Original Research

Endostar, a novel human recombinant endostatin, attenuates liver fibrosis in CCl_4-induced mice

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Abstract

Decreasing hepatic fibrosis remains one of the major therapeutic challenges in hepatology. The present study aims to evaluate the effect of Endostar on both CCl_4-induced liver fibrosis in mice and a hepatic stellate cell (HSC) line. Two main models were studied: (i) a liver fibrosis model was induced in BALB/c mice using CCl_4 by intraperitoneal injection for six weeks. Six animal groups were studied: group 1: normal animals; group 2: CCl_4-induced liver fibrosis; group 3: CCl_4 + Endostar 20 mg/kg/d, six weeks; group 4: CCl_4 + Endostar 10 mg/kg/d, six weeks; group 5: CCl_4 + Endostar 20 mg/kg/d, four weeks; group 6: CCl_4 + Endostar 10 mg/kg/d, four weeks corresponded to different Endostar doses and duration of administration. Liver fibrosis was evaluated by histopathological staining and liver hydroxyproline content. Expressions of *collagen type I*, α-smooth muscle actin (*α-SMA*), *TGF-β1* and *VEGFR* were measured by real-time polymerase chain reaction (PCR). (ii) A liver cell model. HSC-T6 cells were cultured with or without Endostar for 12 h or 24 h. Expressions of *collagen type I*, *α-SMA*, and *TGF-β1* were measured by real-time PCR. Collagen I and transforming growth factor β1 (TGF-β1) contents in cell supernatant were measured by enzyme-linked immunosorbent assay. As compared to the group without Endostar, liver fibrosis scores and hydroxyproline content were decreased in both Endostar groups ($P < 0.05$). Moreover, Endostar inhibited the hepatic expression of *α-SMA*, *TGF-β1*, *Collagen-1*, *VEGFR1*, and *VEGFR2* mRNA ($P < 0.05$). In the HSC-T6 cell line model, Endostar profoundly inhibited the expression of *α-SMA*, *Collagen-1*, and *TGF-β1* mRNA. Expressions of *Collagen-1* and *TGF-β1* protein were decreased in the Endostar group as compared to the normal controls in the supernatant of HSC-T6 cells ($P < 0.05$). Endostar decreased both liver fibrosis in CCl_4-induced mice and collagen synthesis in HSCs *in vitro*. Therefore, this recombinant human endostatin is a promising compound for counteracting liver fibrosis.

Keywords: Carbon tetrachloride, hepatic fibrosis, Endostar, transforming growth factor β, hepatic stellate cell

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Introduction

Endostatin (ES) is an endogenous angiogenesis inhibitor derived from collagen XVIII (C18), a proteoglycan found in vessel walls and basement membranes.[1,2] Studies have shown that ES can release anti-angiogenic fragments to inhibit angiogenesis and tumor growth by restricting endothelial proliferation, migration, and inducing apoptosis of endothelial cells.[3] ES is not only efficient in tumor growth, but also in liver fibrosis. The precursors of ES–C18 transcripts were found in hepatocytes and bile duct epithelia of normal and bile duct ligated (BDL)-induced fibrotic livers and occasionally in arterial myocytes and hepatic stellate cells (HSCs).[4] However, no further studies investigated the relationship between ES and liver fibrosis.

Endostar is a recently introduced recombinant human ES designed by Chinese scientists.[5] Endostar is soluble in the blood and has a long half-life.[6] Endostar was approved by the State Food and Drug Administration, USA (SFDA) in 2005 for the treatment of non-small-cell lung cancer.[5,7,8] Endostar has been considered as a valuable anti-angiogenic agent, which was shown to suppress vascular endothelial growth factor (VEGF)-stimulated proliferation, migration, and tube formation of human umbilical vein endothelial cells (HUVECs) *in vitro*.[5] Liver fibrosis is a reversible scarring response that can occur in all types of chronic liver

injury. Ultimately, liver fibrosis leads to cirrhosis, which is associated with nodule formation and organ contraction.[9] Clinically, cirrhosis is associated with risks of severe liver dysfunction and hepatocellular carcinoma. To date, there is no established anti-fibrotic drug that can be used by clinicians. Recent evidence showed that angiogenesis modulates the formation of liver fibrosis, portal hypertension and hepatic carcinoma.[10,11] Studies have shown that anti-angiogenesis drugs, such as sorafenib and sunitinib, can also inhibit liver fibrosis.[12–14] The key role of HSCs in the development of liver fibrosis has been largely established.[9,15]

The present study aims to explore the possible inhibiting effect of Endostar both in an *in-vivo* model of hepatic fibrosis and in an HSC-derived cell line *in vitro*.

Materials and methods

Mice model of CCl_4-induced liver fibrosis

Endostar was purchased from Simcere Pharmaceutical Research Co., Ltd (China). Male BALB/c mice weighing 18–20 g were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. Liver fibrosis was induced by intraperitoneal (ip) injection of CCl_4 (40% V/V CCl_4 in corn oil, 0.2 mL/100 g body weight, twice weekly) for six weeks (5–8 animals per group). Six groups were studied: group 1: normal mice; group 2: CCl_4-induced liver fibrosis; group 3: CCl_4 + Endostar (20 mg/kg/d for 6 weeks, Endostar was given simultaneously with CCl_4 injection for 6 weeks); group 4: CCl_4 + Endostar (10 mg/kg/d for 6 weeks, Endostar was given simultaneously with CCl_4 injection for 6 weeks); group 5: CCl_4 + Endostar (20 mg/kg/d for 4 weeks, CCl_4 only was given to mice for 2 weeks, then Endostar was given to mice simultaneously with CCl_4 injection for another 4 weeks); group 6: CCl_4 + Endostar (10 mg/kg/d for 4 weeks, CCl_4 only was given to mice for 2 weeks, then Endostar was given to mice simultaneously with CCl_4 injection for another 4 weeks). The mice in group 1 received corn oil ip in the same regimen as CCl_4 treatment mice. The same volume of saline was given to the control groups (groups 1 and 2) and group 5 and 6 by ip compared to the volume of Endostar in group 3. In groups 3 and 4, Endostar was given at the same time as CCl_4 injection. In groups 5 and 6, CCl_4 only was given to mice for two weeks, then Endostar was always administered to mice at the same time as CCl_4 injection for another four weeks. All protocols and procedures were approved by the Animal Care and Use Committee of Harbin Medical University, Harbin, China. The animals were housed in an air-conditioned room at 23–25°C with a 12 h dark/light cycle for one week before initiation of the experiment. All animals received appropriate care during the study with unlimited access to chow and water.

Histological examination

Liver samples were obtained from control mice and mice with CCl_4-induced liver fibrosis, with or without Endostar treatment 48 h after the last CCl_4 induction. Part of the liver was immediately snap-frozen in liquid nitrogen and stored at −80°C until further use. Another was embedded in paraffin and sliced into 4–5 μm sections. Sections were stained with hematoxylin and eosin and Masson staining for histopathological analysis and liver fibrosis evaluation. Each sample was independently assessed and scored by two pathologists, blinded to the study protocol, according to a fibrosis score system.[16] Liver fibrosis was divided into seven stages: stage 0, no fibrosis; stage 1, short fibrous tissue in central venule (C); stage 2, fibrous C-C septa appearance; stage 3, C-C fibrous septa incompletely developed; stage 4, C-C septa completely connected (pseudolobule); stage 5, C-P (portal tract) bridging fibrosis, nodular appearance ≤50%; and stage 6, nodular appearance >50%. Hepatic hydroxyproline was measured using a hydroxyproline detection kit (Jiancheng Institute of Biotechnology, Nanjing, China) according to the manufacturer's instructions. The hydroxyproline content was expressed as mg/g wet liver. Blood was collected from the inferior vena cava. The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, and total bilirubin (TBIL) were assessed using routine laboratory methods.

Immunohistochemistry staining for α-SMA

α-Smooth muscle actin (α-SMA) was detected in paraffin-embedded liver sections using a α-SMA-specific antibody (mouse to monoclonal α-SMA, cat. no. A2547, Sigma, USA) and an avidin–biotin complex immunoperoxidase method. After antigen retrieval by immersion in EDTA (1 mmol/L, pH 8.0) and boiling for 15 min in a microwave oven, sections were pre-blocked with normal goat serum for 10 min and incubated for 2 h at room temperature with α-SMA antibody (final concentration 1:200). Phosphate-buffered saline (PBS) was used in place of the primary antibodies for the negative controls. Following rinsing, the biotinylated secondary antibody, avidin–biotin complex, and horseradish peroxidase (Strept ABComplex; DAKO, USA) were applied. Finally, the sections were washed with PBS, developed with diaminobenzidine tetrahydrochloride substrate for 3 min, and counterstained with hematoxylin.

Computer-assisted semi-quantitative analysis was used to evaluate the areas of positive α-SMA staining using Image-ProPlus version 4.5 (Media Cybernetics, Silver Spring, MD). The data for the α-SMA staining were expressed as the mean percentage of the positively stained area over the total tissue section area.[17]

Cell culture

The HSC-T6 cell line, kindly provided by Dr Scott L. Friedman (Mount Sinai School of Medicine, NY), was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). The cell viability of HSCs was >90% for 24 h in the presence of 500 μg/mL Endostar in DMEM under serum-free conditions. After serum starvation for 16 h, HSC-T6 cells were treated with VEGF (100 ng/mL, R&D systems Inc. Minneapolis, MN, USA)[18] or VEGF (100 ng/mL) plus Endostar (500 μg/mL, 250 μg/mL or 125 μg/mL) in serum-free medium for 12 h or 24 h.

Detection of mRNA levels of collagen I, α-SMA, TGF-β1, VEGFR1, and VEGFR2 by real-time PCR

Total RNA was extracted from liver tissues and HSC-T6 cells by using the TRIzol Reagent kit (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The quality of total RNA was verified by ultraviolet absorbance spectrophotometry at 260 nm and 280 nm. cDNA was reversed transcribed using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Reactions were as follows: 25°C for 10 min, 37°C for 150 min, 85°C for 5 s, and 4°C for 5 min, before chilling on ice. cDNA was stored at −20°C for future use.

Collagen I, α-SMA, TGF-β1, VEGFR1, and *VEGFR2* mRNA levels were quantified by means of real-time PCR. The sequences of the primers used are shown in Tables 1 and 2. *GADPH*, a housekeeping gene, was used as an internal control primer for target genes. All primers were obtained from Invitrogen (Beijing, China). The expression of mRNA was measured by SYBR Green real-time PCR using an ABI 7500 instrument (Applied Biosystems). PCR was performed in 20 μL buffer that contained 2 μg cDNA, 1 μL each primer, and 10 μL SYBR Green PCR Master Mix (Applied Biosystems). Comparative cycle threshold (Ct) calculations were all relative to the control group. The expression of mRNA relative to the control was derived by using the equation $2^{-\Delta\Delta Ct}$.

Determination of collagen I and transforming growth factor β1 (TGF-β1) in HSC-T6 cell supernatant

Cultured HSC-T6 cells were incubated in serum-free medium in the presence or absence of Endostar for 12 h or 24 h. TGF-β1 and collagen I production was determined in culture medium using enzyme-linked immunosorbent assay (ELISA) kits (TGF-β1 ELISA kit, R&D Systems; Collagen I ELISA kit, USCN LIFE, Wuhan, China).

Statistical analysis

The results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using analysis of variance (ANOVA) and unpaired Student's *t*-test as appropriate. The non-parametric data were analyzed by the Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL).

Results

Endostar inhibits liver fibrosis in CCl_4-induced mice

There were no collagen fibers along the central vein in the control group. In the CCl_4 group, collagenous fibers were

Table 1 Primers for quantitative real-time PCR analysis used in animal experiment

Gene	Primer sequence	Accession number
GAPDH (mouse)	F: 5′-GCACAGTCAAGGCCGAGAAT-3′ R: 5′-GCCTTCTCCATGGTGGTGAA-3′	XM_001476707.3
Col I a1 (mouse)	F: 5′-TTCACCTACAGCACGCTTGTG-3′ R: 5′-GATGACTGTCTTGCCCCAAGTT-3′	NM_007742.3
α-SMA (mouse)	F: 5′-TCAGCGCCTCCAGTTCCT-3′ R: 5′-AAAAAAAACCACGAGTAACAAATCAA-3′	NM_007392.2
TGF-β1 (mouse)	F: 5′-CCCGAACCCCCATTGCTGTCC-3′ R: 5′-AGGCGTATCAGTGGGGGTCAG-3′	NM_011577.1
VEGFR1 (mouse)	F: 5′-CCACCTCTCTATCCGCTGG-3′ R: 5′-ACCAATGTGCTAACCGTCTTATT-3′	NM_010228.3
VEGFR2 (mouse)	F: 5′-CAAACCTCAATGTGTCTCTTTGC-3′ R: 5′-AGAGTAAAGCCTATCTCGCTGT-3′	NM_010612.2

GAPDH, glyceraldehyde phosphate dehydrogenase.

Table 2 Primers for quantitative real-time PCR analysis used in HSC-T6 cell experiment

Gene	Primer sequence	Accession number
GAPDH (rat)	F: 5′-CCTGCCAAGTATGATGACATCAAGA-3′ R: 5′-GTAGCCCAGGATGCCCTTTAGT-3′	BC059110.1
Col I (rat)	F: 5′-CCTTTCTCCACCCCCTCTT-3′ R: 5′-TGTGTCTTTGGGGGAGACTT-3′	NM_053304.1
α-SMA (rat)	F: 5′-TGCCATGTATGTGGCTATTCA-3′ R: 5′-ACCAGTTGTACGTCCAGAAGC-3′	NM_001613.2
TGF-β1 (rat)	F: 5′-CCTGGAAAGGGCTCAACAC-3′ R: 5′-CTGCCGTACACAGCAGTTCT-3′	NM_021578.2

GAPDH, glyceraldehyde phosphate dehydrogenase.

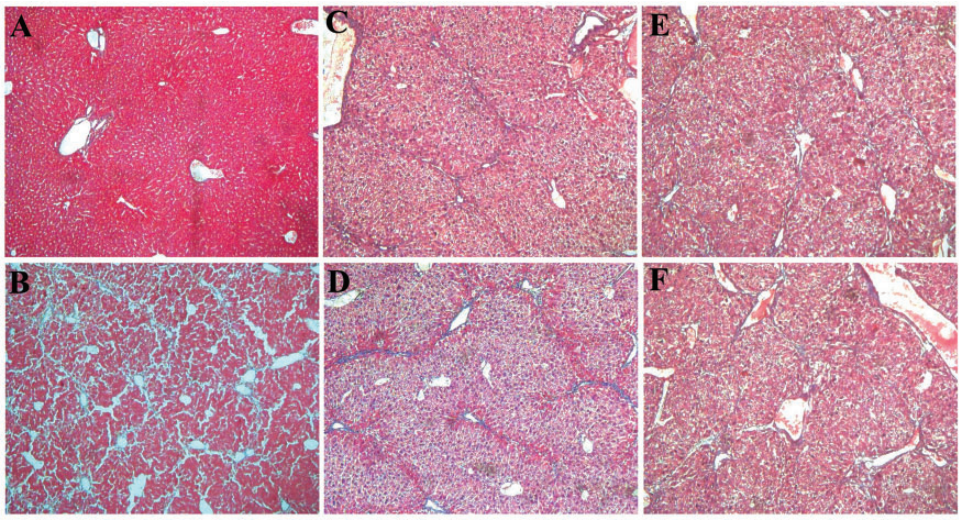

Figure 1 Endostar attenuated the extent of liver fibrosis in CCl_4-stimulated mice. a–f: Representative photomicrograph of Masson staining in the normal group 1 (panel a), CCl_4 model group 2 (panel b), CCl_4 + Endostar (6 weeks 20 mg/kg) group 3 (panel c), CCl_4 + Endostar (6 weeks 10 mg/kg) group 4 (panel d), CCl_4 + Endostar (4 weeks 20 mg/kg) group 5 (panel e), and CCl_4 + Endostar (4 weeks 10 mg/kg) group 6 (panel f), 100x magnification. The fibers are shown in blue. Less fibers were seen in groups treated with Endostar (groups 3, 4, 5, 6) compared with the CCl_4 model group 2. (A color version of this figure is available in the online journal.)

Table 3 Endostar attenuated CCl_4-induced liver fibrosis

	Score of liver fibrosis							
	0	**1**	**2**	**3**	**4**	**5**	**6**	**Mean ± SD**
Group 1 ($n=7$)	7	0	0	0	0	0	0	0
Group 2 ($n=8$)	0	0	0	0	2	5	1	4.88 ± 0.64
Group 3 ($n=5$)	0	0	1	2	2	0	0	3.20 ± 0.84*
Group 4 ($n=5$)	0	0	1	3	0	1	0	3.20 ± 1.10*
Group 5 ($n=6$)	0	0	2	2	1	1	0	3.17 ± 1.17*
Group 6 ($n=7$)	0	0	1	1	3	2	0	3.86 ± 1.07*

Group 1: normal control, group 2: CCl_4 model, group 3: CCl_4 + Endostar (6 weeks 20 mg/kg), group 4: CCl_4 + Endostar (6 weeks 10 mg/kg), group 5: CCl_4 + Endostar (4 weeks 20 mg/kg), and group 6: CCl_4 + Endostar (4 weeks 10 mg/kg).
*$P < 0.01$ (Endostar group *vs.* CCl_4 group).

extended from the central vein and the portal area to the hepatic lobules, with pseudo-lobule formation. With the different doses and administration duration of Endostar (20 and 10 mg/kg/d, 4 or 6 weeks), collagenous fibers were decreased compared to the CCl_4 (alone) group (Figure 1a–f). The fibrosis scores in the four Endostar groups were lower than in the CCl_4 group ($P < 0.05$) (Table 3). Endostar also reduced hydroxyproline concentration in liver tissue, reflecting decreased total hepatic collagen content ($P < 0.01$) (Table 4).

Endostar improved liver function and reduced liver inflammation

Serum ALT and AST levels were significantly decreased by Endostar treatment ($P < 0.01$) while no significant changes in serum TBIL levels were observed (Table 4). The serum albumin level in the Endostar group was not significantly increased versus CCl_4-induced mice ($P > 0.05$). The number of inflammatory cells and extent of liver necrosis were decreased in the liver of Endostar groups compared to the group 2 (CCl_4 treatment alone) (Figure 2a–f).

Endostar inhibited α-SMA protein expression in mice

The expression of α-SMA, a typical marker of activated HSCs, was assessed by immunohistochemistry to evaluate the effect of Endostar on HSC activation during hepatic fibrosis. There was no positive staining on slices from the control group (Figure 3a). Six weeks of CCl_4 induction led to considerable increases in the amount of α-SMA positive cells, which were distributed throughout the fibrotic septa (Figure 3b).

The computer-assisted semi-quantitative analysis revealed that groups 3, 4, 5, and 6 showed significantly decreased α-SMA positive areas as compared with the group 2 ($P < 0.05$), while there was no significant difference between these four groups (Figure 3c, d, e, f, g).

Endostar inhibited collagen I, α-SMA, TGFβ1, VEGFR1, and VEGFR2 mRNA expression in mice

Results showed that CCl_4 increased the mRNA expression of collagen I, α-SMA, TGF-β1, VEGFR1, and VEGFR2 compared to the normal control group ($P < 0.01$) (Figure 4). Endostar inhibited these various mRNA expression compared to the CCl_4 group ($P < 0.01$).

Effect of Endostar on collagen I, α-SMA, and TGF-β1 expression in HSC-T6 cells

To assess the effect of Endostar on ECM production by HSCs, we determined the expression of collagen I mRNA in HSCs and collagen I concentrations in culture medium after adjusting the number of cultured HSC-T6 cells.

Table 4 Effects of Endostar on several markers in mice treated with CCl_4

	Group 1 ($n=7$)	Group 2 ($n=8$)	Group 3 ($n=5$)	Group 4 ($n=5$)	Group 5 ($n=6$)	Group 6 ($n=7$)
Hydroxyproline (mg/g liver)	0.1 ± 0.04	92.0 ± 4.6	40.8 ± 5.9*	46.0 ± 7.4*	38.6 ± 7.8*	40.9 ± 2.4*
ALT (IU/mL)	38.4 ± 5.9	149.5 ± 19.4	110.4 ± 11.6*	125.2 ± 19.6*	108.2 ± 21.6*	114.9 ± 14.2*
AST (IU/mL)	41 ± 8.3	235.5 ± 16.8	174.4 ± 11.9*	179.2 ± 14.5*	162.8 ± 17.9*	181.9 ± 20.6*
TBIL (mg/dL)	1.0 ± 0.1	1.9 ± 0.2	1.8 ± 0.2	1.8 ± 0.3	1.7 ± 0.3	1.8 ± 0.3
Albumin (g/L)	34.8 ± 1.5	26.2 ± 1.9	29.6 ± 1.6	28.9 ± 1.8	30.2 ± 1.4	29.4 ± 1.5

ALT: alanine aminotransferase; AST: aspartate aminotransferase; TBIL: total bilirubin.
Group 1: normal control, group 2: CCl_4 model, group 3: CCl_4 + Endostar (6 weeks 20 mg/kg), group 4: CCl_4 + Endostar (6 weeks 10 mg/kg), group 5: CCl_4 + Endostar (4 weeks 20 mg/kg), and group 6: CCl_4 + Endostar (4 weeks 10 mg/kg). Data are expressed as the mean ± SD.
*$P < 0.01$ (Endostar group *vs*. CCl_4 group).

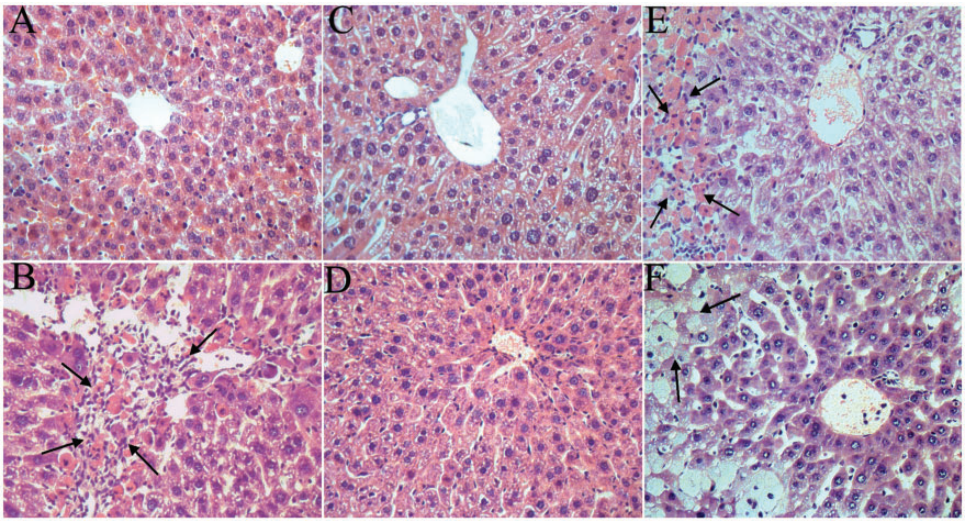

Figure 2 Endostar inhibited apoptosis, necrosis, and inflammation in hepatocytes. a–f: Representative photomicrographs of HE staining in the six groups (as defined in Figure 1), 400x magnification. Endostar reduced CCl_4-induced liver inflammation compared with the CCl_4 model group 2. (Panel a) Normal group 1 mice showed normal liver histology. (Panel b) In the CCl_4 model group 2, large patchy hepatocyte eosinophilic necrosis and groups of inflammatory cells (arrow) were observed. (Panel c, d) In groups 3 and 4, there was little hepatocyte injury and few inflammatory cells were seen. (Panel e) In group 5, focal necrosis and inflammatory cells (arrow) were observed. (Panel f) In group 6, the liver cells only exhibited minor injury or minor edema or plasminic loose (arrow). (A color version of this figure is available in the online journal.)

The level of *collagen I* increased after VEGF treatment and was reduced by Endostar co-treatment compared to normal control ($P < 0.01$) (Figure 5). Treatment of the cells with increasing concentrations of Endostar led to a concentration-dependent suppression of *collagen I* production. After exogenous VEGF (100 ng/mL) stimulation for 12 h, *collagen I* mRNA expression reached 1.58-fold ($P < 0.05$) that of the normal control. Endostar 500 μg/mL, 250 μg/mL, and 125 μg/mL inhibited the expression of 1.18-fold, 1.29-fold, and 1.41-fold, respectively ($P < 0.05$) (Figure 5a). The changes of collagen I protein expression in the supernatant were consistent with mRNA expression data ($P < 0.05$) (Figure 5d).

Changes of α-SMA expression are often used to study the extent of HSC activation. Therefore, in this study, the effect of VEGF and Endostar on the activation of the HSC-T6 cells was investigated by examining changes in *α-SMA* gene expression. Results showed that after exogenous VEGF (100 ng/mL) stimulation for 12 h, *α-SMA* mRNA expression reached 2.03-fold ($P < 0.05$) that of the normal control, whereas Endostar (500 μg/mL) inhibited the expression to 1.17-fold compared to normal control ($P < 0.05$). After 24 h incubation, *α-SMA* mRNA expression reached 2.19-fold ($P < 0.05$) that of the normal control, whereas Endostar (500 μg/mL) inhibited the expression to 1.26-fold compared to normal control ($P < 0.05$) (Figure 5c).

TGF-β1 is one of the important factors in the development of liver fibrosis.[19,20] Therefore, we also detected the level of *TGF-β1* mRNA and protein expression. The results showed that the level of both *TGF-β1* mRNA and protein increased after VEGF treatment compared with normal HSC-T6 cell line and was reduced by Endostar and VEGF co-treatment (Figure 5b, e). Stimulation of HSC-T6 cells for 24 h with exogenous VEGF strongly increased *TGF-β1* gene expression by 1.7-fold versus normal controls, whereas Endostar reduced *TGF-β1* expression to 1.3-fold compared with normal control ($P < 0.05$) (Figure 5b). The changes in TGF-β1 protein expression in the supernatant were consistent with mRNA expression (Figure 5e).

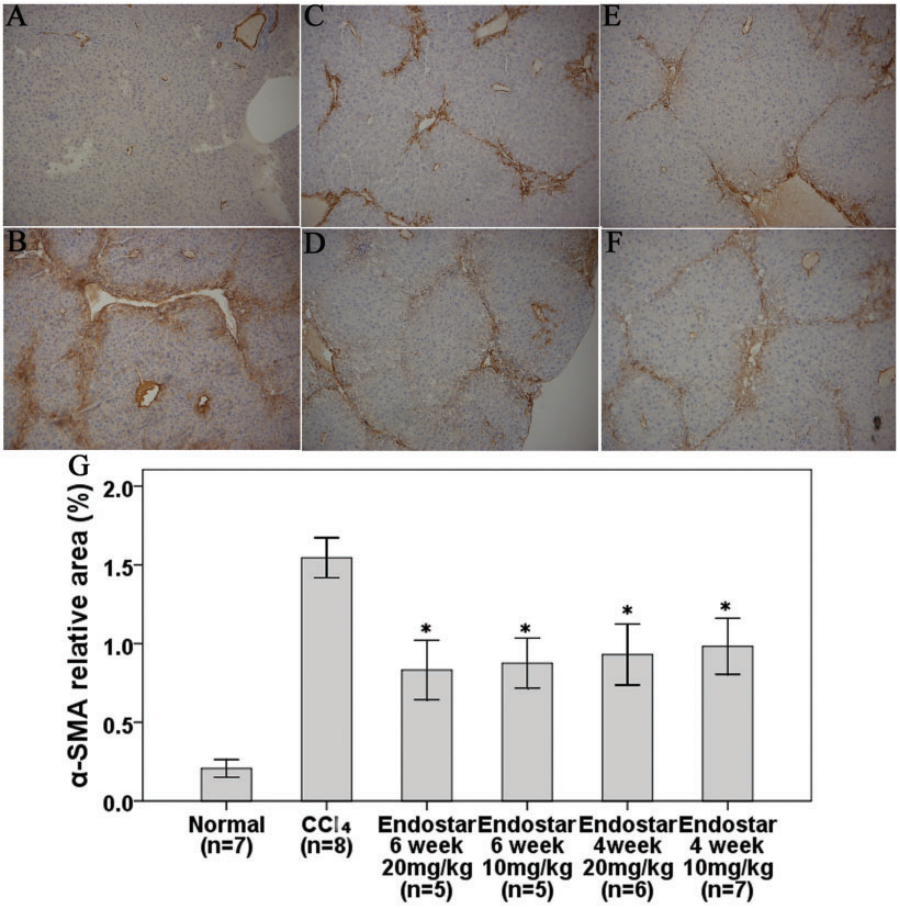

Figure 3 Endostar suppresses α-SMA expression in CCl_4-induced fibrotic mouse livers. Panel a–f: Representative photomicrographs of immunohistochemistry staining in the six groups (as defined in Figure 1), 100x magnification. Endostar reduced α-SMA expression in the CCl_4-induced liver compared with the CCl_4 model group 2. (A color version of this figure is available in the online journal.)

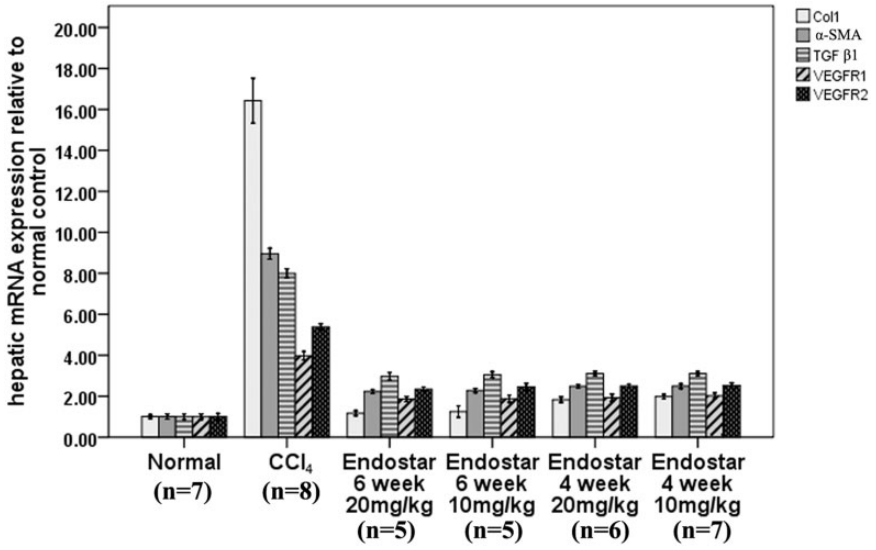

Figure 4 Endostar inhibited the expression of *collagen I*, *α-SMA*, *TGF-β1*, *VEGFR1,* and *VEGFR2* mRNA in fibrotic mice. mRNA expression of *collagen I*, *α-SMA*, *TGF-β1*, *VEGFR1*, and *VEGFR2* were determined by real-time PCR in the liver treated with or without Endostar. Data were normalized to the internal control (GAPDH) and expressed as the mean ± SD. $^*P < 0.01$ (Endostar group *vs*. CCl_4 group). Results showed that the expression of *collagen I*, *α-SMA*, *TGF-β1*, *VEGFR1*, and *VEGFR2* mRNA was increased in the CCl_4 group and decreased after Endostar treatment in the liver of fibrotic mice

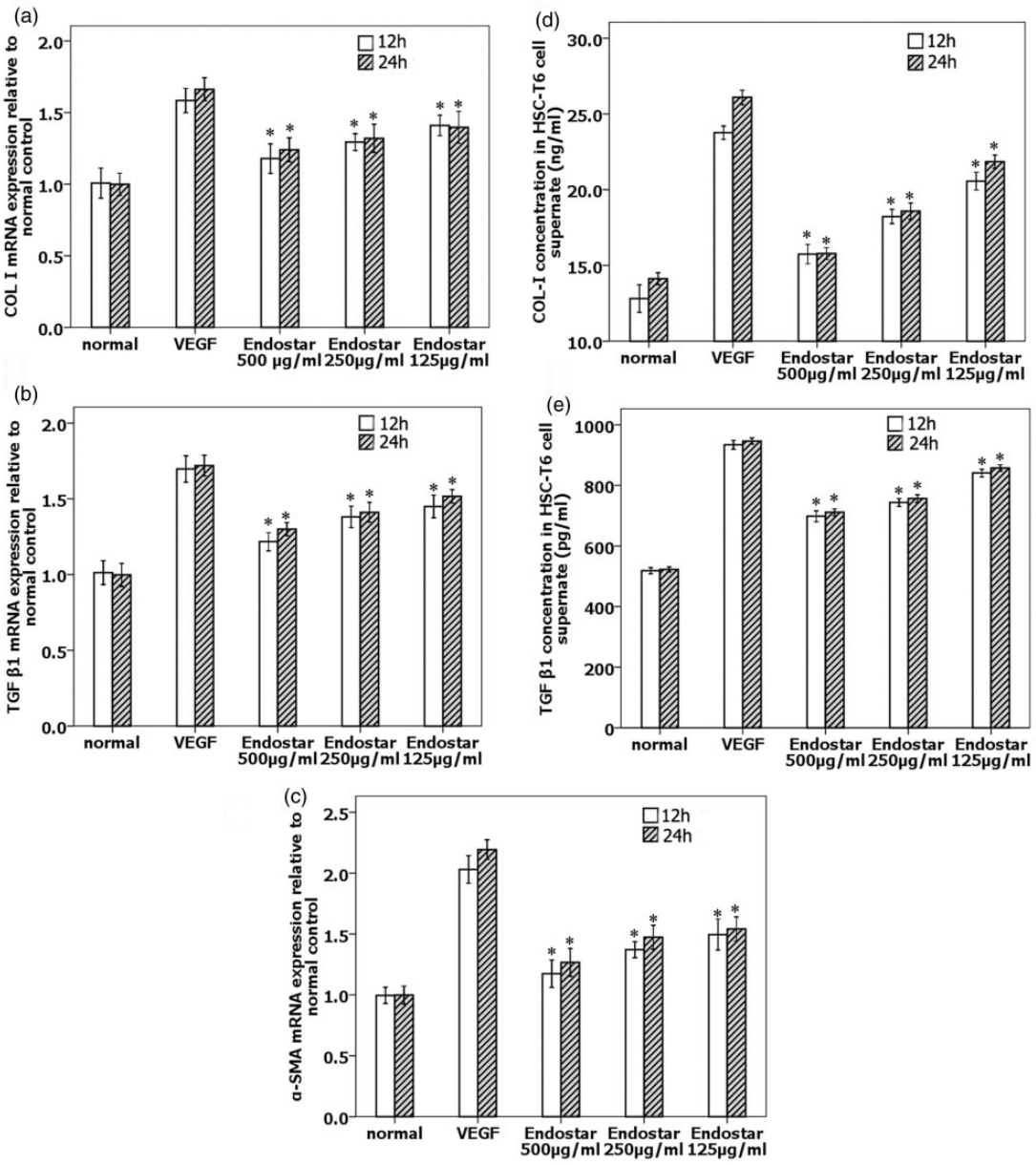

Figure 5 Endostar inhibited the expression of *collagen I*, *α-SMA*, and *TGF-β1* mRNA and the secretion of collagen I and TGF-β1 in HSC-T6 cells. mRNA expression of *collagen I*, *TGF-β1*, and *α-SMA* was determined by real-time PCR in the HSC-T6 cells treated with VEGF and with or without Endostar (a, b, c). Data were normalized to the internal control (GAPDH) and expressed as the mean ± SD. *$P < 0.01$ (Endostar group *vs*. VEGF group) ($n = 6$). Results showed that the expression of *collagen I*, *α-SMA*, and *TGF-β1* mRNA was increased in the VEGF group and decreased after Endostar treatment in HSC-T6 cells. Protein expression of collagen I and TGF-β1 in HSC-T6 cell supernatant was determined by ELISA in HSC-T6 cells treated with VEGF and with or without Endostar (d, e). *$P < 0.01$ (Endostar group *vs*. VEGF group) ($n = 6$). Results showed that production of collagen I and TGF-β1 in HSC-T6 cell supernatant was increased in the VEGF group and decreased after Endostar treatment

Discussion

Hepatic fibrosis is a major complication in chronic liver disease, leading to the risk of cirrhosis and ultimately to hepatic dysfunction and hepatocellular carcinoma. Treating the cause of the liver disease may lead to fibrosis reversal.[21] However, resorting to anti-fibrotic compounds represents an important complementary approach and a major therapeutic challenge.

Angiogenesis and fibrosis are key components in development, growth, wound healing, and regeneration. As anti-angiogenic agents are likely to be delivered earlier in an increasing proportion of patients in the course of life-threatening diseases, probably in combination with additional medications, the safety of anti-angiogenic treatment is an issue of emerging importance.[22] Endostar is a new anti-angiogenic drug which is used in non-small-cell lung cancer and hepatocellular carcinoma patients.[23]

Jia *et al*. have studied the major sources of the precursor of ES–collagen XVIII (C18) in the fibrotic liver due to either CCl_4 injection or bile duct occlusion.[24] The results showed that C18 transcripts were found in hepatocytes and bile

duct epithelia of normal and fibrotic livers, and occasionally in arterial myocytes and HSCs. C18 mRNA levels remained unchanged 72 h after CCl_4 injection, while they increased in biliary fibrosis. C18 expression remained constant in acute fibrogenesis and was upregulated in biliary fibrosis. Another recent study found that C18 mRNA was increased 48 h after a single dose of CCl_4.[25] We are not aware of other studies aiming to detect the *endogenous* ES in the CCl_4-induced fibrotic animals.

A recent study has found that administration of Endostar in healthy mice (14 mg/kg or 2 mg/kg injected intraperitoneally for seven days) showed no detectable signs of injury to the myocardial, lung and kidney tissues, and no differences in the density of microvessels were noticed in these organs.[26] The aim of our study was to detect the effectiveness of Endostar on hepatic fibrosis.

In our study, we found that Endostar administration decreased the liver fibrosis scores and serum markers of liver damage (especially serum transaminases levels), indicating that Endostar exhibited both anti-fibrotic and anti-inflammatory effects *in vivo*.

HSCs are the key cells in collagen accumulation. They correspond to perisinusoidal cells in the subendothelial space between hepatocytes and sinusoidal endothelial cells.[27–29] HSC activation induces the release of VEGF and platelet-derived growth factor (PDGF), which is important for the sustained activation and proliferation of HSCs.[27,30–32] HSCs can be recognized by their vitamin A autofluorescence, perisinusoidal orientation, and expression of the cytoskeletal proteins desmin and α-SMA.[27] In our study, we showed that exogenous VEGF increased the expression of collagen I, α-SMA, and TGF-β1 in HSC-T6 cells, and that these effects could be blocked by Endostar. Therefore, HSCs might be one of the Endostar target cells.

Liver fibrosis is a wound-healing response that is characterized by increased and altered deposition of ECM in the liver.[9,28] The fibrosis process is rigorously controlled by several growth factors and cytokines.[30,33] TGF-β1, VEGF, and its receptor are among the most potent factors in liver fibrosis development.[10,18] Our results reinforce the concept that TGF-β1, VEGF, and VEGFR play a major role in the fibrotic liver. Previous results have shown that Endostar is a multiple receptor tyrosine kinase inhibitor targeting the ERK/MAPK signaling pathways, as well as the VEGF-induced tyrosine phosphorylation of KDR/Flk-1(VEGFR-2).[5] Our present results, showed that Endostar had a significant inhibitory effect *in vivo* on the expression of TGF-β1, VEGFR1, and VEGFR2 and the *in-vitro* expression of TGF-β1 and VEGFR, indicated that Endostar might play a role in counteracting liver fibrosis through the TGF-β and VEGF transduction pathways.

Duncan *et al*. also explored the role of type XVIII collagen, which is the precursor of ES, in acute liver injury.[25] The group found that ES could decrease liver inflammation and hepatocyte necrosis in the acute CCl_4-induced wild type mice liver injury model (24 days), but without similar effects in the col18a1$^{-/-}$ mice. This result was in agreement with our own findings. We also found that ES could decrease liver inflammation and hepatocyte necrosis in the CCl_4-induced liver fibrosis model (6 weeks). However, the main aim of Duncan *et al*.'s study was to show that type XVIII collagen was a functional component of the liver matrix microenvironment and was of primary importance for hepatocyte survival during injury and stress. The group did not explore the role of ES in liver fibrosis. In our study, we examined the role of ES in the CCl_4-induced liver fibrosis mice and HSCs. It should be noted that our experimental design did not allow to explore the Endostar effects on normal mouse liver.

In conclusion, although, the present study demonstrates that Endostar treatment was able to improve the liver histological changes and reduce collagen accumulation in CCl_4-induced liver fibrotic mice. Endostar had a similar inhibitory effect on collagen synthesis in HSCs *in vitro*. TGF-β and VEGFR were found to play a role in the mechanism of Endostar reduction in liver fibrosis. Endostar may pave a new road for the treatment of liver fibrosis.

Author contributions: All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript. LJK, FDL and HYW carried out the animal studies. GY, YJD, FHP, JTS, YJF, AYL and XH W carried out the multiple biochemical studies. JC, DGL and BXL performed the research, analyzed the data and wrote the manuscript. JC and DGL contributed equally.

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REFERENCES

1. Zatterstrom UK, Felbor U, Fukai N, Olsen BR. Collagen XVIII/endostatin structure and functional role in angiogenesis. *Cell Struct Funct* 2000;**25**:97–101
2. Sasaki T, Larsson H, Tisi D, Claesson-Welsh L, Hohenester E, Timpl R. Endostatins derived from collagens XV and XVIII differ in structural and binding properties, tissue distribution and anti-angiogenic activity. *J Mol Biol* 2000;**301**:1179–90
3. Seppinen L, Pihlajaniemi T. The multiple functions of collagen XVIII in development and disease. *Matrix Biol* 2011;**30**:83–92
4. Jia JD, Bauer M, Sedlaczek N, Herbst H, Ruehl M, Hahn EG, Riecken EO, Schuppan D. Modulation of collagen XVIII/endostatin expression in lobular and biliary rat liver fibrogenesis. *J Hepatol* 2001;**35**:386–91
5. Ling Y, Yang Y, Lu N, You QD, Wang S, Gao Y, Chen Y, Guo QL. Endostar, a novel recombinant human endostatin, exerts antiangiogenic effect via blocking VEGF-induced tyrosine phosphorylation of KDR/Flk-1 of endothelial cells. *Biochem Biophys Res Commun* 2007;**361**:79–84
6. Whitworth A. Endostatin: are we waiting for Godot? *J Natl Cancer Inst* 2006;**98**:731–3
7. Tong Y, Zhong K, Tian H, Gao X, Xu X, Yin X, Yao W. Characterization of a monoPEG20000-Endostar. *Int J Biol Macromol* 2010;**46**:331–6
8. Ni Q, Ji H, Zhao Z, Fan X, Xu C. Endostar, a modified endostatin inhibits non small cell lung cancer cell in vitro invasion through osteopontin-related mechanism. *Eur J Pharmacol* 2009;**614**:1–6
9. Friedman SL. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008;**134**:1655–69
10. Fernandez M, Semela D, Bruix J, Colle I, Pinzani M, Bosch J. Angiogenesis in liver disease. *J Hepatol* 2009;**50**:604–20

11. Coulon S, Heindryckx F, Geerts A, Van Steenkiste C, Colle I, Van Vlierberghe H. Angiogenesis in chronic liver disease and its complications. *Liver Int* 2011;**31**:146–62
12. Mejias M, Garcia-Pras E, Tiani C, Miquel R, Bosch J, Fernandez M. Beneficial effects of sorafenib on splanchnic, intrahepatic, and portocollateral circulations in portal hypertensive and cirrhotic rats. *Hepatology* 2009;**49**:1245–56
13. Majumder S, Piguet AC, Dufour JF, Chatterjee S. Study of the cellular mechanism of Sunitinib mediated inactivation of activated hepatic stellate cells and its implications in angiogenesis. *Eur J Pharmacol* 2013;**705**:86–95
14. Thabut D, Routray C, Lomberk G, Shergill U, Glaser K, Huebert R, Patel L, Masyuk T, Blechacz B, Vercnocke A, Ritman E, Ehman R, Urrutia R, Shah V. Complementary vascular and matrix regulatory pathways underlie the beneficial mechanism of action of sorafenib in liver fibrosis. *Hepatology* 2011;**54**:573–85
15. Friedman SL. Hepatic fibrosis - overview. *Toxicology* 2008;**254**:120–9
16. Zhao XY, Wang BE, Li XM, Wang TL. Newly proposed fibrosis staging criterion for assessing carbon tetrachloride- and albumin complex-induced liver fibrosis in rodents. *Pathol Int* 2008;**58**:580–8
17. Fan X, Zhang Q, Li S, Lv Y, Su H, Jiang H, Hao Z. Attenuation of CCl_4-induced hepatic fibrosis in mice by vaccinating against TGF-beta1. *PLoS One* 2013;**8**:e82190
18. Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Hicklin DJ, Wu Y, Yanase K, Namisaki T, Yamazaki M, Tsujinoue H, Imazu H, Masaki T, Fukui H. Vascular endothelial growth factor and receptor interaction is a prerequisite for murine hepatic fibrogenesis. *Gut* 2003;**52**:1347–54
19. Itoh S, ten Dijke P. Negative regulation of TGF-beta receptor/Smad signal transduction. *Curr Opin Cell Biol* 2007;**19**:176–84
20. Baghy K, Iozzo RV, Kovalszky I. Decorin-TGFbeta axis in hepatic fibrosis and cirrhosis. *J Histochem Cytochem* 2012;**60**:262–8
21. Sohrabpour AA, Mohamadnejad M, Malekzadeh R. Review article: the reversibility of cirrhosis. *Aliment Pharmacol Ther* 2012;**36**:824–32
22. Carmeliet P. Angiogenesis in life, disease and medicine. *Nature* 2005;**438**:932–6
23. Deng G, Zhao DL, Li GC, Yu H, Teng GJ. Combination therapy of transcatheter arterial chemoembolization and arterial administration of antiangiogenesis on VX2 liver tumor. *Cardiovasc Intervent Radiol* 2011;**34**:824–32
24. Jia JD, Bauer M, Sedlaczek N, Herbst H, Ruehl M, Hahn EG, Riecken EO, Schuppan D. Modulation of collagen XVIII/endostatin expression in lobular and biliary rat liver fibrogenesis. *J Hepatol* 2001;**35**:386–91
25. Duncan MB, Yang C, Tanjore H, Boyle PM, Keskin D, Sugimoto H, Zeisberg M, Olsen BR, Kalluri R. Type XVIII collagen is essential for survival during acute liver injury in mice. *Dis Model Mech* 2013;**6**:942–51
26. Huang R, Zhan Q, Zhou X, Chu Z, Jiang J, Liang X. Continuous administration of recombinant human endostatin (Endostar): A preclinical safety study. *Exp Ther Med* 2012;**3**:1018–22
27. Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev* 2008;**88**:125–72
28. Jiao J, Friedman SL, Aloman C. Hepatic fibrosis. *Curr Opin Gastroenterol* 2009;**25**:223–9
29. Yin C, Evason KJ, Asahina K, Stainier DY. Hepatic stellate cells in liver development, regeneration, and cancer. *J Clin Invest* 2013;**123**:1902–10
30. Moreno M, Bataller R. Cytokines and renin-angiotensin system signaling in hepatic fibrosis. *Clin Liver Dis* 2008;**12**:825–52, ix
31. Wynn TA. Cellular and molecular mechanisms of fibrosis. *J Pathol* 2008;**214**:199–210
32. Liu Y, Wen XM, Lui EL, Friedman SL, Cui W, Ho NP, Li L, Ye T, Fan ST, Zhang H. Therapeutic targeting of the PDGF and TGF-beta-signaling pathways in hepatic stellate cells by PTK787/ZK22258. *Lab Invest* 2009;**89**:1152–60
33. Parsons CJ, Takashima M, Rippe RA. Molecular mechanisms of hepatic fibrogenesis. *J Gastroenterol Hepatol* 2007;**22**:S79–84

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