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# β-Catenin pathway is required for TGF-β1 inhibition of PPARγ expression in cultured hepatic stellate cells

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ABSTRACT

Hepatic stellate cell (HSC) activation is a key step in process of liver fibrosis. Transforming growth factor-β1 (TGF-β1) is the most powerful mediator of HSC activation and plays a central role in liver fibrosis. Peroxisome proliferator-activated receptor-γ (PPARγ) is an important regulator of adipocyte differentiation and has been proposed as a crucial factor for inhibition of HSC activation. The effect of TGF-β1 on PPARγ in HSCs is largely unknown. This study is aimed to examine whether TGF-β1 can influence PPARγ expression, focusing on the role of β-catenin pathway, a key pathway linked to adipogenesis, in TGF-β1 regulation of PPARγ in cultured HSCs. Our results demonstrated that TGF-β1 evidently inhibited PPARγ expression and activity in cultured HSCs, which were mediated through β-catenin pathway. TGF-β1 promoted β-catenin expression and also increased the stability of β-catenin protein through ERK1/2/glycogen synthase kinase-3β (GSK-3β) axis in cultured HSCs. Moreover, TGF-β1 inhibition of PPARγ expression by β-catenin pathway caused the increase in alpha1(1) collagen and tissue inhibitor of matrix metalloproteinase expression. These results indicated for the first time that TGF-β1 could down-regulate PPARγ expression through β-catenin pathway and subsequently contributed to the increase in alpha1(1) collagen level in cultured HSCs.



## 1. Introduction

Liver fibrosis is a wound healing process characterized by over accumulation of extracellular matrix proteins (ECM) especially collagen types I and III. Hepatic stellate cell (HSC) is the most relevant cell type for the development of liver fibrosis. Its activation is the key step in the process of liver fibrogenesis. Activated HSCs are the main ECM-producing cells in the injured liver. Many studies focus on the mechanisms underlying the process of HSC activation. Interestingly, it has been shown that the process of HSC activation is analogous to the reverse transdifferentiation of adipocyte to preadipocyte (fibroblast) [1]. The nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) is a key regulator of adipocyte differentiation *in vivo* as well as *in vitro* [2] and acts as a transcriptional activator of many adipocyte-specific genes [3].

It has been demonstrated that the level of PPARγ protein is high in quiescent HSCs and its expression and its activity are reduced during HSC activation *in vitro* and *in vivo* [4,5]. Introduction of exogenous PPARγ is sufficient to reverse the morphology of activated HSCs to the quiescent phenotype with reduced expression of activation markers and with the accumulation of retinyl palmitate [6], a characteristic of quiescent HSCs. Furthermore, PPARγ overexpression *in vivo* strongly attenuated stellate cell activation and liver fibrosis [7]. PPARγ has been proposed as a crucial factor for inhibition of HSC activation [1,6,7].

It has been shown that Wnt/β-catenin pathway is a crucial regulator of adipogenesis [8] and β-catenin is a key effector of the Wnt signaling pathway. The absence of Wnts facilitates the phosphorylation of β-catenin protein by glycogen synthase kinase-3β (GSK-3β), leading to β-catenin (a primary substrate of GSK-3β) ubiquitination and degradation [9], whereas Wnts can induce the phosphorylation and inactivation of GSK-3β and subsequent stabilization of β-catenin protein [10]. Importantly, functional Wnt/β-catenin pathway has been demonstrated in HSCs and contributes to HSC activation and liver fibrosis [11].

Transforming growth factor-β1(TGF-β1) is one of the most important cytokines expressed following liver injury and TGF-β1 is considered the most powerful mediator of HSC activation *in vitro* and *in vivo* [12] and [13] and plays a central role in liver fibrosis [14]. Its role in liver fibrosis has been widely studied. However, the effect of TGF-β1 on PPARγ expression is unknown in HSCs. Interestingly, TGF-β1 appears to be associated with β-catenin pathway

*Abbreviations:* TGF-β1, transforming growth factor-β1; PPARγ, peroxisome proliferator-activated receptor-γ; GSK-3β, glycogen synthase kinase-3β; ECM, extracellular matrix proteins; HSC, hepatic stellate cell; ERK1/2, extracellular regulated protein kinase1/2; TIMP, tissue inhibitor of matrix metalloproteinase.

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in human lung fibroblasts [15]. Moreover, TGF-β1 also involves adipocyte differentiation [16].

Based on the observations above, there is a possibility that TGF-β1 could affect PPARγ expression in HSCs and β-catenin pathway might mediate the role of TGF-β1 in PPARγ expression in HSCs. Therefore, this study is aimed to examine whether TGF-β1 could influence PPARγ expression, focusing on the role of β-catenin pathway in TGF-β1 regulation of PPARγ in cultured HSCs.

## 2. Materials and methods

### 2.1. Materials

TGF-β1 and PD98059 (a MEK inhibitor) were purchased from CalBiochem (La Jolla, CA, USA). XAV939 (a potent and specific inhibitor of β-catenin pathway), and all antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Lithium chloride (LiCl, an inhibitor of GSK-3β activation) and GW9662 (a specific PPARγ antagonist) were purchased from Sigma (St. Louis, MO, USA).

### 2.2. HSC isolation and culture

HSCs were isolated from Sprague–Dawley rats as we described previously [17]. Semi-confluent HSCs with 4–8 passages were used for experiments. Cells were serum-starved for 24 h in DMEM with 0.4% FBS before treatment with TGF-β1 for 24 h. Animals received humane care and experimental protocols were conducted according to national and local guidelines.

### 2.3. Western blot analysis

Western blot analysis was performed as we described previously [17]. Target proteins were detected by primary antibodies against PPARγ (diluted 1:500), β-catenin (diluted 1:1000), Ser 9 phosphorylated GSK-3β (diluted 1:500), GSK-3β (diluted 1:1000), alpha1(1) collagen (diluted 1:2000), β-actin (diluted 1:2000), respectively, and subsequently by horseradish peroxidase-conjugated secondary antibodies (diluted 1:4000). β-Actin or total GSK-3β was used as the internal control.

### 2.4. RNA isolation and real-time PCR

Total RNA was extracted by using TRI-Reagent (Sigma, St. Louis, USA) according to the manufacturer's instructions and treated with deoxyribonuclease I. Real-time PCR was performed as we described previously [18]. mRNA levels of target genes relative to the endogenous cyclophilin control were calculated as suggested by Schmittgen et al. [19]. The results were expressed as fold changes relative to the control. The primers used in real-time PCR were as follows:

Rat PPARγ:
- (forward) 5′-ATTCTGGCCCACCAACTTCGG-3′;
- (reverse) 5′-TGGAAGCCTGATGCTTTATCCCCA-3′.

Rat β-catenin:
- (forward) 5′-CCACGACTAGTTCAGCTGCTTGTAC-3′;
- (reverse) 5′-ACTGCACAAACAGTGGAATGGTATT-3′.

Rat alpha1(1) collagen:
- (forward) 5′-TTCCCTGGACCTAAGGGTACT-3′;
- (reverse) 5′-TTGAGCTCCAGCTTCGCC-3′.

Rat tissue inhibitor of matrix metalloproteinase-1 (TIMP-1):
- (forward) 5′-CGCAGCGAGGAGGTTTCTCAT-3′;
- (reverse) 5′-GGCAGTGATGTGCAAATTTCC-3′.

Rat TIMP-2:
- (forward) 5′-GACACGCTTAGCATCACCCAGA-3′;
- (reverse) 5′-CTGTGACCCAGTCCATCCAGAG-3′.

Rat cyclophilin:
- (forward) 5′-TGGATGGCAAGCATGTGGTCTTTG-3′;
- (reverse) 5′-CTTCTTGCTGGTCTTGCCATTCCT-3′.

### 2.5. Plasmids and transient transfection assays

The PPARγ activity reporter plasmid pPPRE × 3-TK-Luc contains three consensus PPARγ-responsive elements linked to the herpes virus thymidine kinase promoter and a Photinus luciferase vector (Addgene Inc., Cambridge, MA, USA). Plasmid pdncatenin encodes dominant-negative β-catenin (a gift from Dr. Jane B. Trepel, Medical Oncology Branch, NCI, NIH, Bethesda, USA).

HSCs in twelve-well plastic plates were transiently transfected with pPPRE × 3-TK-Luc (1 μg DNA/well) plus 30 ng of control vector expressing Renilla luciferase (pRL-TK; Promega, Madison, USA) by using LipofectAMINE reagent (Life Technologies, New York, USA) following manufacturer's instructions. In some experiments, HSCs were cotransfected with pPPRE × 3-TK-Luc (1 μg DNA/well) plus pdn catenin (1 μg DNA/well) or the empty vector and 30 ng Renilla luciferase. The empty vector was used to ensure an equal amount of total DNA in transfection assay. Luciferase activity was quantified fluorimetrically by using the Dual-Luciferase Reporter Assay System (Promega, Madison, USA) and data were expressed as the ratios of Photinus to Renilla luciferase activity for normalization of Photinus luciferase activity. HSCs in 25 $cm^2$ flask were transfected with 8 μg of pdn catenin or the empty vector per flask.

### 2.6. β-catenin and GSK-3β silencing through RNA interference

HSCs in 25 $cm^2$ flask were transfected with a pool of four short interfering RNAs (siRNAs; 250 pmol) targeted specifically to rat β-catenin or rat GSK-3β using LipofectAMINE reagent (Life Technologies, New York, USA) following manufacturer's instructions. Rat β-catenin siRNA was purchased from Santa Cruz (Santa Cruz, CA, USA). Rat GSK-3β siRNA duplexes were synthesized by Life Technologies Corporation (Shanghai, China) to target the rat GSK-3β: (a) 5′-GATCTGCCATCGAGACATT-3′, (b) 5′-CTCAAGAACTGTCAAGTAA-3′, (c) 5′-TCAGAAGTCTAGCCTATAT-3′, and (d) 5′-ACACTAAAGTCATTGGAAA-3′ [20]. As a negative control, cells were transfected with siRNA against GFP (Life Technologies Corporation, Shanghai, China).

### 2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted as we previously described [21]. The sense oligonucleotide of the putative DNA binding site for NFkB was: 5′-GGGGACTTTCCC-3′ [22]. EMSA were performed as we previously reported [21]. Briefly, the probe was end-labeled with [$^{32}$P]dATP and 5 μg of nuclear extract was incubated with the $^{32}$P-labeled probe for 30 min. For the competition assay, 5 μg of nuclear extract was preincubated with 100-fold molar excess of the unlabeled NFkB probe (cold probe) before addition of the labeled probe. For supershift assay, 5 μg of nuclear extract was preincubated with 2 μg of anti-NFkB (p50) antibody (Santa Cruz, CA, USA) before addition of the labeled probe. The samples were then loaded onto a 5% nondenaturating polyacrylamide gel. The gels were subjected to electrophoresis, drying, and autoradiography.

### 2.8. Statistical analysis

Differences between means were evaluated using an unpaired two-sided Student's *t*-test ($P < 0.05$ considered as significant). Where appropriate, comparisons of multiple treatment conditions

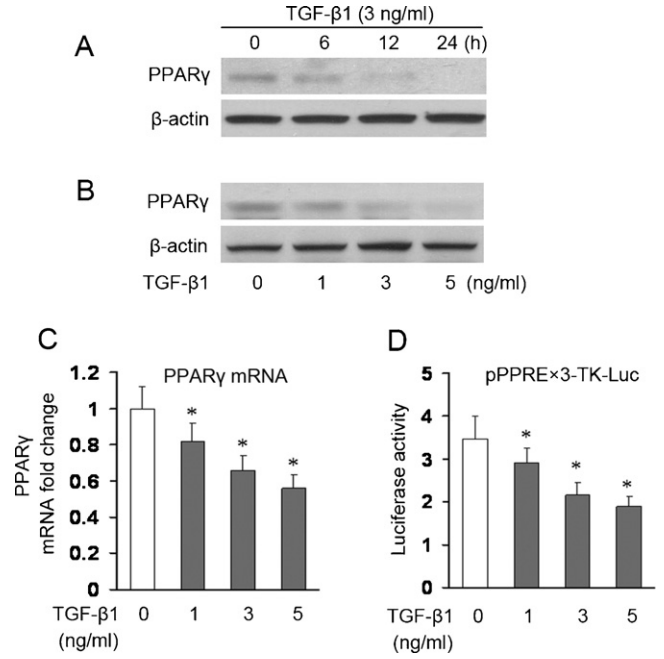


**Fig. 1.** TGF-β1 down-regulates PPARγ expression and trans-activation activity in cultured HSCs. (A and B) Western blot analysis of PPARγ protein level ($n = 3$). HSCs were incubated with 3 ng/ml of TGF-β1 for different time periods (A) or with increasing dose of TGF-β1 for 24 h (B). β-Actin was used as internal control. (C) Real-time PCR analysis of PPARγ mRNA level ($n = 3$). HSCs were incubated with increasing dose of TGF-β1 for 24 h. $^*P < 0.05$ *vs* cells without TGF-β1. (D) Transfection assay for analysis of PPARγ trans-activation activity ($n = 6$). HSCs were transfected with pPPRE × 3-TK-Luc (1 μg/well) plus pRL-TK (30 ng/well) and stimulated with increasing dose of TGF-β1 for 24 h. $^*P < 0.05$ *vs* cells without TGF-β1.

with controls were analyzed by ANOVA with the Dunnett's test for *post hoc* analysis.

## 3. Results

### 3.1. TGF-β1 treatment reduces PPARγ expression in cultured HSCs

To investigate the effect of TGF-β1 on PPARγ expression, serum-starved HSCs were treated with 3 ng/ml of TGF-β1 for different time periods. The level of PPARγ protein was examined by Western blot analysis. Fig. 1A showed that TGF-β1 markedly reduced PPARγ protein level in a time-dependent manner. Next, HSCs were treated with increasing dose of TGF-β1 for 24 h and the levels of PPARγ protein and mRNA were analyzed by Western blot and real-time PCR, respectively. The results demonstrated that TGF-β1 inhibited PPARγ expression at protein (Fig. 1B) and mRNA (Fig. 1C) levels in a dose-dependent manner.

TGF-β1-induced decrease in PPARγ expression might affect its activity. Therefore, HSCs were transfected with pPPRE × 3-TK-Luc (containing three consensus PPARγ-responsive elements) and treated with increasing dose of TGF-β1 for 24 h. Luciferase assay indicated that TGF-β1 treatment led to a dose-dependent decrease in the trans-activation activity of PPARγ (Fig. 1D).

These results strongly suggested that TGF-β1 exerted an inhibitory effect on PPARγ expression and trans-activation activity in cultured HSCs.

### 3.2. TGF-β1 treatment increases β-catenin protein level in cultured HSCs

To test the possibility whether β-catenin pathway might involve the effect of TGF-β1 on PPARγ in cultured HSCs, the influence

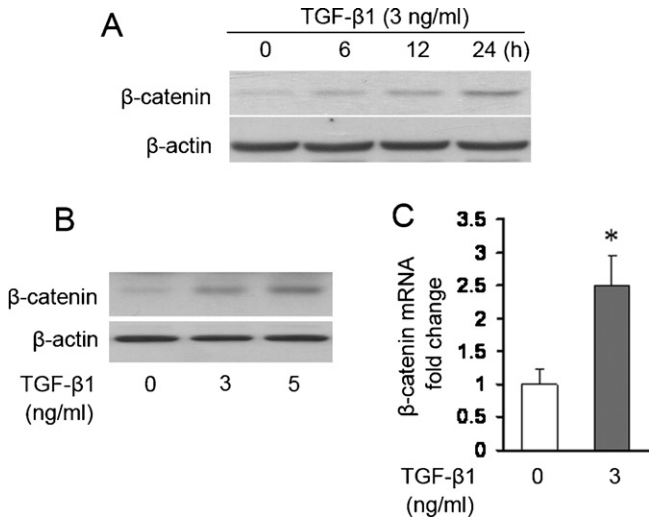


**Fig. 2.** TGF-β1 increases β-catenin expression in cultured HSCs. (A and B) Western blot analysis of β-catenin protein level ($n = 3$). HSCs were incubated with 3 ng/ml of TGF-β1 for different time periods (A) or with increasing dose of TGF-β1 for 24 h (B). β-Actin was used as internal control. (C) Real-time PCR analysis of β-catenin mRNA level ($n = 3$). HSCs were incubated with or without 3 ng/ml of TGF-β1 for 24 h. $^*P < 0.05$ *vs* cells without TGF-β1.

of TGF-β1 on β-catenin protein level was examined. HSCs were stimulated with 3 ng/ml of TGF-β1 for different time periods or stimulated with different doses of TGF-β1 for 24 h. Western blot analysis indicated that TGF-β1 induced β-catenin protein level in a time-dependent manner (Fig. 2A) and 3 or 5 ng/ml of TGF-β1 clearly led to an increase in β-catenin protein level (Fig. 2B). To examine the influence of TGF-β1 on β-catenin mRNA, HSCs were stimulated with or without 3 ng/ml of TGF-β1 for 24 h. Real-time PCR analysis demonstrated that 3 ng/ml of TGF-β1 significantly increased β-catenin mRNA level (Fig. 2C). Fig. 2 indicated that TGF-β1 up-regulated β-catenin expression in cultured HSCs.

Extracellular regulated protein kinase1/2 (ERK1/2) is a member of the mitogen-activated protein kinase (MAPK) family. Its activation leads to the phosphorylation (at Ser9) and inactivation of GSK-3β, subsequently inhibiting the degradation of β-catenin protein (a primary substrate of GSK-3β) in human lung fibroblasts [15]. It has been shown that stimulation of HSCs with 3 ng/ml of TGF-β1 for 20 min is able to induce ERK1/2 activation [23]. Thereby, we examine the effect of TGF-β1-induced activation of ERK1/2 on phosphorylation (at Ser9) of GSK-3β and on β-catenin protein in HSCs. HSCs were pretreated with or without 10 μM of PD98059 for 30 min before stimulation with or without TGF-β1 for an additional 20 min (for analysis of Ser 9 phosphorylated GSK-3β) or 24 h (for analysis of β-catenin protein). Western blot analysis showed that TGF-β1 treatment clearly caused the increase in the levels of Ser 9 phosphorylated GSK-3β (Fig. 3A) and β-catenin protein (Fig. 3B) which were inhibited by pretreatment with 10 μM of PD98059, whereas PD98059 alone had no effect on both types of proteins. These results indicated that TGF-β1-induced activation of ERK1/2 resulted in inactivation of GSK-3β and subsequent stabilization of β-catenin protein in cultured HSCs. To further verify the results, HSCs were incubated with 10 mM of LiCl (an inhibitor of GSK-3β activation) for 24 h or the gene expression of GSK-3β was knocked down by transfection of HSCs with siRNA against GSK-3β. Western blot analysis indicated that β-catenin protein level was up-regulated by LiCl treatment (Fig. 3C) or knockdown of the gene expression of GSK-3β (Fig. 3D), which supported that TGF-β1-induced inhibition of GSK-3β activity led to the stabilization of β-catenin protein in HSCs.

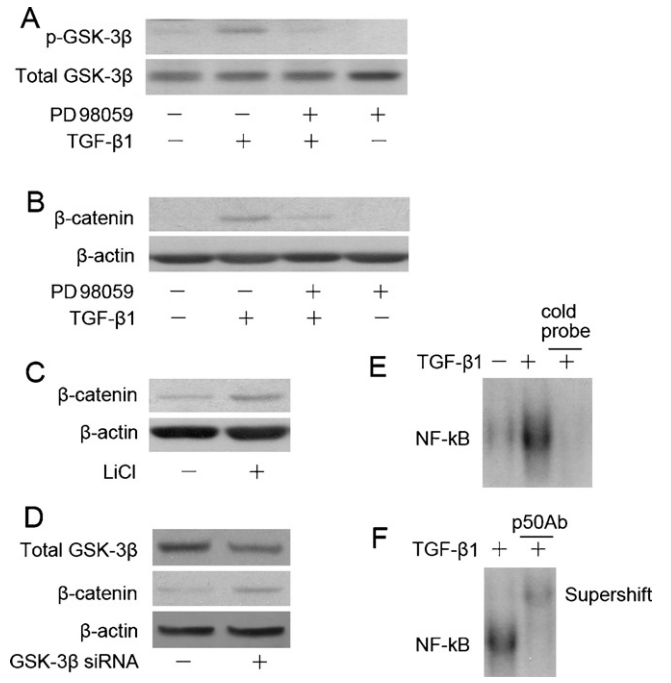


**Fig. 3.** TGF-β1-induced activation of ERK1/2 contributes to the phosphorylation of GSK-3β at Ser9 and the accumulation of β-catenin protein in cultured HSCs. (A and B) Western blot analyses of Ser 9 phosphorylated GSK-3β and β-catenin protein levels ($n = 3$). HSCs were pretreated with or without 10 μM of PD98059 for 30 min before stimulation with or without 3 ng/ml of TGF-β1 for an additional 20 min (for analysis of Ser 9 phosphorylated GSK-3β) (A) or 24 h (for analysis of β-catenin protein) (B). Total GSK-3β or β-actin was used as internal control. (C and D) Western blot analysis of level of β-catenin protein or total GSK-3β protein ($n = 3$). HSCs were treated with or without 10 mM of LiCl for 24 h (C) or transfected with siRNA against GSK-3β or siRNA against GFP (as a negative control) for 24 h (D). β-Actin was used as internal control. (E and F) EMSA of DNA binding activity of NF-kB. Serum-starved HSCs were stimulated with 3 ng/ml of TGF-β1 for 20 min. Nuclear protein was extracted and 5 μg of nuclear protein was incubated with [$^{32}$P]dATP labeled NF-kB probe or nuclear protein from HSCs treated with TGF-β1 was preincubated with100-fold molar excess of the unlabeled NF-kB probe (cold probe) before addition of labeled NF-kB probe (for competition assay, lane 3) (E). For supershift assay, nuclear protein from HSCs treated with TGF-β1 was preincubated with anti-NF-kB(p50) antibody (2 μg) before addition of labeled NF-kB probe (lane 2) (F). A representative EMSA was shown from three independent experiments.

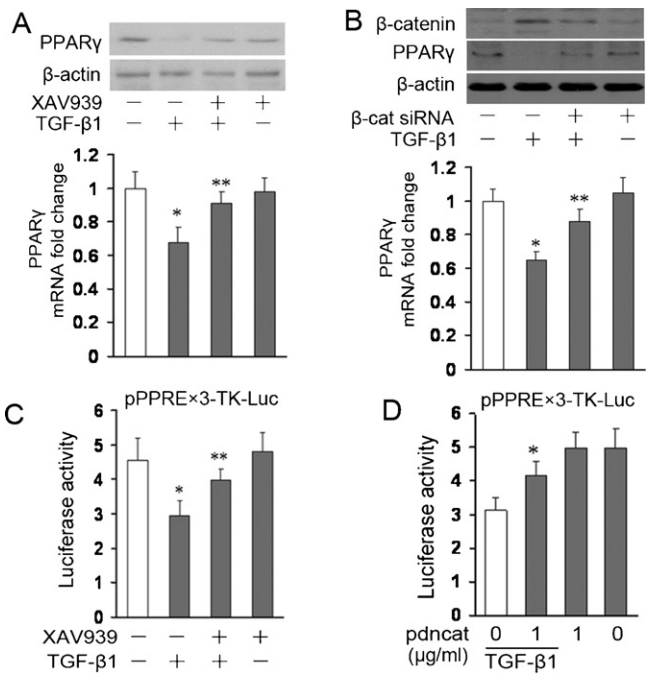


**Fig. 4.** β-Catenin pathway mediates TGF-β1-induced inhibition of PPARγ expression and trans-activation activity in cultured HSCs. (A and B) Analyses of PPARγ protein and mRNA levels or β-catenin protein level ($n = 3$). HSCs were pretreated with or without 5 μM of XAV939 for 30 min before treatment with or without 3 ng/ml of TGF-β1 for an additional 24 h (A), or HSCs were transfected with siRNA against β-catenin (β-cat siRNA) or siRNA against GFP (as a negative control) before treatment with or without 3 ng/ml of TGF-β1 for 24 h (B). The levels of PPARγ protein and mRNA were analyzed by Western blot and real-time PCR, respectively and β-catenin protein level was analyzed by Western blot. $^*P < 0.05$ *vs* the control without treatment (the first column on the left). $^{**}P < 0.05$ *vs* cells with TGF-β1 alone (the second column on the left). (C and D) Transfection assay for analysis of PPARγ trans-activation activity ($n = 6$). HSCs were transfected with pPPRE × 3-TK-Luc (1 μg/well) plus pRL-TK (30 ng per well) and pretreated with or without 5 μM of XAV939 for 30 min before treatment with or without 3 ng/ml of TGF-β1 for an additional 24 h (C), or HSCs were transfected with pPPRE × 3-TK-Luc (1 μg/well), pdn catenin (pdncat, 1 μg/well) or empty plasmid, and pRL-TK (30 ng/well) and treated with or without 3 ng/ml of TGF-β1 for additional 24 h (D). $^*P < 0.05$ *vs* the control (the first column on the left in C or D). $^{**}P < 0.05$ *vs* cells with TGF-β1 alone (the second column on the left in C).

Taken together, these data indicated that TGF-β1 was linked to β-catenin pathway in cultured HSCs.

Since TGF-β1 can induce ERK1/2 activity [23] and the latter leads to NF-kB activation in HSCs [24], we further investigated the effect of TGF-β1 on NF-kB trans-activation activity by EMSA. As shown in Fig. 3E and F, TGF-β1 induced marked DNA binding activity of NF-kB in cultured HSCs.

### 3.3. β-Catenin pathway is required for TGF-β1 inhibition of PPARγ expression and activity in cultured HSCs

Since TGF-β1 was associated with β-catenin pathway and TGF-β1 exerted evident inhibition of PPARγ expression in HSCs, we next examined whether β-catenin pathway mediated TGF-β1-induced decrease in PPARγ expression and activity. Our previous study showed that 5 μM of XAV939 (a specific inhibitor of β-catenin pathway) was enough to inhibit β-catenin pathway in cultured HSCs (data not shown). HSCs were pretreated with or without 5 μM of XAV939 for 30 min before treatment with or without 3 ng/ml of TGF-β1 for an additional 24 h, the levels of PPARγ protein and mRNA were analyzed by Western blot and real-time PCR, respectively. Fig. 4 shows that TGF-β1 significantly reduced PPARγ protein level (Fig. 4A, upper panel) and mRNA level (Fig. 4A, down panel), which were partially reversed by treatment with XAV939 (Fig. 4A, the second band or the second column on the right). To further verify the results, HSCs were transfected with siRNA against β-catenin gene before treatment with 3 ng/ml of TGF-β1 for 24 h. Western blot (Fig. 4B, upper panel) and real-time PCR (Fig. 4B, down panel) analyses indicated that knockdown of β-catenin expression also relieved TGF-β1 inhibition of PPARγ expression, which was consistent with the results in Fig. 4A.

Next, the role of β-catenin pathway in TGF-β1 inhibition of PPARγ activity was tested. HSCs were transfected with pPPRE × 3-TK-Luc and pretreated with or without 5 μM of XAV939 for 30 min before treatment with or without 3 ng/ml of TGF-β1 for an additional 24 h. Luciferase assay (Fig. 4C) revealed that TGF-β1 treatment resulted in the decline in the trans-activation activity of PPARγ, which was also partially inhibited by XAV939. To further verify the data, HSCs were co-transfected with pPPRE × 3-TK-Luc plus pdn catenin (encoding dominant-negative β-catenin to interrupt β-catenin pathway) or empty vector and stimulated with or without 3 ng/ml of TGF-β1 for 24 h. As shown in Fig. 4D by luciferase assays, pdn catenin partially reversed TGF-β1 inhibition of PPARγ trans-activation activity.

Collectively, these results evidently suggested the involvement of β-catenin pathway in TGF-β1 inhibition of PPARγ expression and trans-activation activity in cultured HSCs.

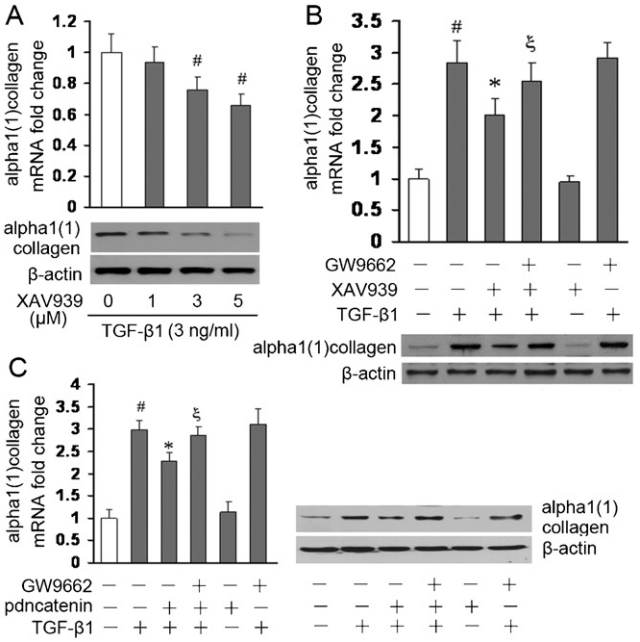


**Fig. 5.** The inhibitory effect of TGF-β1 on PPARγ by β-catenin pathway leads to the increase in alpha1(1) collagen gene expression in cultured HSCs. (A and B) Analyses of alpha1(1) collagen mRNA and protein levels ($n = 3$). HSCs were pretreated with different doses of XAV939 for 30 min before treatment with 3 ng/ml of TGF-β1 for an additional 24 h (A), or HSCs were pretreated with or without 10 μM of GW9662 (a specific PPARγ antagonist) and 5 μM of XAV939 for 30 min before stimulation with or without 3 ng/ml of TGF-β1 for an additional 24 h (B). The levels of alpha1(1) collagen mRNA and protein were analyzed by real-time PCR and Western blot ($n = 3$), respectively. $^{\#}P < 0.05$ *vs* the control (the first column on the left in A or B). $^{*}P < 0.05$ *vs* cells with TGF-β1 alone (the second column on the left in B). $^{\xi}P < 0.05$ *vs* cells with XAV939 plus TGF-β1 (the third column on the left in B). (C) Analysis of alpha1(1) collagen mRNA level ($n = 3$). HSCs were transfected with pdn catenin (8 μg/flask) or empty plasmid and pretreated with or without 10 μM of GW9662 for 30 min before stimulation with or without 3 ng/ml of TGF-β1 for an additional 24 h. The levels of alpha1(1) collagen mRNA and protein were analyzed by real-time PCR (left panel) and Western blot (right panel) analyses, respectively ($n = 3$). $^{\#}P < 0.05$ *vs* the control (the first column on the left). $^{*}P < 0.05$ *vs* cells with TGF-β1 alone (the second column on the left). $^{\xi}P < 0.05$ *vs* cells with pdn catenin plus TGF-β1 (the third column on the left).

### 3.4. The inhibitory effect of TGF-β1 on PPARγ by β-catenin pathway results in the increase of alpha1(1) collagen and TIMP expression in cultured HSCs

Liver fibrosis is characterized by over accumulation of ECM especially collagen types I and III which were mainly produced by the activated HSCs and TGF-β1 is considered the most powerful mediator of HSC activation [12] and [13]. Since TGF-β1 markedly down-regulated PPARγ (a crucial factor for inhibition of HSC activation) expression through β-catenin pathway, it was interesting to examine the influence of TGF-β1-induced inhibition of PPARγ expression by β-catenin pathway on collagen expression in HSCs. First, HSCs were pretreated with different doses of XAV939 for 30 min and then stimulated with TGF-β1 for 24 h for examining the role of TGF-β1-induced β-catenin pathway in alpha1(1) collagen expression. As shown in Fig. 5A, real-time PCR and Western blot analyses demonstrated that XAV939 inhibited TGF-β1-induced increases in levels of alpha1(1) collagen mRNA (upper panel) and protein (down panel). Next, HSCs were pretreated with or without 10 μM of GW9662 (a specific PPARγ antagonist) [25] and 5 μM of XAV939 for 30 min before stimulation with or without TGF-β1 for an additional 24 h. The levels of alpha1(1) collagen mRNA and protein were analyzed by real-time PCR and Western blot analyses, respectively. Fig. 5B demonstrated that TGF-β1 alone evidently increased the levels of alpha1(1) collagen mRNA and protein as compared with the control (the first column or the first band on the left). As expected, pretreatment with XAV939 partially eliminated TGF-β1-induced increase in levels of alpha1(1) collagen mRNA and protein (the third column or the third band on the left), which were attenuated by GW9662 (the fourth column or the fourth band on the left), whereas in the presence of TGF-β1, GW9662 alone did not influence the levels of alpha1(1) collagen mRNA and protein (the first column or the first band on the right) as compared with the control with TGF-β1 alone (the second column or the second band on the left).

To further verify the results, HSCs were transfected with or without 8 μg of pdn catenin (per flask) and then pretreated with or without 10 μM of GW9662 for 30 min before stimulation with or without TGF-β1 for an additional 24 h. The levels of alpha1(1) collagen mRNA and protein were analyzed by real-time PCR and Western blot analyses, respectively. As shown in Fig. 5C, interruption of β-catenin pathway by pdn catenin also partially inhibited TGF-β1-induced increase in alpha1(1) collagen mRNA (the third column on the left in left panel) and protein (the third band on the left in right panel) levels, which were attenuated by GW9662 (the fourth column on the left in the left panel and the fourth band on the left in the right panel).

Results in Fig. 5 suggested that TGF-β1-induced decline in PPARγ expression through β-catenin pathway resulted in the increase in alpha1(1) collagen expression in cultured HSCs.

TIMPs exert the inhibitory effects on matrix metalloproteinase activity, leading to inhibition of the ECM degradation. Therefore, we also examined the role of TGF-β1-induced inhibition of PPARγ expression by β-catenin pathway in the expression of TIMP-1 and TIMP-2 at mRNA level. HSCs were treated as in Fig. 5B. Fig. 6 demonstrated that blockade of β-catenin pathway partially inhibited TGF-β1-induced increase in mRNA levels of TIMP-1 and TIMP-2 (the third black or empty column on the left), which were attenuated by GW9662 (the fourth black or empty column on the left). These results were consistent with that in Fig. 5B and C.

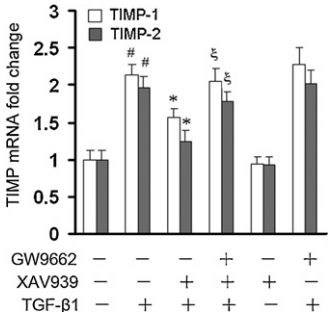


**Fig. 6.** The inhibitory effect of TGF-β1 on PPARγ by β-catenin pathway leads to the increase in mRNA levels of TIMP-1 and TIMP-2. HSCs were treated as in Fig. 5(B). The mRNA levels of TIMP-1 and TIMP-2 were analyzed by real-time PCR ($n = 3$), respectively. $^{\#}P < 0.05$ *vs* the respective control without treatment. $^{*}P < 0.05$ *vs* the respective cells with TGF-β1 alone. $^{\xi}P < 0.05$ *vs* the respective cells with XAV939 plus TGF-β1.

## 4. Discussion

Because quiescent HSC is much like adipocyte and HSC activation is analogous to adipocyte to preadipocyte (fibroblast) transdifferentiation [1], PPARγ, a key regulator of adipocyte differentiation [2], was found to exert a crucial role in inhibition of HSC activation [1,6,7]. TGF-β1 is considered to be a major factor accelerating the progression of organ fibrosis including liver fibrosis [14] and is the most powerful mediator of HSC activation, a key step in the process of liver fibrogenesis, *in vitro* and *in vivo* [12,13]. This study demonstrated that TGF-β1 exerted an

evident inhibitory effect on PPARγ expression and activity in cultured HSCs and TGF-β1-induced β-catenin pathway mediated the effect of TGF-β1 on PPARγ expression and activity. TGF-β1 could up-regulate β-catenin expression at protein and mRNA levels. Furthermore, our results showed that TGF-β1-induced activation of ERK1/2 resulted in the inactivation of GSK-3β and subsequent stabilization of β-catenin protein. The effect of TGF-β1 on β-catenin expression and on β-catenin protein stability led to the decline in PPARγ expression and trans-activation activity, which contributed to the increase in alpha1(1) collagen and TIMP expression in cultured HSCs. These results indicated for the first time that TGF-β1 could down-regulate PPARγ expression and activity through β-catenin pathway in cultured HSCs.

TGF-β1 triggers multiple signaling pathways in HSCs, such as smad pathway [14], ERK1/2 pathway [23], and p38 MAPK pathway [26]. The existence of functional β-catenin pathway has also been demonstrated in HSC and is related to HSC activation and liver fibrosis [11]. We examined the relationship between TGF-β1 and β-catenin pathway in primary cultured HSCs and found that TGF-β1 evidently increased β-catenin protein level, not only by up-regulation of its expression, but also by maintaining its stability. Recently, it was found that TGF-β1 treatment induced β-catenin pathway in airway smooth muscle cells [27] and in fibroblasts [15] and [28], which were in line with our results. Since TGF-β1 is able to induce ERK1/2 pathway [23] and ERK1/2 appears to be associated with the accumulation of β-catenin (a primary substrate of GSK-3β) in human lung fibroblasts [15], our further study revealed a crosstalk between TGF-β1-induced ERK pathway and TGF-β1-induced β-catenin pathway in cultured HSCs. This result was based on the observations: First, TGF-β1 induced phosphorylation of GSK-3β at Ser9, an inactive state of GSK-3β [15], and [29], which was inhibited by interruption of TGF-β1-induced ERK1/2 pathway. Second, TGF-β1-induced ERK1/2 pathway up-regulated the protein level of β-catenin. Third, treatment with the inhibitor for GSK-3β activity or knockdown of GSK-3β expression increased β-catenin protein level in HSCs. Therefore, TGF-β1 seemed to be able to increase β-catenin protein level through inhibition of GSK-3β activity *via* activation of ERK1/2 pathway in cultured HSCs. Our previous results showed that ERK1/2 mediated leptin down-regulation of PPARγ expression in HSCs [17]. The mediators between ERK1/2 pathway and PPARγ expression were unknown in leptin regulation of PPARγ expression, but β-catenin seemed to be a mediator between ERK1/2 pathway and PPARγ expression in TGF-β1-induced decline in PPARγ expression in HSCs. These results did not exclude other mechanisms by which TGF-β1 regulated β-catenin protein level. Interestingly, when our paper was being prepared, Alhmetshina et al. found that β-catenin mediated TGF-β1-induced dermal fibrosis [30] and revealed that TGF-β stimulated β-catenin signaling in a p38-dependent manner by decreasing the expression of the Wnt antagonist Dickkopf-1. Therefore, there is possibility that Dickkopf-1 might also be correlated with TGF-β1-induced decline in PPARγ expression in HSCs.

NF-kB is an essential signaling pathway involved in conveying inflammatory signals and PPARγ exerts anti-inflammatory effects in many cell types, such as smooth muscle cells, endothelial cells, and macrophages [31,32]. Our results also showed TGF-β1-induced increase in NF-kB activity in HSCs. PPARγ appears to inhibit NF-kB activation in skeletal muscle [33]. The relationship between PPARγ and NF-kB in HSCs is worthy of further investigation.

Our study had indicated that β-catenin pathway mediated TGF-β1 inhibition of PPARγ expression and trans-activation activity in cultured HSCs. As PPARγ is a crucial factor for inhibition of HSC activation [1,6,7], these results might therefore partially explain the mechanisms underlying the involvement of β-catenin pathway in HSC activation and liver fibrosis [11].

Although the results in this report demonstrated β-catenin pathway mediated TGF-β1 inhibition of PPARγ expression and activity in HSCs, induction of β-catenin pathway by LiCl was shown to elevate PPARγ expression and activity in colon cancer cells [34]. Our data in present study cannot explain the discrepancy in HSCs and colon cancer cells. The discrepancy might be duo to the different types of cells. Increased production of type I collagen is a common hallmark of fibrotic diseases in various organs including liver [14]. Activated HSCs are the principal cells to produce type I collagen in fibrotic liver and contribute to the development of liver fibrosis through TGF-β1-stimulated collagen production [14]. Our results further demonstrated that TGF-β1 down-regulation of PPARγ expression through β-catenin pathway led to the increase in alpha1(1) collagen expression at mRNA and protein levels in cultured HSCs. Moreover, TGF-β1-induced decline in PPARγ expression by β-catenin pathway also up-regulated the mRNA levels of TIMP-1 and TIMP-2 (the inhibitors of matrix metalloproteinase).

TGF-β1 is the most powerful mediator of HSC activation [12,13] and PPARγ is a key inhibitor of HSC activation [1,6,7]. TGF-β1 negative-regulation of the expression and activity of PPARγ in cultured HSCs might extend the mechanisms underlying the role of TGF-β1 in HSC activation and liver fibrosis. It should be emphasized that these results in this report were generated from cultured HSCs, and that they might not necessarily and comprehensively reflect the situation in HSCs *in vivo*. Gong et al. demonstrated that disruption of TGF-β1 pathway significantly attenuated chronic hypoxia-induced down-regulation of PPARγ in mouse lung [35], implying that TGF-β1 seems also to exert an inhibitory effect on PPARγ expression *in vivo*. The mechanisms by which TGF-β-induced β-catenin exerts its inhibitory effect on PPARγ expression in HSCs deserves further investigation. Interestingly, ectopic expression of PPARγ can also inhibit TGF-β1 expression in HSCs [6]. This result suggested that there may be a reciprocal relationship between TGF-β1 and PPARγ in HSCs.

## 5. Conclusions

In summary, this report indicated that TGF-β1 down-regulated PPARγ expression and activity through increasing β-catenin expression and stabilizing β-catenin protein in cultured HSCs. The ERK1/2/GSK-3β axis mediated TGF-β1-induced stabilization of β-catenin protein. TGF-β1 inhibition of PPARγ led to increase in the expression of collagen and TIMPs in cultured HSCs. Since TGF-β1 is the most powerful mediator of HSC activation *in vitro* and *in vivo* [12,13] and plays a central role in liver fibrosis [14], the data in this report might provide novel insights into the molecular mechanisms underlying the role of TGF-β1 in HSC activation and liver fibrosis.

## Conflict of interest

The authors have no any conflicts of interest to disclose.

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