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# Effects of p-CREB-1 on Transforming Growth Factor-β3 Auto-Regulation in Hepatic Stellate Cells

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## ABSTRACT

Previous studies have demonstrated that transforming growth factor-β3 (TGF-β3) protected liver against fibrosis in vivo and vitro, but its regulation is poorly understood. In addition, the cAMP-responsive element (CRE) in TGF-β3 promoter is recognized as an important regulatory site for TGF-β3 auto-regulation. Thus, we hypothesize that transcription factor CRE-binding protein-1 (CREB-1) regulates the auto-induction of TGF-β3 in hepatic stellate cells (HSCs). We used exogenous TGF-β3 to activate the signal pathway of TGF-β3 auto-regulation in HSCs, results indicated that exogenous TGF-β3 could up-regulate the protein and mRNA expressions of TGF-β3, and provoke the phosphorylation of CREB-1 on Ser-133, besides, it could induce the DNA binding activity of p-CREB-1 and activate TGF-β3 promoter as well. Additionally, we used pGenesil-1.1-shRNA-CREB-1 and pRSV-CREB-1 expression vector to silence and up-regulate CREB-1 gene expression respectively, and the results indicated that inhibition of CREB-1 suppressed exogenous TGF-β3 stimulation of TGF-β3 mRNA and protein expressions in HSCs, whereas up-regulation of CREB-1 induced this stimulation. Our results indicate that exogenous TGF-β3 up-regulates the activity of TGF-β3 promoter by activating CREB-1, then induces the mRNA and protein expressions of TGF-β3. Especially, p-CREB-1 is a critical transcription factor in mediating TGF-β3 auto-induction. J. Cell. Biochem. 112: 1046–1054, 2011. 

**KEY WORDS:** HEPATIC STELLATE CELLS; TRANSFORMING GROWTH FACTOR-β3; PHOSPHORYLATION OF cAMP-RESPONSIVE ELEMENT BINDING PROTEIN-1; TGF-β3 PROMOTER; cAMP-RESPONSIVE ELEMENT

Hepatic fibrosis is a common response to many chronic hepatic injuries, and the occurrence of that is strongly associated with an increased and altered deposition of extracellular matrix in liver. At the cellular and molecular level, this progressive process is mainly characterized by cellular activation of hepatic stellate cells (HSCs) and aberrant activity of transforming growth factor-β1 (TGF-β1) [Wallace et al., 2008; Wells, 2008]. In contrast, inhibiting HSCs activation or blocking TGF-β1 activity can reverse the fibrosis progression [Povero et al., 2010].

TGF-β super family has three homologous forms in mammals: TGF-β1, TGF-β2 and TGF-β3, which have different biological functions [Leask and Abraham, 2004]. TGF-β1 has traditionally been considered as the key fibrogenic and proliferative stimuli to HSCs, and TGF-β3 has antagonistic effect to TGF-β1. Furthermore, TGF-β3 has been implicated in wound healing, and exogenous injection of TGF-β3 into cutaneous wounds in rat reduces inflammation, scarring, and extracellular matrix generation [Shah et al., 1995; Bauer and Schuppan, 2001; Lu et al., 2005; Murata et al., 1997]. However, the function of TGF-β3 in hepatic fibrosis has been considerably less well studied. In our previous researches, we had proved that recombinant TGF-β3 obviously inhibited the mRNA and protein expressions of TGF-β1, suppressed the synthesis of collagen protein, and up-regulated the expression of matrix metalloproteinase-9 (MMP-9) in HSCs [Li et al., 2008; Zhou et al., 2008]. Besides, rAAV2-TGF-β3 reduced the histopathologic damage of liver fibrosis in rats [Zhang et al., 2010]. Although TGF-β3 has been shown to resist hepatic fibrosis in previous studies, the regulation of TGF-β3 expression was poorly understood. Therefore, it is of interest to investigate the mechanisms of TGF-β3 auto-regulation and to find a regulatory factor that could induce a high expression of TGF-β3 in HSCs.

Cyclic adenosine 3′,5′-monophosphate (cAMP)-responsive element (CRE) binding protein-1 (CREB-1) binds as a dimer to a conserved CRE site, which is found in the promoters of numerous eukaryotic genes. Phosphorylation of Ser-133 is a critical event in CREB-1 activation and induces an increase in CREB-1 transactivation potential by allowing the recruitment and binding to coactivators such as CREB-binding protein (CBP) [Johannessen et al.,

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2004]. It has been established that TGF-β3 promoter contains a CRE site, which is critical in response to TGF-β stimulation, and phosphorylation of CREB-1 (p-CREB-1) is recognized as one of transcription factor for TGF-β3 auto-regulation in rat intestinal epithelium [Lafyatis et al., 1990; Liu et al., 2006a]. Furthermore, phosphorylation of CREB-1 on Ser-133 could inhibit the proliferation of HSCs and reduce the histopathologic damage of liver fibrosis [Houglum et al., 1997; Tan et al., 2006]. Accordingly, we hypothesis p-CREB-1 is an important transcription factor for TGF-β3 auto-regulation in HSCs.

Here we used exogenous TGF-β3 (recombinant human TGF-β3) to activate the signal pathway of TGF-β3 auto-regulation in HSCs, in order to observe the spectacular changes in this signal, such as the expressions of endogenous TGF-β3 and p-CREB-1, the activity of TGF-β3 promoter, and the DNA binding activity of p-CREB-1. Besides, we used silenced or expressed vector of CREB-1 to investigate the functions of p-CREB-1 in mediating TGF-β3 auto-induction. In this manuscript, we describe a schematic model for TGF-β3 auto-regulation in HSCs, and prove that p-CREB-1 plays an important role in this auto-regulation.

## MATERIALS AND METHODS

### CELL CULTURES AND TREATMENTS

The rat HSCs cell line, which is the phenotypically activated HSC, was obtained from Hepatopathy Institute of Shanghai University of Traditional Chinese Medicine. HSCs were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in 5% $CO_2$. All experiments were conducted when cells were at an exponential stage of growth. Cells were seeded into 25 $cm^2$ plastic culture flask, 6-well plates or 24-well plates until 70–80% confluent, and treated with or without exogenous TGF-β3 (recombinant human TGF-β3). Exogenous TGF-β3 was purchased from PEPRO TECH., INC., and the purity was over 98% through the measurement of SDS–PAGE.

### CONSTRUCTION OF TGF-β3 PROMOTER LUCIFERASE REPORTER PLASMID

TGF-β3 promoter luciferase reporter plasmid (PGL3-TGF-β3-P) encodes a TGF-β3 promoter region from −221 to +110. CRE-mutated TGF-β3 promoter luciferase reporter plasmid (PGL3-TGF-β3-MP) contains a mCRE site, which was changed from (CRE site, −45 to −39) 5′-GACGTCA-3′ into 5′-GACactA-3′ [Lafyatis et al., 1990; Liu et al., 2006a]. TGF-β3 promoter sequence was synthesized according to GenBank (Gene ID: 25717) by Shanghai Generay Biotech Corporation. Synthetic TGF-β3 promoter and mCRE promoter fragments were subcloned to pGH vectors, and then were inserted into the basic pGL3 luciferase reporter vector (Promega), respectively, after digestion with *Mlu*I and *Bgl*II. The sequence of the resulting luciferase reporter plasmid was confirmed by sequencing.

### EXTRACTION OF CYTOPLASMIC AND NUCLEAR PROTEINS

HSCs were incubated in the absence or presence of exogenous TGF-β3 (10 ng/ml), and were harvested at the time which depended on different experiments. Then, cells were washed with cold PBS and disrupted in 100 μl lysis buffer I (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.2% Nonidet P-40, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1% phosphatase inhibitors mixture) for 10 min. After a 5-min centrifugation (12,000*g*), the supernatant containing cytoplasmic proteins were collected and stored at −70°C, and the pellet containing nuclei was resuspended in 20–30 μl extraction buffer II (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1% phosphatase inhibitors mixture) for 30 min. Nuclei were pelleted again by centrifugation (12,000*g*) for 15 min, and the supernatant containing nuclear proteins were collected and stored at −70°C. All manipulations were carried out at 4°C. Protein concentrations were determined using the BCA™ Protein Assay Kit (Pierce).

### TRANSIENT TRANSFECTION WITH pGENESIL-1.1-shRNA-CREB-1 AND pRSV-CREB-1 EXPRESSION VECTOR

HSCs were seeded in 6-well plates and grown to 80–90% confluence. Then, cells were transiently transfected with 2 μg of pGenesil-1.1-shRNA-CREB-1 (Wuhan GeneSil Biotechnology, China), pRSV-CREB-1 expression vector (presented by Greenberg Lab, Harvard) and control plasmid (pGenesil-1.1-shRNA-control) per well, respectively, using Lipofectmine™ 2000 (Invitrogen). Each well contained 5 μl liposome, 250 μl Opti-MEM®I Reduced Serum Medium (Gibco) and 2 ml DMEM. Five hours after transfections, culture mediums (CMs) were changed with fresh DMEM, and cells were incubated for an additional 17 h. Then, 10 ng/ml exogenous TGF-β3 was added into the treatment wells, and HSCs were incubated for 2 h continuously. Lastly, total RNA were harvested, CREB-1 mRNA, TGF-β3 mRNA and TGF-β3 protein were detected by real-time RT-PCR and ELISA described below.

### REAL-TIME RT-PCR

RNA was extracted from HSCs, which were treated with or without exogenous TGF-β3 (10 ng/ml), using TRIzol reagent (Invitrogen) according to the manufacturers' protocol. Total cDNA was prepared by RT-PCR using oligo$(dt)_{18}$ primers (Invitrogen) and extraction of RNA as the template. Real-time PCR was performed using SYBR Green I agent (Invitrogen), with the cDNA prepared above as the template. Rat specific forward and reverse primer sequences were applied for TGFβ3 (5′-TGCGCCCCCTCTACATTG-3′ and 5′-GGTTCGTGGACCCATTTCC-3′), CREB-1 (5′-CA GTGCCAACCCC-GATTTA-3′ and 5′-TTGCTCCTCCCTGGGTAATG-3′) and GAPDH (5′-GTATGACTCTACC CACGGCAAGT-3′ and 5′-TTCCCGTTGAT-GACCAGCTT-3′). All PCRs were performed under the conditions of 10 min at 95°C, followed by 40 cycles of 5 s at 95°C, and 60 s at 60°C on an ABI StepOne™ (Applied Biosystems). The reactions were analyzed in triplicate and normalized to GAPDH. Expression level was calculated by $2^{-\Delta\Delta CT}$ method.

### WESTERN BLOTTING

Proteins (30–60 μg), collected as described above, were separated by 12% SDS–PAGE and subsequently transferred to NC membrane. Then, the membranes were blocked with 5% non-fat milk for 1 h and incubated individually with primary antibody of phospho-CREB-1 (1:1,000, dilution; Cell Signaling Technology), CREB-1 (1:1,000, dilution; Cell Signaling Technology), TGF-β3 (1:250, dilution; R&D

Systems) or GAPDH (1:500, dilution; BOSTER, China) overnight at 4°C. After that, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:8,000, dilution; BOSTER) for 1 h at room temperature. Target proteins were detected by enhanced chemiluminescence (ECL) detection kit (Thermo Scientific). The primary antibody of p-CREB-1 detects endogenous levels of CREB-1 only when phosphorylated at Ser-133.

### LUCIFERASE REPORTER ASSAYS

HSCs were seeded in 24-well plates and grown to 75% confluence. Cells were transiently transfected with 0.8 μg of PGL3-TGF-β3-P, 0.8 μg of PGL3-TGF-β3-MP and 0.02 μg of Renilla luciferase control reporter (pRL-SV40) per well, respectively, using Lipofectmine™ 2000. Twenty hours after transfections, treatment groups were exposed to exogenous TGF-β3 (10 ng/ml) for 6, 12, 24, and 48 h, while control group was not treated. Then HSCs were harvested and dual-luciferase reporter assay was conducted using 20 ml cell extract, 100 ml Luciferase Assay Reagent II (LAR II) and 100 ml Stop & Glo™ Reagent (Promega). Transfection efficiencies were determined by co-transfecting with Renilla luciferase.

### ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear proteins from HSCs were prepared as described above. Double-stranded oligonucleotide probes used in the EMSA assay (5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′) were labeled with biotin, and the CRE consensus binding motif was GACGTCA. Ten to 20 μg of nuclear extract was incubated with 10× binding buffer (100 mM Tris, 500 mM KCl, 10 mM dithiothreitol, pH 7.5), 1 μg/μl poly (dI–dC), 50% glycerol, 100 mM $MgCl_2$, 1% Nonidet-P-40 and 50 fM biotin-CRE probe for 30–60 min at room temperature. Unlabeled CRE probe was carried out with a 200-fold molar excess of the biotin-CRE probe. Nuclear extracts-DNA complexes were resolved in 6% polyacrylamide gels using 0.5% TBE, and transferred electrophoretically to nylon membrane. Then the biotin-labeled DNA was detected by chemiluminescence. Densitometry analyses were performed to confirm the significance of the differences. EMSA kit was purchased from Thermo Scientific and CRE probes were ordered form Invitrogen.

### EUZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

HSCs were incubated in the absence or presence of TGF-β3 (10 ng/ml) and CMs were collected at the time points indicated in figures. In addition, we changed the CMs with fresh DMEM to remove the exogenous TGF-β3. Quantitative determination of endogenous TGF-β3 was detected by a semi-product ELISA kit (R&D Systems) according to the manufacturer's protocol. Briefly, 96-well microtiter plate (Greiner, Germany) was coated with 4 μg/ml of cell wall capture antibody overnight. One hundred microliters sample was pipetted into wells and incubated for 1 h. After a subsequent washing, 100 μl 0.2 μg/ml of biotinylated detection antibody was added and incubated for an additional 1 h. Repeated the wash as above operation, added substrate solution and incubated for 20 min. Then, reaction was stopped and extinction values were read at 450 nm. All manipulations were carried out at room temperature. Continuous determination of antibody activity was done by using the provided standard curve.

### STATISTICAL ANALYSIS

Data were analyzed and statistically significant differences were determined using the one-way analysis of variance (ANOVA) by SPSS 13.0 (SPSS, Inc., USA). All values were expressed as mean ± standard deviation (SD). Differences with a $P < 0.05$ were considered statistically significant.

## RESULTS

### EXOGENOUS TGF-β3 UP-REGULATES THE EXPRESSION OF ENDOGENOUS TGF-β3

We have demonstrated previously that recombinant TGF-β3 resists the progression of hepatic fibrosis [Li et al., 2008; Zhou et al., 2008; Zhang et al., 2010]. Accordingly, it is of interest to study how to induce the autocrine of TGF-β3. Here we used recombinant human TGF-β3 as exogenous TGF-β3 to activate the signal pathway of TGF-β3 auto-induction in HSCs. There are some differences between human TGF-β3 and rat TGF-β3, but they have the same biological function. Additionally, it has been reported that human recombinant TGF-β3 reduces scar formation in an incised and sutured mouse lip in vivo, and induces the expression of endogenous TGF-β3 [Hosokawa et al., 2003].

In order to find a concentration that could induce the TGF-β3 autocrine effectively, we examined the expression of endogenous TGF-β3 in response to different concentrations of exogenous TGF-β3. As shown in Figure 1A, there was a dose-dependent increase of endogenous TGF-β3 following treatment of exogenous TGF-β3 in HSCs, and the most effective concentration of exogenous TGF-β3 was at 10 ng/ml, and the secretion of endogenous TGF-β3 at that concentration was sixfold higher (0.98 ± 0.09 ng/ml, $P < 0.05$) compared to control. Therefore, we chose the 10 ng/ml as the inducing concentration.

To determine whether exogenous TGF-β3 continually increased the expression of endogenous TGF-β3, we examined the endogenous TGF-β3 secretion in response to 10 ng/ml exogenous TGF-β3 at different times. Most unexpectedly, exogenous TGF-β3 up-regulated the secretion of endogenous TGF-β3 rapidly, but did not increase it in a time-dependent manner or a sustainable situation. As shown in Figure 1B, exogenous TGF-β3 increased the secretion of total TGF-β3 (exogenous TGF-β3 plus endogenous TGF-β3) at 2 h, maximized at 4 h (18.93 ± 2.90 ng/ml, 1.9-fold higher compared to control, $P < 0.05$), descended at 8 h and returned to basal level in 24 h. Similarly, exogenous TGF-β3 increased the secretion of endogenous TGF-β3 at 15 min after changing CM, peak at 1 h (0.84 ± 0.03 ng/ml, 32.4-fold higher than control group, $P < 0.05$), decreased steadily out to 10 h (0.22 ± 0.06 ng/ml, 8.5-fold higher than control group, $P < 0.05$) (Fig. 1C).

We also examined the expression of TGF-β3 protein in cytoplasm in response to exogenous TGF-β3. As shown in Figure 1D, exogenous TGF-β3 had no effect on the protein secretion of TGF-β3 in cytoplasm ($P > 0.05$).

### EXOGENOUS TGF-β3 UP-REGULATES THE EXPRESSION OF TGF-β3 mRNA

As we have demonstrated that exogenous TGF-β3 up-regulated endogenous TGF-β3 secretion in HSCs, it is necessary to study the

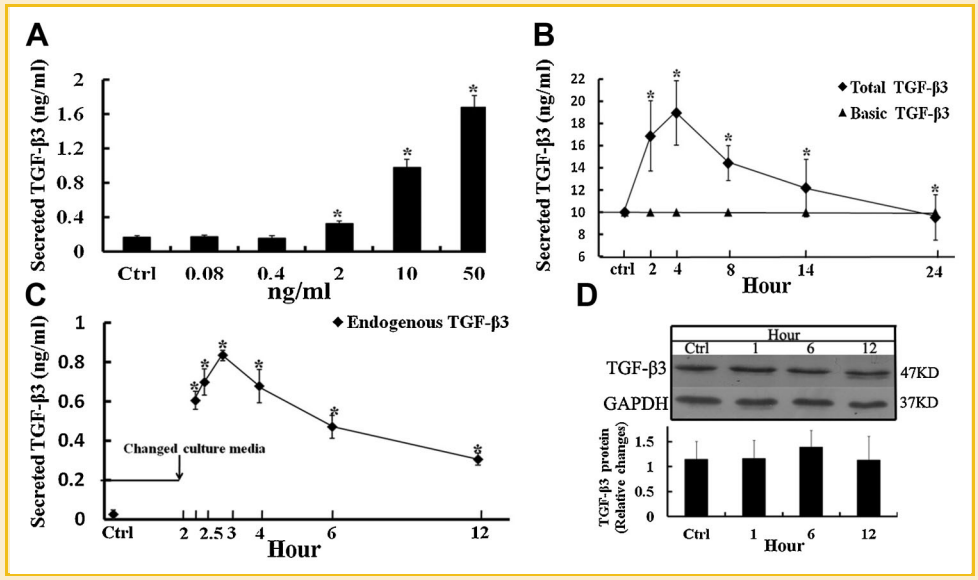


Fig. 1. The expression of endogenous TGF-β3 was up-regulated by exogenous TGF-β3. A: HSCs were seeded in 24-well plates and grown to 70–80% confluence. Cells were incubated in different concentrations of exogenous TGF-β3 at 0, 0.08, 0.4, 2, 10, and 50 ng/ml. Each concentration of exogenous TGF-β3 group had a parallel contrast, which just has CMs with different concentrations of exogenous TGF-β3, but without HSCs. Two hours after treatment, CMs were changed with DMEM, then HSCs and parallel contrasts were incubated for an additional 1 h. After that, CMs were harvested and TGF-β3 protein was detected by ELISA. The value of each treatment group minus the value of corresponding parallel contrast group represented the production of endogenous TGF-β3. Data are plotted as mean ± SE of triplicate samples for each of three independent experiments. B: HSCs were incubated in the absence or presence of exogenous TGF-β3 (10 ng/ml), then CMs were collected at the time points indicated, and total TGF-β3 (exogenous TGF-β3 plus endogenous TGF-β3) was detected by ELISA. Data are plotted as mean ± SE of triplicate samples for each of three independent experiments. C. HSCs were incubated in the absence or presence of exogenous TGF-β3 (10 ng/ml) for 2 h, then CMs were changed with DMEM and HSCs were incubated continuously. CMs were collected at the time points indicated, and TGF-β3 (endogenous TGF-β3) was detected by ELISA. Data are plotted as mean ± SE of triplicate samples for each of three independent experiments. D: HSCs were incubated in the absence or presence of exogenous TGF-β3 (10 ng/ml), cytoplasm proteins were harvested at the time points indicated, and production of TGF-β3 was detected by Western blotting. Three independent experiments were performed, and representative figure is shown. The densitometric data are plotted below the representative figure. Asterisks indicate a statistically significant difference ($P < 0.05$) in the fold changes between treatment groups and control group.

effect of exogenous TGF-β3 on the expression of TGF-β3 mRNA. As shown in Figure 2, 2 h after treatment, maximal value of TGF-β3 mRNA expression (3.76 ± 0.67, 3.1-fold higher than control group, $P < 0.05$) was observed, then expression levels decreased and maintained from 4 to 24 h, and there were still significantly statistical differences between treatment groups and control group ($P < 0.05$).

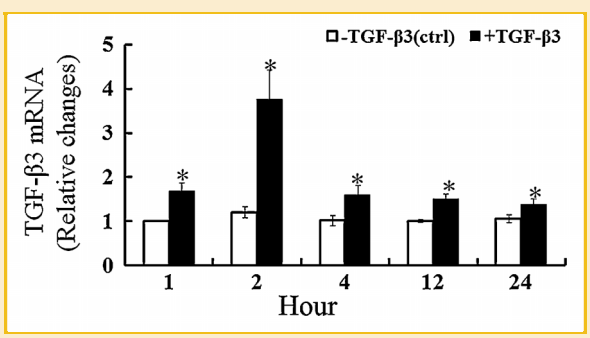


Fig. 2. The expression of TGF-β3 mRNA was up-regulated by exogenous TGF-β3. HSCs were incubated in the absence or presence of exogenous TGF-β3 (10 ng/ml), and total RNA were collected at the time points indicated, then TGF-β3 mRNA was detected by real-time Quantitative RT-PCR. Data are plotted as mean ± SE of triplicate samples for each of three independent experiments. Numbers on top of each bar indicate the fold increase compared with control. Asterisks indicate a statistically significant difference ($P < 0.05$) in the fold changes between treatment groups and control group.

## CRE SITE IS REQUISITE FOR EXOGENOUS TGF-β3 INDUCTION OF TGF-β3 PROMOTER ACTIVITY

It has been reported that a sequence from −490 to +100 in the 5′-flanking region of the TGF-β3 gene, which contains two Sp-1 sites, an AP-2 site and a CRE site, might be important in regulating TGF-β3 expression [Lafyatis et al., 1990]. Although previous report has suggested that CRE site, but not Sp-1, SBE and AP-2 sites, in the TGF-β3 promoter region is important to mediate the TGF-β3 gene transcription in rat intestinal epithelium [Liu et al., 2006a], there is no definitive role of this CRE site in mediating TGF-β3 gene transcription in HSCs. To determine whether the CRE site in the TGF-β3 promoter region was requisite for exogenous TGF-β3 induction of TGF-β3 promoter activity in HSCs, we constructed PGL3-TGF-β3-P and PGL3-TGF-β3-MP, which were transiently transfected into HSCs. Our results indicated that exogenous TGF-β3 induced the activity of PGL3-TGF-β3-P at 6 h, peaked at 24 h (10.68 ± 0.57, 2.2-fold higher than control group, $P < 0.05$), and decreased at 48 h (Fig. 3A). Furthermore, as showed in Figure 3B, the promoter activity of PGL3-TGF-β3-MP containing the mutational CRE site was completely blocked in the presence of exogenous TGF-β3, and there was no significantly statistical difference between treatment group and control group ($P > 0.05$). Additionally, TGF-β3 promoter

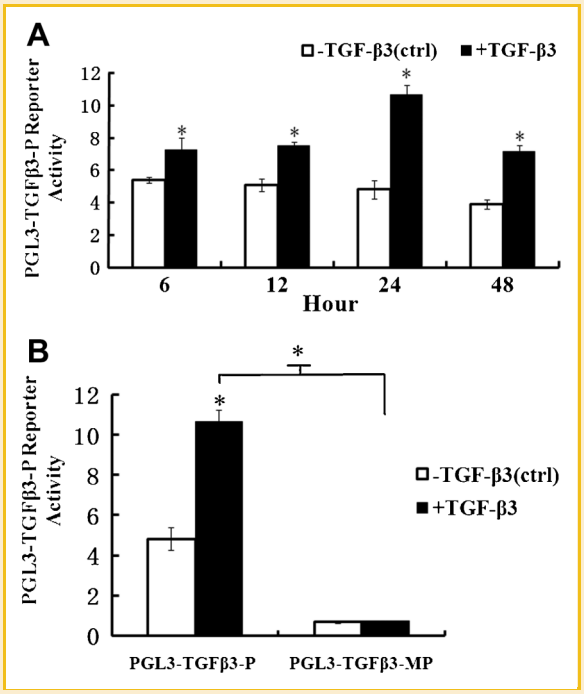


Fig. 3. Exogenous TGF-β3 increases PGL3-TGF-β3-P activity and the CRE site in the TGF-β3 promoter region is requisite for TGF-β3 gene transcription. A: HSCs were seeded in 24-well plates and grown to 75% confluence. Cells were individually transfected with 0.8 μg of PGL3-TGF-β3-P and 0.02 μg of Renilla luciferase control reporter (pRL-SV40) per well. Twenty hours after transfection, cells were incubated in the absence or presence of TGF-β3 (10 ng/ml) for 6, 12, 24, and 48 h. Then cells were harvested, and luciferase assays were performed as described under the Materials and Methods Section. Data plotted are the mean ± SE of triplicate samples from one of three independent experiments. Asterisks indicate a statistically significant difference ($P < 0.05$) in the fold changes between treatment groups and control group. B: HSCs were individually transfected with 0.8 μg of PGL3-TGF-β3-P, 0.8 μg of PGL3-TGF-β3-MP and 0.02 μg of Renilla luciferase control reporter (pRL-SV40) per well. Twenty hours after transfection, cells were incubated in the absence or presence of TGF-β3 (10 ng/ml) for 24 h. Then, cells were harvested, and luciferase assays were performed as described under the Materials and Methods Section. Data plotted are the mean ± SE of triplicate samples from one of three independent experiments. Asterisks indicate a statistically significant difference ($P < 0.05$) in the fold changes between PGL3-TGF-β3-P transfection group and (control group or PGL3-TGF-β3-MP transfection group).

activity was decreased by 85% compared with PGL3-TGF-β3-P, when the CRE site was mutated.

## EXOGENOUS TGF-β3 ACTIVATES CREB-1

It is known that phosphorylation of CREB-1 can recognize and bind to CRE consensus site in various gene promoters [Hosokawa et al., 2003], and the results described above demonstrated that the CRE site was critical in TGF-β3 promoter for TGF-β3 auto-induction. Accordingly, we conjectured whether exogenous TGF-β3 activated CREB-1 in HSCs, which was phosphorylated at Ser-133. To determine whether exogenous TGF-β3 induce the transcription activity of p-CREB-1 in HSCs, we examined the DNA binding activity of it in response to exogenous TGF-β3. As shown in Figure 4A, CRE probe showed a good specificity in binding with p-CREB-1, and 200-fold molar excess of unlabelled CRE probe blocked the binding of bio-CRE probe with p-CREB-1 completely. Besides, exogenous TGF-β3 significantly increased the complex formation at CRE site in a time-dependent manner, with a peak level occurring at 1 h (14.02 ± 0.83, 2.4-fold higher than control group, $P < 0.05$) after TGF-β3 treatment, and maintained for the next 11 h (Fig. 4B). Furthermore, phosphorylation levels of CREB-1 in HSCs stimulated by exogenous TGF-β3 for 0–6 h were detected by Western blot analysis with an antibody specific for the activated form of p-CREB-1 at Ser-133. Results showed that exogenous TGF-β3 increased CREB-1 phosphorylation at 15 min, maximized at 1 h, and decreased steadily out to 6 h, while CREB-1 protein levels remained unchanged in all time points, and the relative value of p-CREB-1/CREB-1 at the peak time was 2.9-fold greater compared to control (Fig. 4C). Also, we examined the expression of CREB-1 mRNA by exogenous TGF-β3 stimulation in HSCs, and results suggested that there were no significantly statistical differences between the groups treated with exogenous TGF-β3 and the control group ($P > 0.05$).

## PHOSPHORYLATION OF CREB-1 IS AN IMPORTANT TRANSCRIPTION FACTOR IN MEDIATING TGF-β3 AUTO-INDUCTION

Although we have demonstrated that p-CREB-1 was involved in exogenous TGF-β3 induction of TGF-β3 promoter binding and transcription activities, it is of interest to examine whether CREB-1 was necessary in mediating TGF-β3 auto-induction. In order to investigate this supposition, pGenesil-1.1-shRNA-CREB-1 was used to silence CREB-1 gene expression via RNA interference, while pRSV-CREB-1 expression vector was used to induce CREB-1 expression via gene translation. As shown in Figure 5A, results indicated that the expression of CREB-1 mRNA in HSCs transfected with pGenesil-1.1-shRNA-CREB-1 were significantly suppressed compared to the cells transfected with pGenesil-1.1-shRNA-control (0.54 ± 0.07, $P < 0.05$), while the mRNA expression of CREB-1 in HSCs transfected with pRSV-CREB-1 expression vector were excessively up-regulated compared to the cells transfected with pGenesil-1.1-shRNA-control (610.30 ± 131.87, $P < 0.05$). In addition, as shown in Figure 5B,C, exogenous TGF-β3 induced a 5.1- and a 9.1-fold increase in the mRNA and protein expressions of TGF-β3 in control group respectively, and pGenesil-1.1-shRNA-control had no effect on this stimuli ($P > 0.05$). Besides, pGenesil-1.1-shRNA-CREB-1 individually decreased the exogenous TGF-β3 induction of the mRNA and protein expressions of TGF-β3 to levels of only 2.6- and 4.5-fold, statistically significant decrease compared with that for pGenesil-1.1-shRNA-control ($P < 0.05$), while pRSV-CREB-1 expression vector individually induced a 7.4- and a 12.8-fold increase of the mRNA and protein expressions of TGF-β3 compared to pGenesil-1.1-shRNA-control ($P < 0.05$).

## SCHEMATIC MODEL FOR TGF-β3 AUTO-REGULATION IN HSCs

TGF-β initiates its signals by binding and activating a tetrameric receptor complex (Type I and II serine/threonine kinase receptors). The active receptor complex then transduces signals to downstream cellular transcription factors, which bind with special DNA site and activate TGF-β promoter, and induce the expression of TGF-β [Piek et al., 1999]. In this study, we described the signal transduction pathway that mediates TGF-β3 auto-regulation upon the results in

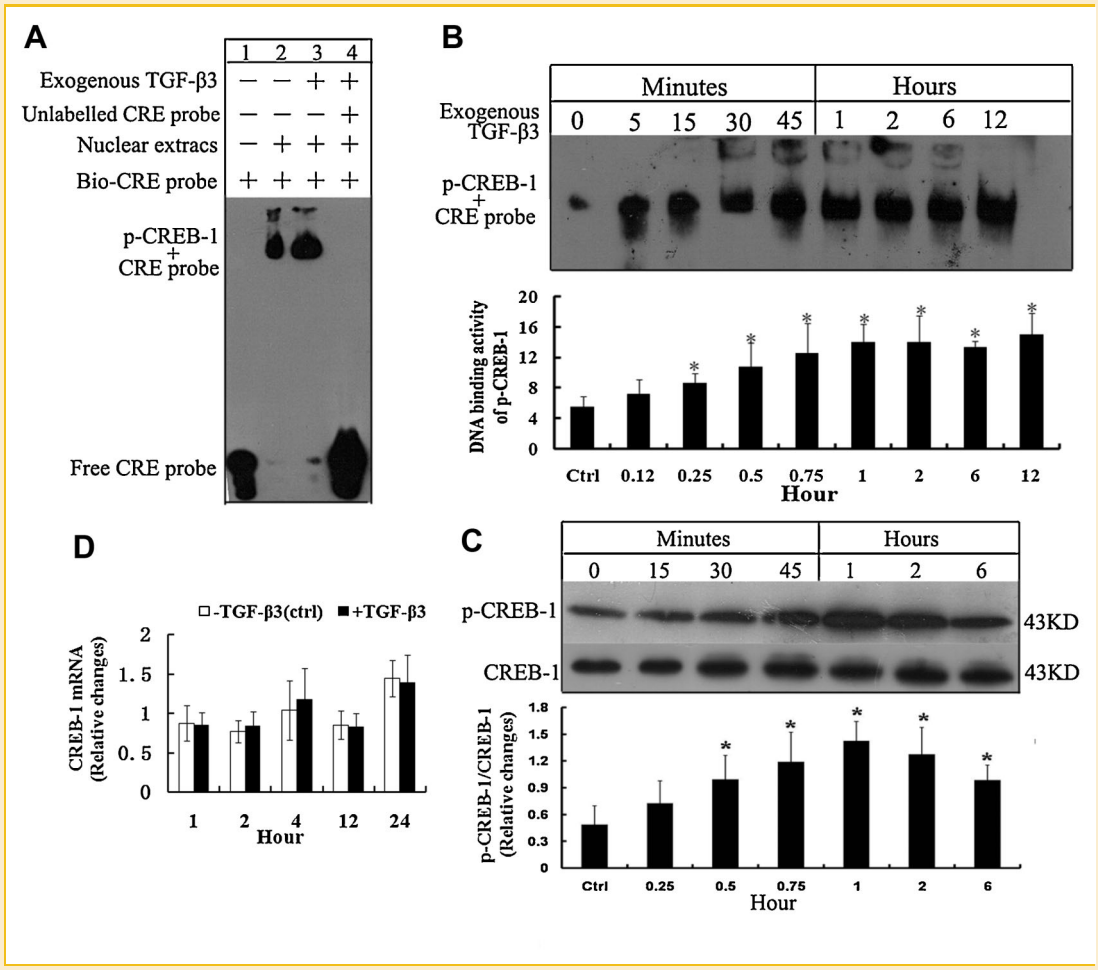


Fig. 4. CREB-1 was activated by exogenous TGF-β3. A: HSCs were incubated in the absence or presence of TGF-β3 (10 ng/ml) for 1 h, then nuclear proteins were harvested and DNA binding activity of p-CREB-1 was detected by EMSA. A 200-fold excess of unlabeled CRE probe (cold probe) was used to compete with bio-CRE probe (lane 4). Three independent experiments were performed, and representative figure is shown. B: HSCs were incubated in the absence or presence of TGF-β3 (10 ng/ml). Nuclear proteins were harvested at the time points indicated, and DNA binding activity of p-CREB-1 was detected by EMSA. Three independent experiments were performed, and representative figure is shown. The DNA binding activity of p-CREB-1 is shown as histogram below the representative figure. C: HSCs were incubated in the absence or presence of TGF-β3 (10 ng/ml). Nuclear proteins were harvested at the time points indicated, and production of p-CREB-1 and CREB-1 were detected by Western blotting. Three independent experiments were performed, and representative figure is shown. The densitometric data are plotted below the representative figure. D: HSCs were incubated in the absence or presence of exogenous TGF-β3 (10 ng/ml), the total RNA were collected at the time points indicated, and CREB-1 mRNA was detected by real-time quantitative RT-PCR. Data are plotted as mean ± SE of triplicate samples for each of three independent experiments. Asterisks indicate a statistically significant difference ($P < 0.05$) in the fold changes between treatment groups and control group.

the preceding figures. As shown in Figure 6, CREB-1 is phosphorylated at Ser-133 by exogenous TGF-β3, then p-CREB-1 binds at the CRE site in TGF-β3 promoter, activates TGF-β3 promoter and up-regulates the mRNA and protein expression of TGF-β3. Overall, phosphorylation of CREB-1 is an important transcription factor in mediating TGF-β3 auto-induction.

## DISCUSSION

As a member of TGF-β super family, TGF-β3 is involved in cell differentiation, embryogenesis and development. It has been reported that TGF-β3 promotes scarless repair of cleft lip in mouse fetuses, and inhibits fibrosis in vivo under certain experimental conditions such as pancreatic fibrosis and wound healing [Hakvoort et al., 2000; Kohama et al., 2002; Hosokawa et al., 2003]. Furthermore, our previous results indicate that TGF-β3 has the ability to inhibit hepatic fibrosis in vivo and vitro, but the regulation of TGF-β3 is poorly understood in hepatic fibrosis. In this work, we confirmed that TGF-β3 auto-regulation was mediated by the CRE site in TGF-β3 promoter and that p-CREB-1 was an important transcription factor in mediating TGF-β3 auto-induction. Besides, we described a schematic model for TGF-β3 auto-regulation in HSCs.

In this study, we found that a high expression of endogenous TGF-β3 in extracellular fluid was significantly increased by exogenous TGF-β3, while no significant increase happened in cytoplasm, and these data indicated that TGF-β3 is an autocrine

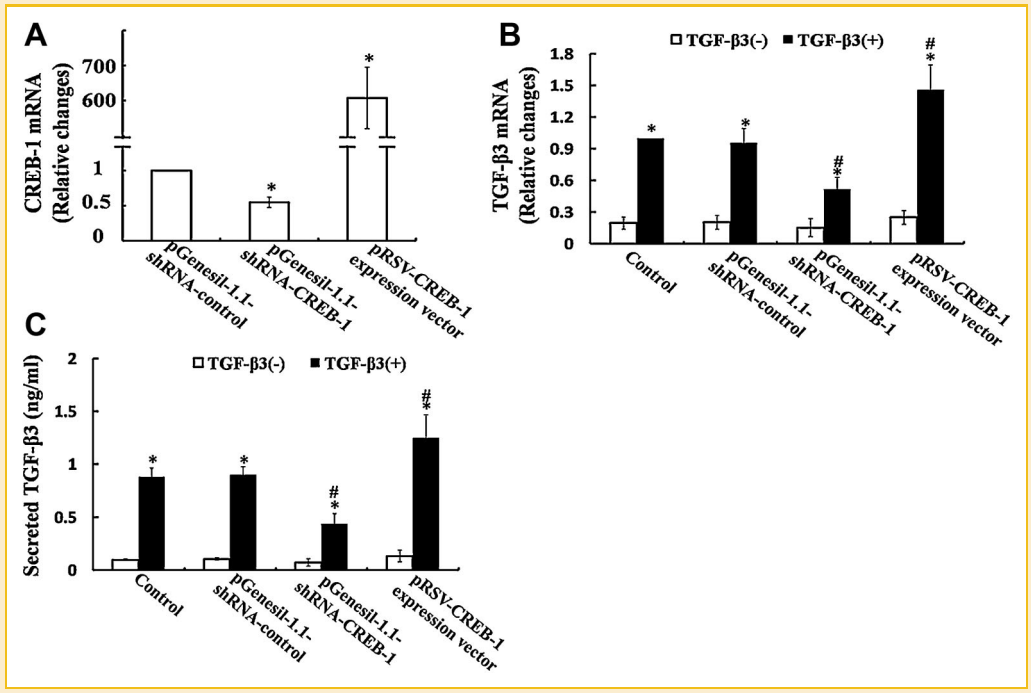


Fig. 5. Phosphorylation of CREB-1 is an important transcription factor in mediating TGF-β3 auto-induction. HSCs were seeded in 6-well plates and grown to 70–80% confluence. Then, cells were transiently transfected with 2 μg of control plasmid (pGenesil-1.1-shRNA-control), 2 μg of pGenesil-1.1-shRNA-CREB-1 and pRSV-CREB-1 expression vector per well, respectively. A: Twenty-four hours after transfection, total RNA were harvested and prepared. CREB-1 mRNA was detected by real-time RT-PCR. B: Twenty-two hours after transfection, added 10 ng/ml exogenous TGF-β3 to the treatment wells and HSCs were incubated for 2 h continuously. TGF-β3 mRNA was detected by real-time RT-PCR. C: Twenty-two hours after transfection, added 10 ng/ml exogenous TGF-β3 to the treatment wells and HSCs were incubated for 2 h. Then, CMs were changed with DMEM and HSCs were incubated continuously for 1 h, CMs were collected and TGF-β3 protein was detected by ELISA. Data plotted are the mean ± SE of triplicate samples from one of three independent experiments. *Significantly different from the control, and # significantly different from the pGenesil-1.1-shRNA-control ($P < 0.05$).

protein. Theoretically, administration of an exogenous protein inhibits the endogenous expression [Langlois et al., 1998], but our studies obtained the opposite result in HSCs, thereby we conjecture (1) that exogenous TGF-β3 is to activate the signal pathway of TGF-β3 and (2) that there are bidirectional regulations of TGF-β3 across the cell membrane of HSCs, shown a synergetic effect on exogenous and endogenous protein. Furthermore, the high expression of endogenous TGF-β3 protein and mRNA could not maintain following the peak time, and reduced slowly until the production kept at a weak level. An interesting report demonstrated that TGF-β mRNA was down-regulated by the activation of the spontaneously secreted TGF-β, and that TGF-β3 gene is most sensitive to repression by TGF-β [Taimor et al., 1999]. In keeping with these findings, and our results herein, it is conceivable that endogenous TGF-β3 released from HSCs and activated in the extra-cellular medium, exerts a negative feedback on TGF-β3 mRNA expression within the HSCs, then accompanied by decrease of TGF-β3 protein. These results demonstrate that exogenous TGF-β3 activates the TGF-β3 signal pathway and induces TGF-β3 expression rapidly, but there are some factors which restrict the expression of endogenous TGF-β3.

To study the regulation of TGF-β3 in HSCs, we examined the TGF-β3 promoter activity in response to exogenous TGF-β3, and found that exogenous TGF-β3 enormously induced the promoter activity. Interestingly, the activity of TGF-β3 promoter was completely abolished by mutation of CRE site. It has been recognized that CRE is found in the promoters of numerous eukaryotic genes, and CREB-1 binds with it in order to regulate the activity of those promoters [Montminy, 1997; Goren et al., 2001]. The data indicates that CRE site in TGF-β3 promoter region is requisite for TGF-β3 auto-induction in HSCs.

It has been reported that phosphorylation of CREB-1 on Ser-133 is a key event in the regulation of CREB-1 mediated transcription, and PKA plays an essential role on stellate cell activation through the induction of CREB-1 phosphorylation on Ser-133, which indicated that CREB-1$^{pSer133}$ might be indispensable for the quiescent stellate cell phenotype [Yamamoto et al., 1988; Houglum et al., 1997; Servillo et al., 2002]. Furthermore, Kathleen M. Mulder has convinced that CREB-1 and Smad3, as well as JNKs and p38, but not PKA, ERKs, or Smad4, were the critical activators of TGF-β3 expression, and CREB-1 is phosphorylated at Ser-133 by an activated JNK and p38 [Liu et al., 2006]. Generally, Smad3 is recognized as a key factor in TGF-β1 signal pathway and binds to Smad binding element (SBE) in the promoters of numerous eukaryotic, while p-CREB-1 is an important transcription factor involved in fibrosis, such as corneal wound-healing process, pulmonary fibrosis, cardiac fibroblasts, and hepatic fibrosis [Houglum et al., 1997; Liu et al., 2005, 2006b; Zhao et al., 2006; Tan et al., 2006; Xing and Bonanno, 2009]. Since our previous data indicate that TGF-β3 resists hepatic fibrosis in vivo and vitro, we

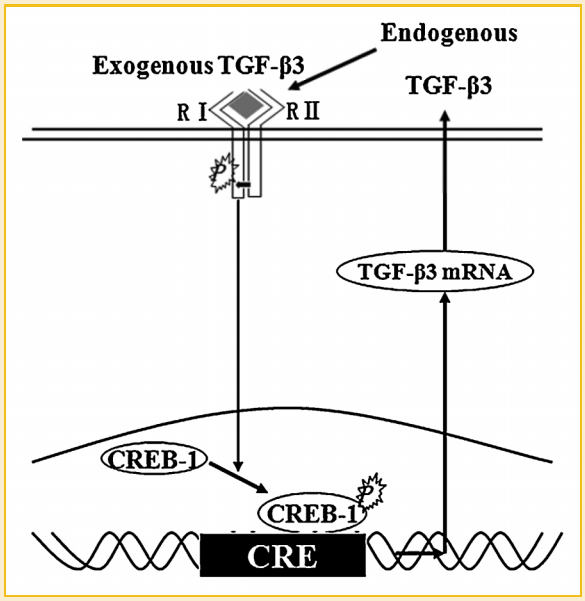


Fig. 6. Schematic model for TGF-β3 auto-regulation in HSCs.

focus on the relationship between p-CREB-1 and TGF-β3. We found that exogenous TGF-β3 phosphorylated CREB-1 at Ser-133, and increased the DNA binding activity of p-CREB-1 in a time-dependent manner, but had no effect on the mRNA and protein expressions of CREB-1. Strangely, the expression of p-CREB-1 decreased after 1 h in response to TGF-β3. Maybe this decrease is associated with phosphatase, which attenuates p-CREB-1 activity by dephosphorylation. The data suggest that p-CREB-1 is involved in TGF-β3 auto-regulation.

To confirm that p-CREB-1 is a critical transcription factor in mediating TGF-β3 auto-regulation, we constructed shRNA-CREB-1 and pRSV-CREB-1 vectors. Results shown that pGenesil-1.1-shRNA-CREB-1 decreased half of the mRNA and protein expressions of TGF-β3 by exogenous TGF-β3 stimulation, while pRSV-CREB-1 expression vector induced a 1.5- and a 1.4-fold increase of the mRNA and protein expressions of TGF-β3. However, pGenesil-1.1-shRNA-CREB-1 could not inhibit the production of TGF-β3 completely, and the siRNA-resistant CREB-1 has still effect on TGF-β3 secretion. These findings suggest that p-CREB-1 is a critical factor of TGF-β3 expression.

It should be pointed out that TGF-β3 suppresses synthesis of collagen protein and reverses fibrosis progression by inhibiting the expression of TGF-β1 in hepatic fibrosis [Li et al., 2008; Zhou et al., 2008; Zhang et al., 2010]. Interestingly, phosphorylation of CREB-1 at Ser-133 allows recruitment of the CREB-binding protein (CBP) or its paralogue p300, which causes Smad3/4–p300/CBP complex disruption and transcriptional suppression of TGF-β1 function [Liang et al., 2008]. In addition, p-CREB-1 binds to CRE site and cooperates with BMP-stimulated Smad signaling to enhance promoter transcription and protein expression of Smad6, which has been documented to play a key role in inhibiting signal transduction of TGF-β1 [Ionescu et al., 2004; Park, 2005]. In our report, p-CREB-1 is an important transcription factor in mediating TGF-β3 auto-regulation, thus our findings suggest that TGF-β3 may inhibit TGF-β1-inducible hepatic fibrosis by activating CREB-1.

In summary, we provide evidences that exogenous TGF-β3 up-regulates the expression of endogenous TGF-β3 protein, TGF-β3 mRNA, p-CREB-1, as well as the activity of TGF-β3 promoter and the DNA binding activity of p-CREB-1, and we described a schematic model that expounded some known biological functions of TGF-β3 in HSCs. Besides, we also demonstrate p-CREB-1 is a critical transcription factor in mediating TGF-β3 auto-induction. What is more, TGF-β3 may suppress TGF-β1 biological function by activating CREB-1, which guiding our future research. Our studies provide important insights into the molecular mechanisms modulating hepatic fibrosis.

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