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BRIEF ARTICLE

Specific shRNA targeting of *FAK* influenced collagen metabolism in rat hepatic stellate cells

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Abstract

AIM: To investigate the effects and mechanism of disruption of focal adhesion kinase (*FAK*) expression on collagen metabolism in rat hepatic stellate cells (HSC).

METHODS: The plasmids expressing *FAK* short hairpin RNA (shRNA) were transfected into HSC-T6 cells, and the level of *FAK* expression was determined by both real-time quantitative polymerase chain reaction (Q-PCR) and Western blotting analysis. The production of type Ⅰ collagen and type Ⅲ collagen in *FAK*-disrupted cells was analyzed by real-time Q-PCR. The level of collagen metabolism proteins, including matrix metalloproteinases-13 (MMP-13) and tissue inhibitors of metalloproteinases-1 (TIMP-1) was also determined by both real-time Q-PCR and Western blotting analysis.

RESULTS: The transfection of *FAK* shRNA plasmids into HSC resulted in disrupted *FAK* expression. Compared with the HK group, the levels of type Ⅰ collagen and type Ⅲ collagen mRNA transcripts in *FAK* shRNA plasmid group were significantly decreased (0.69 ± 0.03 *vs* 1.96 ± 0.15, $P = 0.000$; 0.59 ± 0.07 *vs* 1.62 ± 0.12, $P = 0.020$). The production of TIMP-1 in this cell type was also significantly reduced at both mRNA and protein levels (0.49 ± 0.02 *vs* 1.72 ± 0.10, $P = 0.005$; 0.76 ± 0.08 *vs* 2.31 ± 0.24, $P = 0.000$). However, the expression of MMP-13 mRNA could be significantly up-regulated by the transfection of *FAK* shRNA plasmids into HSC (1.74 ± 0.20 *vs* 1.09 ± 0.09, $P = 0.000$).

CONCLUSION: These data support the hypothesis that shRNA-mediated disruption of *FAK* expression could attenuate extracellular matrix (ECM) synthesis and promote ECM degradation, making *FAK* a potential target for novel anti-fibrosis therapies.

Key words: Focal adhesion kinase; Hepatic stellate cells; Matrix metalloproteinases; RNA interference; Type Ⅰ collagen; Type Ⅲ collagen; Tissue inhibitors of metalloproteinases

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INTRODUCTION

Liver fibrosis results from excessive deposition of extracellular matrix (ECM) components[1]. These components, which are mainly composed of type I collagen and type Ⅲ collagen, are produced by hepatic stellate cells (HSC). The activation, proliferation and migration of HSC play a central role in liver fibrogenesis[2,3]. Activated HSCs are the main producers of collagens and matrix metalloproteinases (MMPs) in the fibrotic liver. The MMP which is able to degrade type I collagen and type Ⅲ collagen is MMP-13. However, this can be specifically inhibited by tissue inhibitors of metalloproteinases-1 (TIMP-1), and its level is found to be high in the fibrotic liver of rats.

Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase, whose phosphorylation can promote the proliferation and collagen synthesis of HSC[4-8]. It had previously been shown that when FAK related non-kinase (FRNK) plasmids were transfected into fibronectin (FN)-stimulated HSC using liposome transfection, the over-expression of FRNK significantly decreased the collagen synthesis of HSC *in vitro*[9,10]. This led us to speculate that suppression of FAK expression may provide a new target in the treatment of liver fibrosis.

To date, RNA interference has been the most effective gene silencing technology. It can specifically inhibit the transcription of target genes, and in turn reduce the expression and function of the corresponding proteins[11]. We aim to inhibit FAK expression by transfecting FAK short hairpin RNA (shRNA) plasmids into HSC. To our knowledge, this is the first report that FAK expression is specifically inhibited in HSC cells. This allows us to further analyze the role of FAK in collagen synthesis and degradation in this cell type, and find out how FAK regulates the expression of MMP-13 and TIMP-1.

MATERIALS AND METHODS

Reagents

The shRNA-expressing plasmids, pEGFP-*FAK* shRNA, were purchased from Wuhan Genesil Biotechnology Co. Ltd. (Wuhan, China). One additional plasmid, p-EGFP-HK, was used to express nonsense shRNA and served as the control. Sofast™ Transfection Reagent was purchased from Xiamen Sunma Biological Engineering Co. Ltd. (Xiamen, China).

Cell line and cell culture

The cell line HSC-T6, which is the phenotypically activated HSC, was donated by Professor Xu LM, from Hepatopathy Institute of Shanghai University of Traditional Chinese Medicine. HSCs were cultured in HG-DMEM medium supplemented with 8% FBS, 100 IU/mL penicillin, 100 g/mL streptomycin, 4 mmol/L glutamine and 1 mol/L HEPES. Cells were cultured in a 5% CO_2 humidified incubator at 37℃. All experiments were conducted when cells were at an exponential stage of growth. Cells were seeded into a 25 cm^2 plastic culture flask with a total of 2-3 × 10^5 cells or were seeded in 96-well plates to a density of 3 × 10^4/mL × 200 μL/well. When cells were approximately 70%-80% confluent, shRNA plasmid was transfected into FN-stimulated HSC using a cationic polymer. The cells were divided into five groups: (1) blank control group (control); (2) FN stimulation group (FN); (3) transfection reagent group (Sofast); (4) pEGFP-HK shRNA group (HK); and (5) pEGFP-*FAK* shRNA group (*FAK* shRNA). FN was added to groups 2-5 at a concentration of 10 mg/L.

Efficiency of transfection

At 48 h after transfection, the cells were analyzed by fluorescence microscopy and flow cytometry (FCM) to obtain the efficiency of transfection.

Semiquantitative real-time quantitative polymerase chain reaction

The expressions of the gene *FAK*, *type I collagen* and *type Ⅲ collagen*, *MMP-13* and *TIMP-1* were characterized by semiquantitative real-time quantitative polymerase chain reaction (Q-PCR). Briefly, total RNA was extracted from the cells that had been transfected with the plasmid expressing the *FAK* or HK shRNA and reversely transcripted into cDNA, which was used as the template for PCR. Using the primer design software, Primer Express 2.0, the specific primers for each gene were synthesized by Beijing Saibaisheng Gene Technique Co., Ltd. and the following primers were generated: *FAK*-Forward 5'-ACTTGGACGCTGTATTGGAG-3', *FAK*-Reverse 5'-CTGTTGCCTGTCTTCTGGAT-3' (833 bp amplicon); Collagen type I -Forward 5'-TACAGCACGCTTGTGGATG-3', Collagen type I -Reverse 5'-TTGAGTTTGGGTTGTTGGTC-3' (256 bp amplicon); Collagen type Ⅲ-Forward 5'-ATGGTGGCTTTCAGTTCACC-3', Collagen type Ⅲ-Reverse 5'-TGGGGTTTCAGAGAGTTTGG-3' (425 bp amplicon); MMP-13-Forward 5'-GCGGGAATCCTGAAGAAGTCTAC-3', MMP-13-Reverse 5'-TTGGTCCAGGAGGAAAAGCG-3' (424 bp amplicon); TIMP-1-Forward 5'-TCCCCAGAAATCATCGACAC-3', TIMP-1-Reverse 5'-ATCGCTGAACAGGGAAACAC-3' (329 bp amplicon); *GAPDH*-Forward 5'-GAGGACCAGGTTGTCTCCTG-3', *GAPDH*-Reverse 5'-GGATGGAATTGTGAGGGAGA-3' (298 bp amplicon). Reaction system: 10 μL 2.5 × real master Mix, 1.25 μL 20 × SYBR solution, 0.5 μL upstream primer, 0.5 μL downstream primer and 2 μL DNA template were brought up to 25 μL with purified water. Reaction conditions: 93℃ 5 min, 1 cycle; 93℃ 45 s, 55℃ 1 min, 10 cycles; 93℃ 30 s, 55℃ 45 s, 30 cycles. The PCR reactions were subjected to 93℃ for 5 min, 1 cycle; and then 10 cycles of 93℃ 45 s, 55℃ 1 min, and 30 cycles of 93℃ 30 s, 55℃ 45 s. The size and quantity of amplified prod-

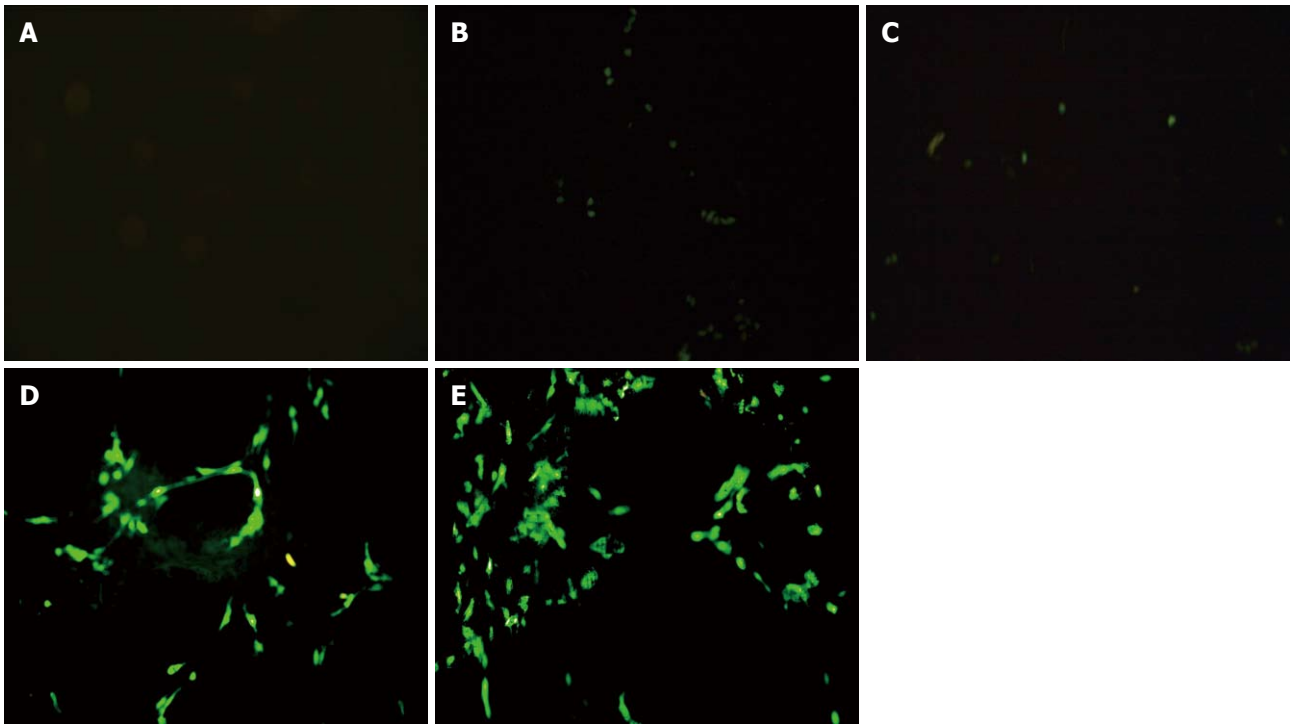

Figure 1 Expression of enhanced green fluorescent protein at 48 h after treatment (fluorescent images, original magnification × 200). A: Control group; B: Fibronectin group; C: Sofast group; D: HK group; E: Focal adhesion kinase (FAK) short hairpin RNA group. *FAK* short hairpin RNA plasmids were successfully transfected into hepatic stellate cells. The results from fluorescence microscopy and flow cytometry showed that the transfection efficiency was 40% at 48 h.

ucts were confirmed by 2% agarose gel electrophoresis. Fluorescent quantitative analysis was performed with the thermal cycler's software package to calculate the ΔCt value. The expression levels of *FAK*, type I collagen and type Ⅲ collagen, *MMP-13* and *TIMP-1* were calculated by the $2^{-\Delta\Delta Ct}$ analysis. The $2^{-\Delta\Delta Ct}$ was presented as the relative expression of the gene expression[12].

Western blotting

At 24 or 48 h after transfection of *FAK* shRNA, HSCs were harvested, washed with phosphate-buffered saline (PBS), and lysed in the improved RIPA buffer (50 mmol/L Tris-HCL, pH 7.5; 100 mmol/L NaCl; 1% NP-40; 0.5% sodium deoxycholate; 2 μg/mL leupeptin; 1% SDS; 2 mmol/L EDTA; 1 mmol/L PMSF; 50 mmol/L HEPES; 1 mmol/L sodium orthovanadate). The supernatant was collected and the protein concentration was determined using comassie brilliant blue assay. Cell extracts containing equal quantities of proteins (100-110 μg) were electrophoresed in 8% or 10% polyacrylamide gel. Subsequently, the separated proteins were transferred to nitrocellulose membrane. The membrane was blocked for non-specific binding for 30 min (5% skimmed milk in PBS), and then incubated overnight at 4℃ with rabbit anti-FAK polyclonal antibody (1:400), rabbit anti-MMP-13 polyclonal antibody (1:200), rabbit anti-TIMP-1 polyclonal antibody (1:200) or mouse anti–GAPDH monoclonal antibody (1:100). The membrane was subsequently incubated at room temperature for 2 h with goat anti-rabbit IgG (1:2000). Blots were developed with enhanced chemiluminescence detection reagents (Santa Cruz Biotechnology Inc.), exposed on Kodak Xdmat blue XB-1 film and quantified by Bandscan 5.0 software using GAPDH as internal control. Densitometry is reported using the integral optical density value (IOD). The results were represented in the form of IOD ratio of the target protein to GAPDH.

Statistical analysis

All the data were expressed by mean ± SD and analyzed with SPSS 13.0 software. The comparison of mean variability among all groups was conducted by one-way ANOVA analysis and two group comparison with LSD test. Student's *t* test was carried out for independent samples. Statistical significance was considered at $P < 0.05$.

RESULTS

Expression of FAK effectively down-regulated by FAK shRNA in HSC

FAK shRNA plasmids were successfully transfected into HSC. The results from fluorescence microscopy and FCM showed that the transfection efficiency was 40% at 48 h (Figure 1). The levels of *FAK* mRNA transcripts and protein expression were determined by real-time Q-PCR and Western blotting analysis. The expression of *FAK* mRNA and FAK protein in the FN group was significantly higher than that of the control group, $P = 0.000$ and $P = 0.024$, respectively. There was no difference between the FN group, Sofast group and HK group. In comparison with the HK group, the expression of *FAK* mRNA and FAK

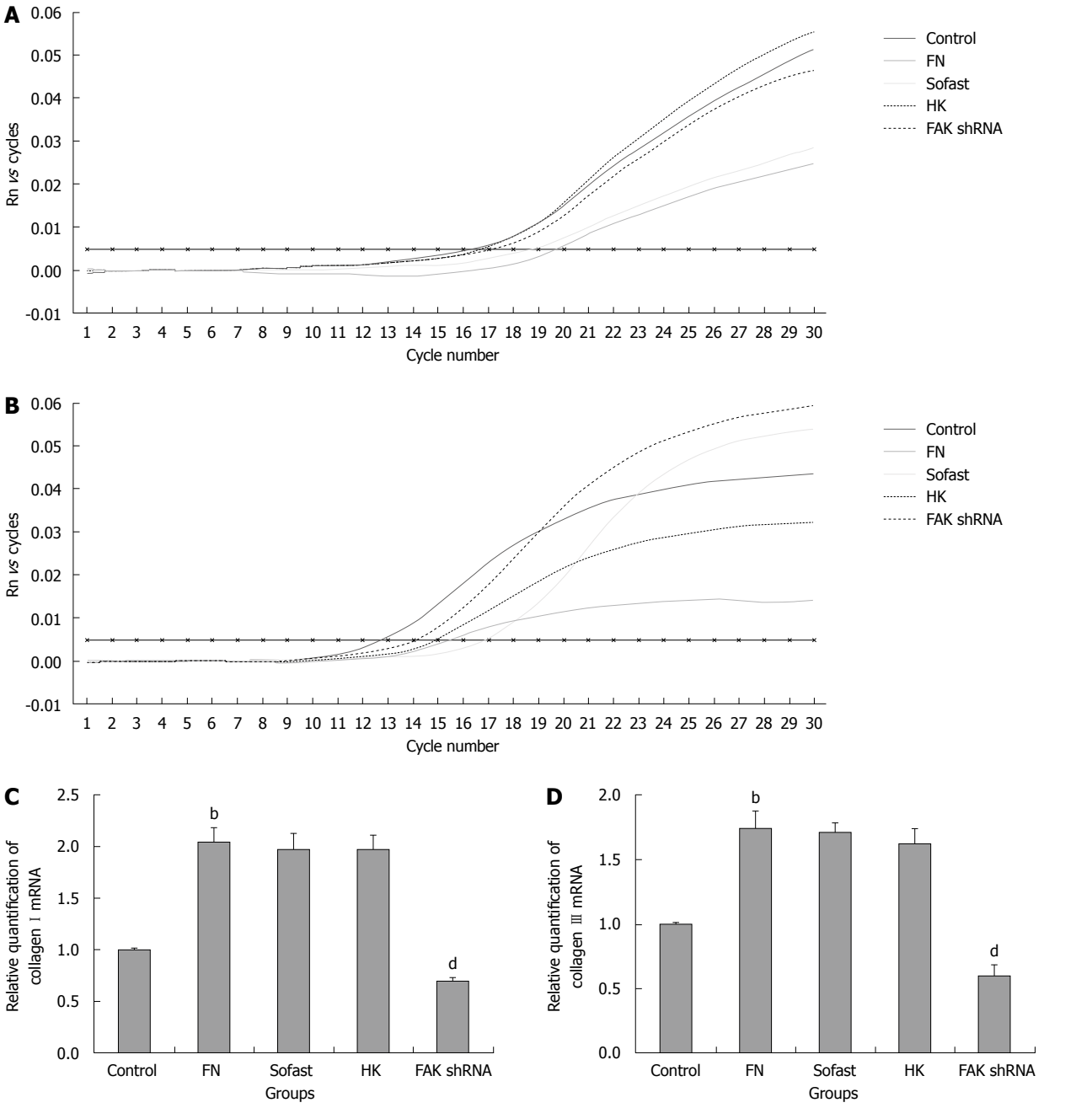

Figure 2 Focal adhesion kinase short hairpin RNA selectively inhibited the expressions of collagen Ⅰ mRNA and collagen Ⅲ mRNA in hepatic stellate cells after focal adhesion kinase short hairpin RNA transfection. A, B: Real-time polymerase chain reaction SYBR Green Ⅰ fluorescence history *vs* cycle number of target gene 1 (collagen Ⅰ, A) and target gene 2 (collagen Ⅲ, B) in sample cDNA. The cycle threshold (Ct) is shown by the darker horizontal line; C, D: The relative quantification of collagen Ⅰ mRNA (C) and collagen Ⅲ mRNA (D) are calculated according to $2^{-\Delta\Delta C(t)}$, [$\Delta\Delta$Ct = ($Ct_{Collagen\ I\ or\ III}$ - Ct_{GAPDH}) experimental group - ($Ct_{Collagen\ I\ or\ III}$ - Ct_{GAPDH}) control group] and shown in the bar graphs (n = 3, ^{b}P < 0.01 *vs* control, ^{d}P < 0.01 *vs* HK). It showed that the levels of type Ⅰ collagen and type Ⅲ collagen mRNA transcripts in fibronectin (FN) group was significantly higher than in the control group. FAK: Focal adhesion kinase; shRNA: Short hairpin RNA.

protein in the *FAK* shRNA plasmid group was significantly decreased (0.37 ± 0.03 *vs* 1.59 ± 0.06, P = 0.000; 0.77 ± 0.03 *vs* 2.24 ± 0.20, P = 0.000), and the rates of down-regulation were 70.51% and 72.53%, respectively.

Effects of FAK by shRNA on the collagen synthesis in HSC

Investigation was carried out in the influence of disruption of FAK expression mediated by *FAK* shRNA on ECM synthesis in HSC. The levels of type Ⅰ collagen and type Ⅲ collagen mRNA transcripts were determined by real-time Q-PCR. The levels of type Ⅰ collagen and type Ⅲ collagen mRNA transcripts in FN group were significantly higher than that of the control group. The levels of type Ⅰ collagen and type Ⅲ collagen mRNA transcripts in *FAK* shRNA plasmid group were significantly decreased compared with the HK group (0.69 ± 0.03 *vs* 1.96 ± 0.15, P = 0.000; 0.59 ± 0.07 *vs* 1.62 ± 0.12, P = 0.020) and the down-regulated rates were 64.80% and 63.58%, respectively (Figure 2).

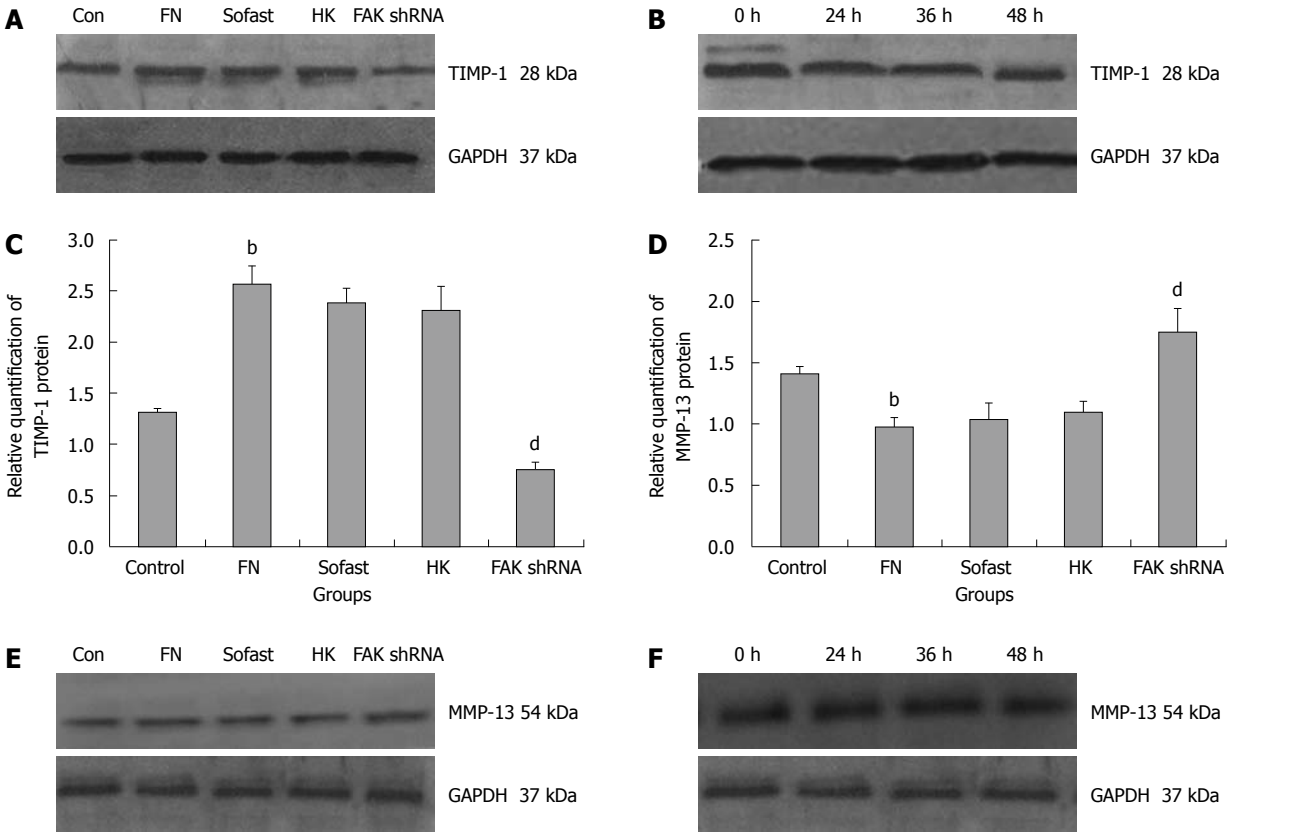

Figure 3 Focal adhesion kinase short hairpin RNA specifically inhibits the expressions of tissue inhibitors of metalloproteinases-1 protein and promotes the expressions of matrix metalloproteinases-13 protein in hepatic stellate cells. A: Cells were harvested, lysed and total protein extracts were separated by SDS-PAGE and analyzed by Western blotting with polyclonal anti-tissue inhibitors of metalloproteinases-1 (TIMP-1) antibody. GAPDH served as a loading control; B: Western blotting analysis was used to detect the expressions of TIMP-1 at different time points; C: TIMP-1 expression levels obtained from scanning densitometry were expressed as a ratio of integral optical density value (IOD) TIMP-1/IOD GAPDH (n = 3, $^{b}P < 0.01$ *vs* Con, $^{d}P < 0.01$ *vs* HK); D: Matrix metalloproteinases-13 (MMP-13) expression levels obtained from scanning densitometry were expressed as a ratio of IOD MMP-13/IOD GAPDH (n = 3, $^{b}P < 0.01$ *vs* Con, $^{d}P < 0.01$ *vs* HK); E, F: Western blotting analysis was carried out at different groups (E) and different time points (F) using polyclonal anti-MMP-13 antibody and monoclonal anti-GAPDH antibody. FN: Fibronectin; FAK: Focal adhesion kinase; shRNA: Short hairpin RNA.

Effects of FAK by shRNA on the collagen degradation in HSC

To further explore the effects of *FAK* shRNA on the ECM degradation in HSC, the levels of MMP-13 and TIMP-1 were determined by real-time Q-PCR and Western blotting analysis. The transfection of HK shRNA did not modulate the levels of MMP-13 and TIMP-1, the cells expressing HK shRNA were similar to that in FN group and Sofast group, $P > 0.05$. However, the knockdown of FAK expression by the *FAK* shRNA significantly reduced the levels of *TIMP-1* mRNA and TIMP-1 protein (0.49 ± 0.02 *vs* 1.72 ± 0.10, $P = 0.005$; 0.76 ± 0.08 *vs* 2.31 ± 0.24, $P = 0.000$), and the down-regulated rates were 69.78% and 67.10%, respectively (Figure 3A-C). The results of real-time Q-PCR and Western blotting analysis showed that the levels of MMP-13 of FN group were significantly down-regulated compared with that of control group. Compared with the HK group, the expression of MMP-13 mRNA was significantly up-regulated by 56.96% at 36 h after transfection of *FAK* shRNA plasmids into HSC (1.24 ± 0.04 *vs* 0.79 ± 0.03, $P = 0.020$), and the expression of MMP-13 protein could be increased by 59.63% at 48 h after transfection (1.74 ± 0.20 *vs* 1.09 ± 0.09, $P = 0.000$) (Figure 3D-F).

DISCUSSION

The current knowledge on the pathophysiology of liver fibrogenesis refers to the increased synthesis and decreased degradation of ECM, mainly type I collagen and type Ⅲ collagen, thereby ECM was overproduced and deposited in the liver. Although several hepatic cell types can synthesize ECM proteins, HSCs are the major source of increased ECM in chronic liver diseases. They can undergo a proliferative and phenotypic change. Excessive deposition of ECM, mainly type I collagen and type Ⅲ collagen, results in liver fibrosis; and the up-regulation of TIMPs blocks activity of MMPs and inhibits the degradation of ECM, thereby aggravating liver fibrosis.

The interaction of HSC and ECM mainly lies between integrins, and FAK plays an integral role in the integrin signal pathway. Activated FAK has been implicated in a diverse array of cellular behaviors, such as cell proliferation[4,5], apoptosis, cell migration[6], collagen metabolism[7,8] and the transfer of tumor cells. It is closely related to numerous fibrotic diseases and it plays a vital role in the occurrence and development of liver fibrosis[13]. This is consistent with our previous studies, which indicated that FAK phosphorylation could promote collagen synthesis

of HSC *in vivo*. Furthermore, using *in vitro* cell culture techniques, we found that the synthesis of total collagen and type I collagen in HSC could be inhibited by the endogenous inhibitor FRNK[9]. We hypothesized that FAK gene silencing may represent a novel method for the treatment and reversal of liver fibrosis. Therefore, in this study, *FAK* shRNA plasmids were transfected into HSC transiently to test our hypothesis, and the expressions of FAK mRNA and FAK protein were significantly decreased, the down-regulation rates being 70.51% and 72.53%, respectively. We have found that *FAK* shRNA can effectively and specially suppress the expression of FAK.

A substantial change in liver fibrosis or liver cirrhosis is the deposition of ECM, which is mainly composed of type I collagen and type Ⅲ collagen, covering approximately 80%-90% of the increased total collagen. The increase of type I collagen and type Ⅲ collagen is an important symbol of liver fibrosis or liver cirrhosis. Therefore, in this study, *FAK* shRNA plasmids were transfected into FN-stimulated HSC transiently and the expression of type I collagen mRNA and type Ⅲ collagen mRNA was significantly down-regulated by 64.80% and 63.58%, respectively. These data show that *FAK* shRNA can effectively suppress the synthesis of collagen and *FAK* gene silencing may, therefore, represent a novel direction for the treatment and reversal of liver fibrosis.

Furthermore, we attempted to assess the role of FAK in the regulation of collagen metabolism in HSC. In the liver, ECM is regulated by MMPs and their specific inhibitors, TIMPs. A principal feature of hepatic fibrosis is a disturbance in the balance between MMPs and TIMPs. Collagenases such as MMP-1 and MMP-13 are able to degrade fibrillar collagens, mainly type Ⅰ, Ⅱ and Ⅲ collagen. These may be responsible for key events in the degradation of ECM. MMP-13 is the interstitial collagenase in rats and its specific inhibitor is TIMP-1. Although the expression of MMP-13 was increased in the liver tissues of CCl_4-induced rat liver fibrosis models, fibrosis still occurred as there was also a corresponding increase in the expression of TIMP-1[14]. This strongly suggests that a disruption in the balance between MMP-13 and TIMP-1 is possibly an important factor in liver fibrogenesis[15]. According to some studies, FAK is closely related to the expression of TIMP-1 and MMP-13[16,17]. In this study, *FAK* shRNA plasmids were transfected into FN-stimulated HSC transiently and the expression of MMP-13 mRNA and MMP-13 protein was significantly up-regulated by 56.96% and 59.63%. Correspondingly, the levels of TIMP-1 mRNA and TIMP-1 protein were significantly down-regulated by 69.78% and 67.10%, respectively. *FAK* shRNA inhibited the ratio of TIMP-1/MMP-13 expression in mRNA and protein levels in HSC after transfection. The data indicate that *FAK* shRNA regulated the collagen metabolism in HSC by disturbing the balance between MMP-13 and TIMP-1.

In summary, we have effectively disrupted the expression of FAK by *FAK* shRNA. The knockdown of FAK expression significantly reduced the synthesis of type I collagen and type Ⅲ collagen, which may be related to the up-regulation of MMP-13 and down-regulation of TIMP-1. These data support the hypothesis that *FAK* disruption by shRNA may be an efficient and specific approach for treatment of liver fibrosis. Future studies will address the signal transduction pathway by which FAK regulates the collagen metabolism in HSC.

COMMENTS

Background

Focal adhesion kinase (FAK) plays an essential role in the activation of hepatic stellate cells (HSCs) which are the major source of collagens and matrix metalloproteinases in the fibrotic liver. Liver fibrosis results from excessive deposition of extracellular matrix components, composed of mainly type Ⅰ collagen produced by HSC.

Research frontiers

The central events in the liver fibrogenesis have been proved to be the activation, proliferation and migration of HSC, and their proliferation and collagen synthesis are promoted by phosphorylation of FAK, a non-receptor protein tyrosine kinase. In the area of knockdown or inhibition of FAK with various molecular biological technologies, an area of intense research is to establish a method to knockdown or inhibit FAK expression thoroughly so as to enhance the collagen metabolism.

Innovations and breakthroughs

Recent reports have highlighted the importance of HSC including activation, proliferation and migration in pathogenesis of liver fibrosis. The collagen metabolism in HSC, particular in activated HSC, is currently an area of intense research. This is the first study to report that shRNA-mediated disruption of *FAK* expression can attenuate extracellular matrix (ECM) synthesis and promote ECM degradation. This represents a potential target for novel anti-fibrosis therapies.

Applications

The results of this study indicated that suppression of *FAK* expression may represent a novel method and direction for the treatment and reversal of hepatic fibrosis.

Peer review

The study focuses on modification of hepatic stellate cell metabolism by shRNA mediated inhibition of FAK, a non-receptor protein tyrosine kinase involved in proliferation and collagen synthesis. The authors demonstrate that FAK inhibition is associated with a decrease in collagen synthesis by HSCs.

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