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Mechanism of the transforming growth factor-β induction of fibronectin expression in hepatic stem-like cells

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Abstract

Transforming growth factor-β1 (TGF-β1) plays an important role in the fibrogenic process in the liver. The aim of the present study was to explore the action of TGF-β1 on fibronectin expression in rat hepatic stem-like cells and the underlying mechanisms. The level of fibronectin expression was determined in hepatic stem-like cells (WB cells) before and after TGF-β1 stimulation by RT-PCR and Western blot methods. Using immunogold transmission electron microscopy and the Western blot method, we observed the result of the expression and the distribution of cAMP, phosphorylated Smad3 and Smad7 before and after TGF-β1 treatment. The levels of fibronectin expression in both mRNA and protein increased 4- to 5-fold after TGF-β1 stimulation, reaching an optimum level after 8 h and then gradually falling back. Similarly, TGF-β1 stimulation resulted in an increase of cAMP in WB cells, peaking at 8 h. After treatment with TGF-β1 for 24 h, the expression of cAMP gradually decreased. In addition, we found that TGF-β1 treatment also contributed to the increased expression and to changes in cellular distribution of phosphorylated Smad3 (translocation from the cytoplasm to the nucleus) and Smad7 (translocation from the nucleus to the cytoplasm) in WB cells. The present study demonstrates that TGF-β is involved in the fibrogenic process in hepatic stem cells through up-regulation of fibronectin expression, and the mechanisms underlying this process may be associated with the activation of cAMP and Smad pathways.

Key words: TGF-β; Fibronectin; Fibrogenesis; Hepatic stem cell; cAMP; Smad

Introduction

The remarkable regenerative capacity of the human liver is usually provided by the presence of hepatocytes and cholangiocytes (1). Sometimes, when the ability of hepatocytes to divide and replace damaged tissue is compromised, bipotent progenitor cells will be activated, giving rise to both new hepatocytes and biliary epithelial cells.

While the function and nomenclature of stem cells located in the liver is still an area of dispute, it is nevertheless generally agreed that liver oval cells are 'stem-like' cells descending from a population of progenitor cells involved in liver regeneration (2,3). More importantly, recent reports hint that hepatic stellate cell (HSC) activation may contribute to hepatic stem cell behavior (4). Thus, 'stem-like' cells in the liver may play a significant role in hepatic fibrogenesis.

It is firmly established that the fibrogenic process in the liver is prominently regulated by transforming growth factor-β1 (TGF-β1) (5). TGF-β1 signaling is initiated following ligand binding to the TGF-β type II receptor, TβR-II. This allows the recruitment of the TGF-β type I receptor, TβR-I, into a heteromeric complex, resulting in transphosphorylation of TβR-I by TβR-II. Following phosphorylation of Smad2 or Smad3 by the activated TβR-I, a heteromeric complex is formed with Smad4, resulting in translocation of the complex to the nucleus where the complex can directly or indirectly, by interactions with other transcription factors, regulate the involvement of gene transcription in fibrosis (5-7). Besides HSC, which are induced by TGF-β to transdifferentiate to myofibroblasts and to synthesize an array of extracellular matrix molecules such as fibronectin (FN), hepatocytes are also increasingly recognized as an important cell type involved in the TGF-β-regulated fibrogenic process (6,7). However, it is still unclear how TGF-β

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regulates the fibrogenic process in hepatic stem cells. The rat liver epithelial stem-like WB-F344 cell line, isolated from the liver of an adult male Fischer 344 rat, is considered to be an *in vitro* model of bipotent oval cells since it shares their phenotype (8,9). In the present study, using the WB-F344 cell line, we demonstrate that TGF-β is involved in the fibrogenic process by regulating FN in hepatic stem cells, and show that the cAMP and Smad pathways may be required for this process.

Material and Methods

Cell culture

The rat hepatic stem-like cell line WB-F344 was generously provided by Dr. Han Rui (Chinese Academy of Medical Sciences, Beijing) and maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc., USA) supplemented with 10% FCS, 200 mM glutamine, and 0.05% gentamicin.

Western blot analysis

Cells were treated with 5 ng/mL TGF-β1 for various times (0, 2, 4, 8, 12, and 24 h) followed by incubation in lysis buffer on ice for 30 min. Denatured proteins were then separated by SDS-PAGE, transferred to PVDF membranes, and incubated at room temperature for 1 h or at 4°C overnight with a rabbit-anti-rat FN antibody (1:100; Santa Cruz Biotechnology, USA), or a rabbit monoclonal anti-rat P-Smad3 antibody (1:500; Santa Cruz Biotechnology), or a rabbit monoclonal anti-rat Smad7 antibody (1:500; Santa Cruz Biotechnology). After washing in Tris-buffered saline with 0.05% Tween 20 (TBS-Tween), the blots were reacted with alkaline phosphorylated-linked goat anti-rabbit IgG (1:500) at room temperature for 2 h. Pre-stained molecular mass was used to estimate the position of various proteins on the gel. Blots were stripped and incubated with a monoclonal antibody against β-actin (1:1000; Santa Cruz Biotechnology) to confirm that an equal amount of protein was loaded. The target was then stained with 5-bromo-4-chloro-indolyl phosphate/nitroblue tetrazodium and the bands were analyzed with the Tanon-Gis system.

RNA extraction and reverse transcription-PCR

After treating WB cells with TGF-β1 (5 ng/mL) for 0, 4, 8, and 24 h, total RNA was extracted from the cells with the TRIzol Reagent (Invitrogen-Life Technologies, Inc., USA) according to manufacturer instructions. RNA was reverse-transcribed with Superscript II Transcriptase (Invitrogen-Life Technologies, Inc.) in the presence of oligo-dT and random primers. Primer sequences and PCR conditions are shown in Table 1. PCR products were analyzed with 1% agarose gels stained with ethidium bromide.

Immunogold transmission electron microscopy

After treatment with TGF-β1, WB cells were first collected and prefixed with 2.5% glutaraldehyde in PBS for 120 min, washed three times with 0.1 M PBS, postfixed in 1% osmium tetroxide fixative (Millonigs buffer, pH 7.3) for 30 min and dehydrated in an ethanol series. They were then saturated and embedded in epoxy resin (Emitech, Ashford, Kent, UK) overnight. The cell blocks were subsequently cut on a Leica Ultracut machine to an ultrathin thickness of 70 nm. The ultrathin sections were loaded on nickel nets, which were then blocked for 30 min with 1% bovine serum albumin (BSA) and washed with 0.1 M PBS. The primary antibodies (rabbit monoclonal anti-rat cAMP, 1:80; P-Smad3, 1:80; Smad7, 1:60) were incubated in incubation buffer [1% goat serum (British Biocell International, UK), 0.1% Tween 20 (British Biocell International) and 1% BSA (British Biocell International) in PBS, adjusted to pH 8.2 by the addition of sodium hydroxide and hydrochloric acid] for 2 h, and then washed twice with the incubation buffer. Next, the cells were incubated with the secondary gold-labelled antibodies (gold-conjugated goat anti-rabbit IgG 1:25, 6 nm) in the incubating buffer (as above) for 1.5 h and then washed three times in PBS. Finally, the ultrathin sections were stained with uranyl acetate and areas were selected for viewing by transmission electron microscopy (TEM).

Results

TGF-β induces fibronectin expression in WB cells

In order to investigate the role of TGF-β in the fibrogenic process in hepatic stem cells, we first detected the expres-

Table 1. Primers, amplicon size, and protocol details for PCR.

	Primers (5' to 3')	Size (bp)	T (°C)	Extension time (s)	No. of cycles
Fibronectin	F: GTGTCCTCCTTCCATCTTC	290	55	60	35
	R: CAGACTGTCGGTACTCACG				
β-actin	F: CCACACAGATGACTTGCGCTCAGG	690	55	60	35
	R: CACCCTGTGCTGCTCACCGAGGCC				

F = forward; R = reverse; T(°C) = annealing temperature.

sion of FN, an extracellular matrix glycoprotein, at 0, 4, 8, and 24 h after TGF-β stimulation by Western blot analysis. The results showed that FN levels increased gradually and reached their optimum yield (5-fold increasing) after 8 h, decreasing thereafter (Figure 1A). Next, to ascertain the up-regulation of FN transcription induced by TGF-β, we performed RT-PCR. The results showed a pattern similar to that of the immunoblot data (Figure 1B). When combined, the results demonstrated that TGF-β1 stimulation can promote FN expression at both the levels of mRNA and protein.

Involvement of Smad signaling in TGF-β-induced up-regulation of FN

To determine whether stem cells signal in response to TGF-β and to explore the potential mechanism of FN up-regulation, we examined Smad signaling using immunogold TEM and the Western blot method. The immunogold TEM data showed that, after TGF-β1 treatment, WB cells exhibited a substantial increase in phospho-Smad3 at 4 h (Figure 2B), especially in the nucleus, indicating that stem cells contain sufficient quantities of receptors and Smads to signal in response to TGF-β. Smad3 phosphorylation peaked in WB cells at 8 h, and began to decrease after 24 h (Figure 2C,D). The Western blot results showed a pattern similar to that of immunogold TEM data (Figure 3). The phospho-Smad3 content increased within the initial 2 h following TGF-β stimulation and then fell to baseline by 24 h. In addition, we also observed alteration of Smad7 after TGF-β stimulation in WB cells. There was low expression of Smad7 in WB cells before TGF-β treatment (Figure 4A). The cytoplasmic expression of Smad7 was increased in WB cells after TGF-β treatment for 4 h (Figure 4B). Smad7 began to diminish in WB cells at 24 h (Figure 4D). To confirm the data described above, we performed a Western blot. The content of Smad7 increased within the initial 4 h following TGF-β stimulation and then fell by 24 h, in agreement with the results of immunogold TEM (Figure 3).

cAMP involvement in TGF-β-induced up-regulation of FN

cAMP is a second messenger that is important in many biological processes. To determine whether cAMP is involved in the FN up-regulation induced by TGF-β, we determined cAMP expression using immunogold TEM. As shown in Figure 5A, there was low expression of cAMP in the cytoplasm and nucleus of WB cells before TGF-β1 treatment. In contrast, when WB cells were exposed to TGF-β1 for 4 h, the expressions of cAMP in cytoplasm and nucleus increased significantly (Figure 5B) and peaked after 8 h, especially in the cytoplasm (Figure 5C). After treatment with TGF-β1 for 24 h, the expression of cAMP gradually decreased in WB cells (Figure 5D). The alterations of cAMP induced by TGF-β1 in WB cells had a pattern similar to that of FN.

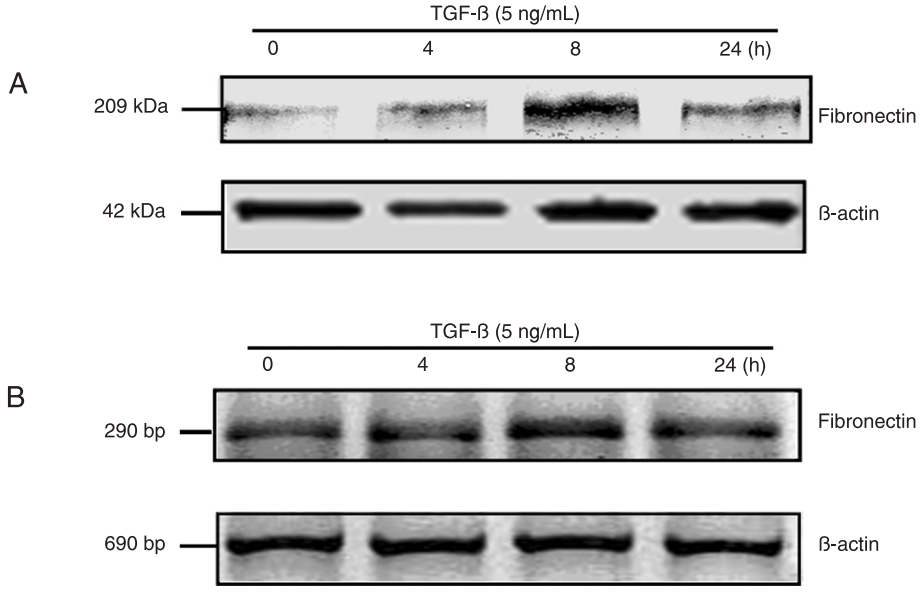

Figure 1. Effects of stimulation with transforming growth factor-beta (TGF-β) for various times on the expression of fibronectin (FN) in hepatic stem-like cells. *A*, Immunoblot analysis of FN protein. FN levels increased at 4 h, reached a peak near 8 h, and decreased after 24 h. *B*, RT-PCR analysis of the fibronectin gene. The target protein was stained with 5-bromo-4-chloro-indolyl phosphate/nitroblue tetrazodium. PCR products were analyzed with 1% agarose gels stained with ethidium bromide. β-actin was used to normalize protein and mRNA levels.

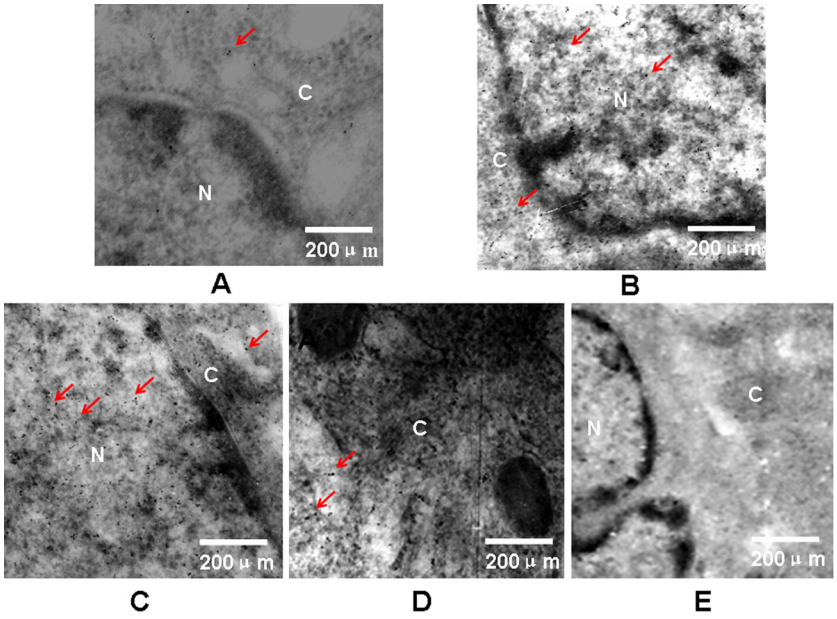

Figure 2. Electron micrographs of phosphorylated Smad3 expression and cellular distribution before and after stimulation with transforming growth factor-beta (TGF-β) in hepatic stem-like cells. *A*, Before TGF-β stimulation, weak nuclear and cytoplasmic staining. *B*, TGF-β stimulation for 4 h, strong nuclear staining with medium cytoplasm staining. *C*, TGF-β stimulation for 8 h, strong nuclear staining with medium cytoplasm staining. *D*, TGF-β stimulation for 24 h, with weak cytoplasm staining. *E*, Negative control, negative nuclear and cytoplasmic staining. The primary antibody of rabbit monoclonal anti-rat P-Smad3 was diluted eighty times, and the secondary antibody of goat anti-rabbit IgG (6 nm) was diluted twenty-five times. Red arrows indicate positive staining. Magnification: 4000X. N = nucleus; C = cytoplasm.

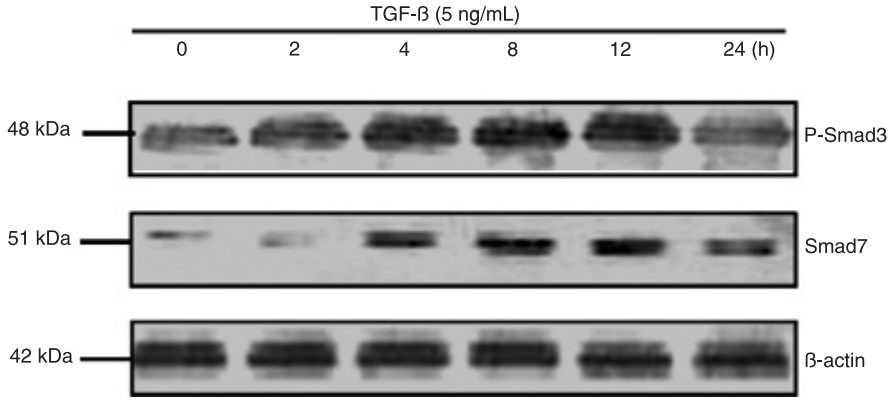

Figure 3. Effects of stimulation with transforming growth factor-beta (TGF-β) for various times on the expression of phosphorylated Smad3 and Smad7 in hepatic stem-like cells determined by immunoblot. For phosphorylated Smad3, the content increased within the initial 2 h following TGF-β stimulation and then fell to baseline by 24 h. For Smad7, the expression level increased within the initial 4 h following TGF-β stimulation and then fell by 24 h. The primary antibodies of rabbit monoclonal anti-rat P-Smad3 and anti-rat-Smad7 were diluted five hundred times, and the secondary antibodies of monoclonal antibody against β-actin was diluted one thousand times. β-actin was used to normalize protein levels.

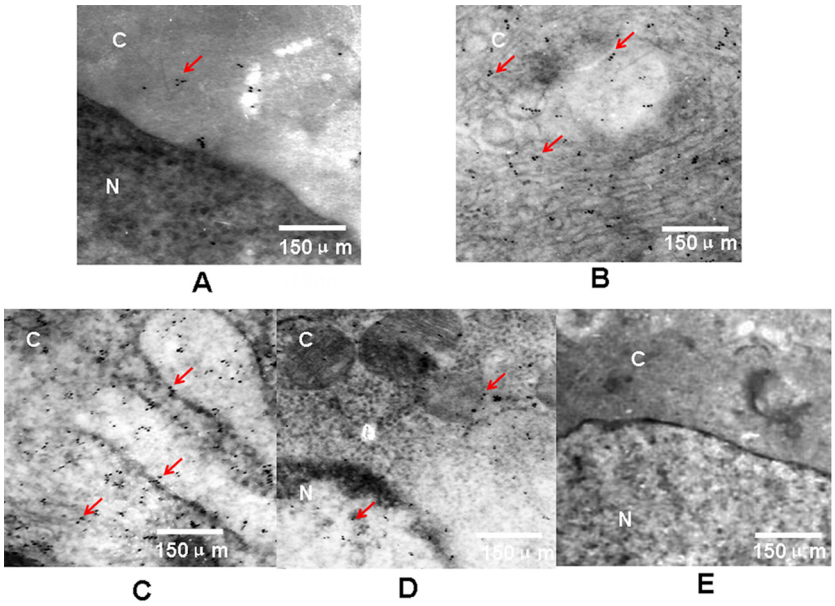

Figure 4. Electron micrographs of Smad7 expression and cellular distribution before and after stimulation with transforming growth factor-beta (TGF-β) in hepatic stem-like cells. *A*, Before TGF-β stimulation, weak nuclear and cytoplasm staining. *B*, TGF-β stimulation for 4 h, strong cytoplasm staining. *C*, TGF-β stimulation for 8 h, strong cytoplasm staining. *D*, TGF-β stimulation for 24 h, weak nuclear and cytoplasm staining. *E*, Negative control, negative nuclear and cytoplasm staining. The primary antibody of rabbit monoclonal anti-rat Smad7 was diluted sixty times, and the secondary antibody of goat anti-rabbit IgG (6 nm) was diluted twenty-five times. Red arrows indicate positive staining. Magnification: 4000X. N = nucleus; C = cytoplasm.

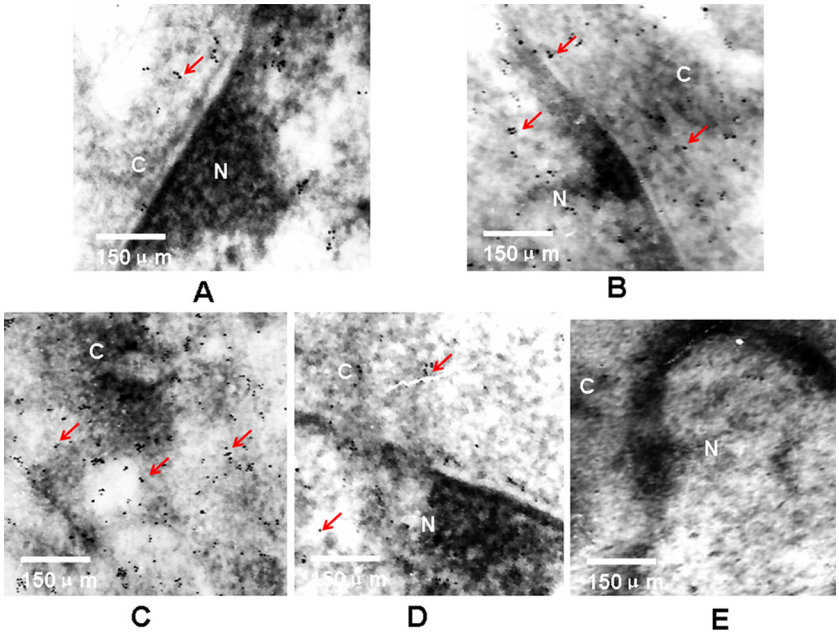

Figure 5. Electron micrographs of cAMP expression and cellular distribution before and after stimulation with transforming growth factor-beta (TGF-β) in hepatic stem-like cells. *A*, Before TGF-β stimulation, weak nuclear and cytoplasm staining. *B*, TGF-β stimulation for 4 h, middle cytoplasm and nuclear staining. *C*, TGF-β stimulation for 8 h, strong cytoplasm staining. *D*, TGF-β stimulation for 24 h, weak nuclear and cytoplasm staining. *E*, Negative control, negative nuclear and cytoplasmic staining. The primary antibody of rabbit monoclonal anti-rat cAMP was diluted eighty times, and the secondary antibody of goat anti-rabbit IgG (6 nm) was diluted twenty-five times. Red arrows indicate positive staining. Magnification: 4000X. N = nucleus; C = cytoplasm.

Discussion

Coordinated regulation of the production and turnover of components of the extracellular matrix (ECM) is essential for normal tissue homeostasis. The composition of the ECM can influence cell growth, state of differentiation and specific gene induction. FN, a major component of the ECM, plays an important role during development and wound healing by promoting cell adhesion, migration and cytoskeletal organization (10,11). Overproduction of matrix components including FN is the main pathological finding in tissue fibrosis (12). Decreased FN production, however, is often observed following oncogenic transformation, leading to decreased adhesion and increased metastatic potential (11). TGF-β, which is released by degranulating platelets at a wound site, has been shown to potently induce FN expression at both the mRNA and protein levels (5,13,14).

Recent research has shown that TGF-β induces liver fibrosis by promoting FN expression. Ha et al. (15) reported that high glucose levels may increase FN expression in peritoneal mesothelial cells, leading to ECM deposition (15). Kitamura and Ninomiya (16) showed that TGF-β1 causes liver fibrosis by promoting Smad 2/3, 4, and 7 expression in HSC. Schnur et al. (17) used transgenic mice to study the relationship between TGF-β and liver fibrosis and proved that TGF-β is somehow related to liver fibrosis. These studies demonstrate that HSC and hepatocytes are the major cells which produce ECM including FN. TGF-β causes HSC and hepatocytes to produce FN and, as a result, leads to liver fibrosis. But it is not known whether TGF-β can cause hepatic stem cells to express FN.

Here, we used TGF-β to stimulate hepatic stem-like cells *in vitro* and measured FN protein and mRNA expression at different intervals after exposure to TGF-β1. Our results indicated that the expression of FN increased gradually and peaked at 8 h after TGF-β exposure. It is suggested that TGF-β can cause ECM deposition by promoting not only HSC and hepatocytes, but also hepatic stem cells during the process of liver fibrosis.

In the presence of the TGF-β ligand, the receptor-activated Smads (R-Smads), Smads2 and 3, are phosphorylated directly by the TGF-β receptor I kinase, bind to the common mediator Smad, Smad4, and are translocated into the nucleus (6,7). In a recent experiment using microarrays to compare gene expression profiles of fibroblasts taken from adult *Smad3*$^{-/-}$ and *Smad3*$^{+/+}$ mice, TGF-β did not induce transcription in *Smad3*$^{-/-}$ fibroblasts (18). Thus, in adult fibroblasts Smad3 is required for TGF-β-induced gene expression. On the basis of these findings, we measured the expression levels and cellular distribution of phosphorylated Smad3 to identify the mechanism underlying the TGF-β-induced up-regulation of FN. We observed that WB cells presented a substantial increase in phospho-Smad3 levels after 4 h, peaking at 8 h, before decreasing after 24 h. These results suggest that phospho-Smad3 might be involved in the TGF-β-induced up-regulation of FN. Zhao et al. (19) reported that TGF-β induced-phospho-Smad3 reached a peak at 1.5 h and then quickly fell by 3 h in HSC. Compared with these data, our result is relatively late and persistent, indicating that the duration of response to TGF-β is cell-type dependent.

In normal cells, TGF-β directly stimulates the transcription of inhibitory Smad7. Thus, Smad7 induction serves as a critical intracellular "brake" of TGF-β-induced responses. The physiologic importance of this Smad7-mediated autoinhibitory feedback loop is highlighted by recent observations with cultured cells in *in vivo* animal models. For example, tumor necrosis factor-α (TNF-α)-induced abrogation of TGF-β-dependent profibrotic responses was linked to the induction of endogenous Smad7 (20). On the other hand, defective Smad7 induction is implicated in the exaggerated TGF-β responsiveness characteristic of hepatic cells and myofibroblasts from chronically injured livers (21,22). In the present study, we observed that the expression of Smad7 began to increase after TGF-β treatment for 4 h. These data agree well with previous results obtained for skin fibroblasts (23). In addition, our result showed an obvious Smad7 translocation from nucleus to cytoplasm after treatment with TGF-β for 4 h. In fact, Smad7 has been shown to inhibit TGF-β signaling by associating with the type I receptor and thereby interfering with Smad2/3 phosphorylation by the activated receptor complex in the cytoplasm (6,7). Thus, it is possible that the elevated levels of Smad7 in the cytoplasm of stem cells provide a means to inhibit the signaling response to the ligand, which can prevent the phosphorylation and translocation to the nucleus of Smad2/3, and consequently inhibit the TGF-β-induced expression of FN. This hypothesis is supported by the finding of the alteration process of phospho-Smad3 and Smad7 induced by TGF-β in WB cells.

In order to determine the mechanism underlying this response to TGF-β, we also examined the levels of cAMP in hepatic stem-like cells. cAMP is a second messenger that has been implicated as a modulator of cell proliferation and cell growth in several fibroblastic cell types (24). Recently, it was demonstrated that increased cAMP levels inhibit cardiac myofibroblast formation and collagen synthesis (25). In addition, β-adrenergic receptor agonists, which stimulate cAMP production, attenuate the fibroblast-mediated contraction of collagen gels (26). Nonetheless, in hepatic stem cells, the role of cAMP in TGF-β-induced FN expression remains undefined. The present study indicates that when WB cells are exposed to TGF-β for 4 h, the expression of cAMP in cytoplasm and nucleus increases significantly and peaks at 8 h, especially in the cytoplasm. After treatment with TGF-β for 24 h, the expression of cAMP gradually decreased in WB cells. The process of cAMP alteration induced by TGF-β in WB cells showed a pattern similar to that of FN. These findings indicate that cAMP may be involved in the TGF-β-induced up-regulation of FN. It is well known that the activa-

tion of cAMP could result in the phosphorylation of cAMP response element binding protein (CREB), a transcription factor known for its ability to stimulate FN expression (27). Thus, it is possible that TGF-β-induced up-regulation of FN occurs in part through the cAMP-CREB pathway. However, the detailed roles of cAMP in TGF-β-induced up-regulation of FN in hepatic stem-like cells merit further investigation.

The present study demonstrated for the first time that TGF-β is involved in the fibrogenic process in hepatic stem-like cells through up-regulation of FN expression, and the mechanisms underlying this process seem to be associated with the activation of cAMP and Smad pathways.

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