the same effect with

200 μ M Zn treatment and further proved that TGF- β RI inhibition played a key role in collagen synthesis ability decrease mediated by Zn in LX-2 cells.

Discussion

Hepatic fibrosis is the key factor that leading to cirrhosis and liver failure in the final. Zn is an essential trace element for human body, which plays roles in many aspects such as regulating antioxidant, anti-inflammatory and anti apoptosis [22]. Besides, Zn has an important role on the regulation of liver fibrosis [23]. In this study, it was found that with the concentration of added Zn increased, the activity of LX-2 cells was inhibited. The proliferation ability decreased significantly, meanwhile, α SMA and type I collagen transcription and protein content decreased significantly, which, demonstrated that collagen synthesis was inhibited. This inhibitory effect was most obvious when the added Zn was up to a final concentration of 200 μ M.

MMP-13 plays an important role in the degradation of collagen [10]. This study showed that, MMP-13 transcription and protein content were increased by Zn added and this significantly inhibited the expression of α SMA and type I collagen. MMP-13 is a matrix metalloproteinase involved in the regulation of hepatic fibrosis. This study failed to detect the influence of Zn on the expression of other MMPs, however, related experiments will be carried out in follow-up works. TIMP1 plays an inhibitory effect on matrix metalloproteinases, imbalance between them is an important reason that leads to liver fibrosis, in this study, it was shown that Zn did not affect the protein content of TIMP1. These results demonstrated that MMP-13 played a key role in the process of collagen synthesis inhibition by Zn in LX-2 cells.

TGF- β is the most powerful fibrosis promoting cytokine in HSCs [15] and it is demonstrated that the TGF- β signaling pathway can regulate the expression of many proteins. In the present study, it was found that 200 μ M Zn could significantly decrease the protein content TGF- β RI, but did not affect protein content of TGF- β RII. Further study found that, TGF- β expression was similarly promoted while TGF- β RI was silenced using siRNA. Meanwhile, proliferative capacity of LX-2 cells and the expression of α SMA and type I collagen were significantly restrained. Zn

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might functions through inhibiting TGF- β RI gene expression or increasing TGF- β RI protein degradation, unfortunately, the specific molecular mechanism had not been elucidated. Related problems remain to be explored in the future.

In summary, in this study, human hepatic stellate cell line LX-2 was used as study model, Zn with a concentrate of 200 μ M could significantly inhibit the activity of LX-2 cells for cell proliferation ability was significantly decreased. TGF- β signaling pathway played a key role in this process, by inhibiting TGF- β RI protein content and promoting the expression of MMP-13, Zn significantly inhibited expression of α SMA and type I collagen in LX-2 cells. Meanwhile, TGF- β RI inhibition by siRNA silencing had the same effect of α SMA and type I collagen expression inhibition.

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Disclosure of conflict of interest

None.

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