

LPS-Induced Epithelial-Mesenchymal Transition of Intrahepatic Biliary Epithelial Cells

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Background. Recent studies have revealed that the epithelial-mesenchymal transition (EMT) of bile duct epithelial cells is engaged in hepatic fibrogenesis. However, the association between etiological factors of liver disease such as virus or bacterial infection and EMT remains to be investigated. The present study focuses on the inductive role of endotoxin, the main component of the cell wall's ectoblast of gram-negative bacteria, in the EMT of human intrahepatic biliary epithelial cells (HIBEpiCs).

Methods. The expressions of E-cadherin, S100A4, α -SMA, TGF- β 1, and Smad2/3 in HIBEpiCs cultured with or without lipopolysaccharide LPS, were detected by real-time PCR and Western blotting. We blocked the expression of TGF- β 1 using paclitaxel and knocked down Smad2/3 by siRNA to explore the role of TGF- β 1/Smad2/3 pathway in the EMT of HIBEpiCs.

Results. Resting HIBEpiCs showed epithelioid cobblestone morphology, and underwent a phenotypic change to produce bipolar cells with a fibroblastic morphology when co-cultured with LPS. After LPS stimulation and the up-regulation of mRNA and protein expression of TGF- β 1 and Smad2/Smad3, the mRNA and protein expression of mesenchymal markers (S100A and α -SMA) increased significantly. Paclitaxel inhibited the mRNA and protein expression of TGF- β 1 *in vitro*. Knock-down of Smad2/3 by siRNA led to up-regulation of epithelial markers E-cadherin and down-regulation of S100A and α -SMA, indicating a reversal of the EMT.

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Conclusions. LPS can induce the expression of TGF- β 1 and a subsequent EMT in HIBEpiCs, and the inhibition of TGF- β 1 or Smad 2/3 could reverse this EMT, suggesting that LPS may play a potential role in the EMT of HIBEpiCs. © 2010 Elsevier Inc. All rights reserved.

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INTRODUCTION

Repeated biliary infection is a characteristic feature of a range of chronic inflammatory liver diseases including hepatolithiasis (HL) and biliary stricture or obstruction. Biliary chronic inflammation plays an important role in liver fibrosis or even cirrhosis. The causative organism of biliary infection is often gram-negative bacteria, which could release endotoxin and induce inflammatory reaction. Studies have showed that when the IBECs are exposed to a high level of lipopolysaccharide during bacterial infections, IBECs often show cellular damage and pathologic proliferation. This response of IBECs plays a role in the progression of liver fibrogenesis or even cirrhosis [1].

Although the activation of hepatic stellate cells remains a central event in liver fibrosis, the role of epithelial-mesenchymal transition (EMT) of both hepatocytes and cholangiocytes in liver fibrosis is attracting great attentions. EMT has been implicated in a variety of biological processes such as fibrogenesis, embryonic development, and tumor progression. Recent clinical and animal studies have demonstrated that bile duct epithelial cells can undergo EMT, thereby contributing to hepatic fibrosis [2–5]. Consistent with these studies, our previous retrospective clinical study shown that we observed the stimulation of the TGF- β 1/smad2/3



TABLE 1
Primer Sequences Used for RT-PCR Amplification

Primers (mRNA)	Sequences	Tm(°C)	Size (bp)
E-cadherin	Sense:5'-GCCCCGCCTTATGATTCTCTGC-3' Antisense:5'-CTCGCCGCCTCCGTACATGTC-3'	53°C	171 bp
S100A4	Sense:5'-GGCTGCCAACC GGAACAATG-3' Antisense:5'-GCAGGCGCAATAGTGTCT-3'	53°C	250 bp
α-SMA	Sense:5'-CGGGCATCCACGAAACCACC-3' Antisense:5'-CTGGGTGCGAGGGCTGTGAT-3'	58°C	154 bp
Smad2/3	Sense:5'-TCCACCAGGCTGTAATCTGAAGA-3' Antisense:5'-GACATGCTTGAGCAACTGACT-3'	53°C	273 bp
GAPDH	Sense:5'-ACCCATCACCATCTCCAGGAG-3' Antisense:5'-GAAGGGGCGGAGATGATGAC-3'	58°C	159 bp

signaling pathway followed by the loss of epithelial markers and the acquirement of mesenchymal markers in bile duct epithelial cells from patients with primary hepatolithiasis (Table 1).

Transforming growth factor beta1 (TGF-β1) is known to be the most potent inducer of EMT, and it initiates morphological transition of the cells from an epithelial to a fibroblastic appearance, accompanied by a loss of epithelial cell markers such as E-cadherin and a gain of mesenchymal cell markers such as vimentin, fibronectin, and N-cadherin [6, 7] However, how the TGF-β1 is induced in chronic liver diseases remains unclear. As infection is a common pathogenic process of chronic liver disease that mainly involves bile duct injury, and also participates in liver fibrosis, we hypothesized that biliary infection is related to the induction of TGF-β1-mediated EMT and subsequent fibrogenesis. Therefore, in this study, primary IBECs were cultured with LPS to investigate whether these can activate TGFβ/Smads signaling pathway and induce EMT.

MATERIALS AND METHODS

Antibodies and Reagents

Antibodies and reagents included anti-TGF-β1, anti-E-cadherin, anti-S100A4, anti-p-Smad 2/3, and anti-α-SMA (DAKO, Glostrup, Denmark); Smad2/3 siRNA and control siRNA (Santa Cruz Technology, Santa Cruz, CA); paclitaxel and LPS (Sigma Chemical Co.); Takara PrimeScript TMRT-PCR Kit (Takara Biotechnology Co., Dalian, China); RIMP1640 (Sigma Chemical Co.); and Trizol (Invitrogen).

Cell Culture

Human intrahepatic Biliary Epithelial Cells (HIBEpiCs) were purchased from the Sciencell Research Laboratories. HIBEpiCs were cultured in RIMP1640 supplemented with 10% fetal bovine serum (FBS), at 37 °C in a 5% CO₂ humidified atmosphere. Because TGF-β1 mRNA was markedly up-regulated when the concentration of LPS reached 2 μg/mL *in vitro* [8], cells were stimulated with LPS at a concentration of 2 μg/mL at different time points.

Small Interference RNA Transfection

One day before transfection, cells were plated in a 24-well plate at the density of 2 × 10⁵/well. When the cells were 80%–90% confluent, the Smad siRNA and control siRNA were transfected according to the manual of Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the medium was changed with fresh RIMP1640 containing 10% FBS. Then, the cells were incubated at 37 °C in a CO₂ incubator for 24 h before the cells were collected for further investigation. Efficiency of transfection was detected according to the fluorescence density of enhanced green fluorescent protein (EGFP).

Real-Time PCR

Quantitative PCR analyses were performed using SYBR Green detection. The transcript level of each gene was calculated using the $\Delta\Delta CT$ method. The formula used is: fold induction = 2^{-[$\Delta\Delta CT$]}, where $\Delta\Delta CT = [Ct \text{ gene (unknown sample)} - Ct \text{ GAPDH (unknown sample)}] - [Ct \text{ gene (calibrator sample)} - Ct \text{ GAPDH (calibrator sample)}]$. Here, we used untreated HIBEpiCs as the calibrator sample. The primer sets used to detect E-cadherin, S100A4, α-SMA, TGF-β1, and Smad2/3 in HIBEpiCs and the relevant PCR conditions are shown in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. Each experiment was repeated for four times.

Western Blotting

Protein concentration was determined by BCA Assay Kit (Beyotime Institute Biotechnology, Haimen, China). An equal amount of protein was loaded and separated on a 12% SDS polyacrylamide gel. After electrophoresis, proteins were transferred to a PVDF membrane. The membrane was incubated with 5% defatted milk overnight at 4 °C. Then, it was incubated with a primary antibody (E-cadherin 1:200, S100A4 1:200 or α-SMA1:200) for 2 h, followed by incubation in a secondary antibody for 2 h at room temperature. After incubating with luminescent reagents for 5 min, the membrane was exposed to Kodak film. Images were scanned for quantitative analysis of each band. The results were determined by taking the ratios of the gray values of target genes and β-actin. Each experiment was repeated at least three times.

Statistical Analysis

All data were analyzed using the software SPSS 13.0 (Statistical Package for Social Sciences; SPSS, Munich, Germany). Statistical analysis was performed using one-way analysis of variance (ANOVA), and if the null hypothesis was rejected, *post hoc* Turkey's tests were performed for multiple comparisons. A *P* value < 0.05 was considered statistically significant.

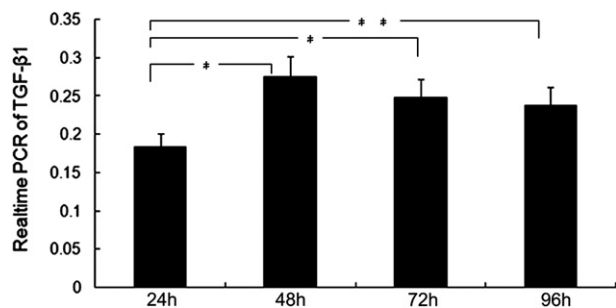


FIG. 1. Effects of LPS on TGF- β 1 mRNA expression by HIBEpiCs. After exposure to LPS (2 μ g/mL) for the indicated time, the TGF- β 1 mRNA level in HIBEpiCs was determined by real-time PCR. After more than 48 h treated with LPS, TGF- β 1 mRNA level was significantly higher than that within 24 h. The data represent results of four independent experiments. * $P < 0.01$; ** $P < 0.05$.

RESULTS

Effects of LPS on TGF- β 1 mRNA Expression by HIBEpiCs

Twenty-four hours after the administration of LPS, marked TGF- β 1 mRNA was detected. The expression of TGF- β 1 mRNA reached the peak at 48 h, but the mRNA level decreased after 72 h. These results suggest that the expression of TGF- β 1 was induced by LPS and reached a peak value at 48 h (Fig. 1 * $P < 0.01$; ** $P < 0.05$). Therefore, we detected the expression of proteins involved in the EMT process at 48 and 72 h in the following experiments.

LPS-induced expression of E-cadherin, S100A4, and α -SMA in HIBEpiCs

Resting HIBEpiCs showed epithelioid cobblestone morphology, and underwent a phenotypic change to produce bipolar cells with a fibroblastic morphology when co-cultured with LPS. Along with the exposure to LPS, the mRNA of epithelial markers E-cadherin decreased gradually (Fig. 2A, $P < 0.01$), whereas the mesenchymal markers (S100A and α -SMA) increased significantly (Fig. 2B and C, $P < 0.01$). However, the expression of markers S100A4 and α -SMA at different time points did not show any difference ($P > 0.05$).

Consistent with the results of Western blotting, the protein level of E-cadherin decreased (Fig. 3A, $P < 0.05$), whereas S100A and α -SMA increased (Fig. 3B and C, $P < 0.05$).

Effects of PT on TGF- β 1/Smad signaling

Administration of paclitaxel alone did not change the expression of TGF- β 1 mRNA. However, pretreatment with paclitaxel before administration of LPS significantly down-regulated the mRNA of TGF- β 1, Smad2/3, especially at 48 h, when the mRNA of TGF- β 1, Smad2/3 decreased by 60%, 36%, and %28%, respectively

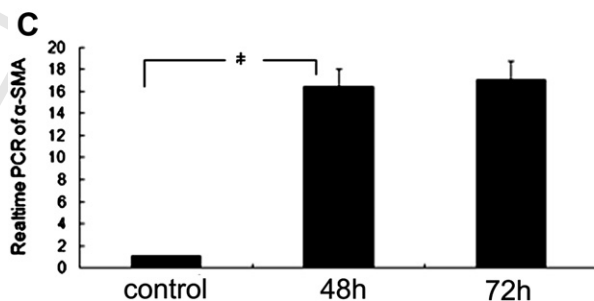
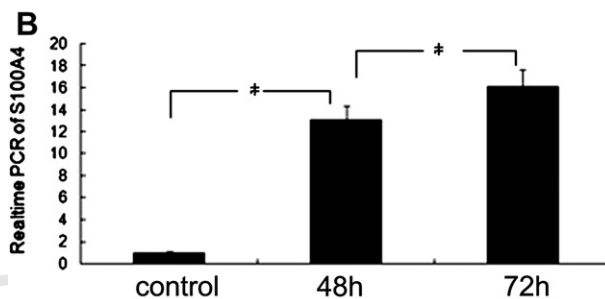
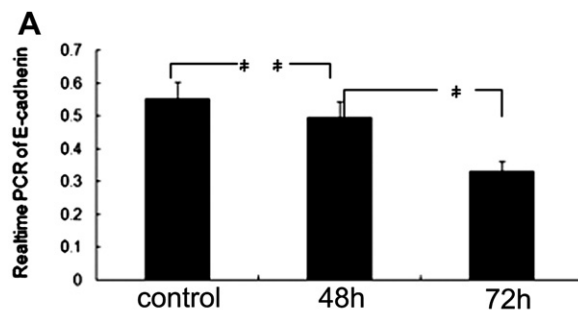


FIG. 2. LPS-induced mRNA expression of E-cadherin (A), S100A4 (B), and α -SMA (C) in HIBEpiCs. Along with the exposure to LPS, the mRNA of epithelial markers E-cadherin decreased gradually, whereas the mesenchymal markers (S100A and α -SMA) increased significantly. The data represent results of four independent experiments. * $P < 0.01$; ** $P < 0.05$.

(Fig. 4A, B, and C, $P < 0.05$). These results indicated that PT was not a specific antagonist of Smad2/3 because the blockage of Smad2/3 by PT was not as efficient as that of TGF- β 1 ($P > 0.05$). We assumed that there were other proteins besides Smad 2/3 involved in the reduced transcription of TGF- β 1 that resulted from PT treatment.

Knock-Down of Smad2/3 Affected the Expression of EMT Markers

Forty-eight hours after transfection with siRNA Smad2/3, the fluorescence intensity reached its peak, but the fluorescence intensity gradually weakened after 72 h. The transfection efficiency of HIBEpiCs was 78% (data not shown). The results of real-time PCR showed that the levels of Smad 2/3 mRNA in transfected cells were significantly decreased by more than 90% at both 48 and 72 h (Fig. 5A, $P < 0.05$) and that

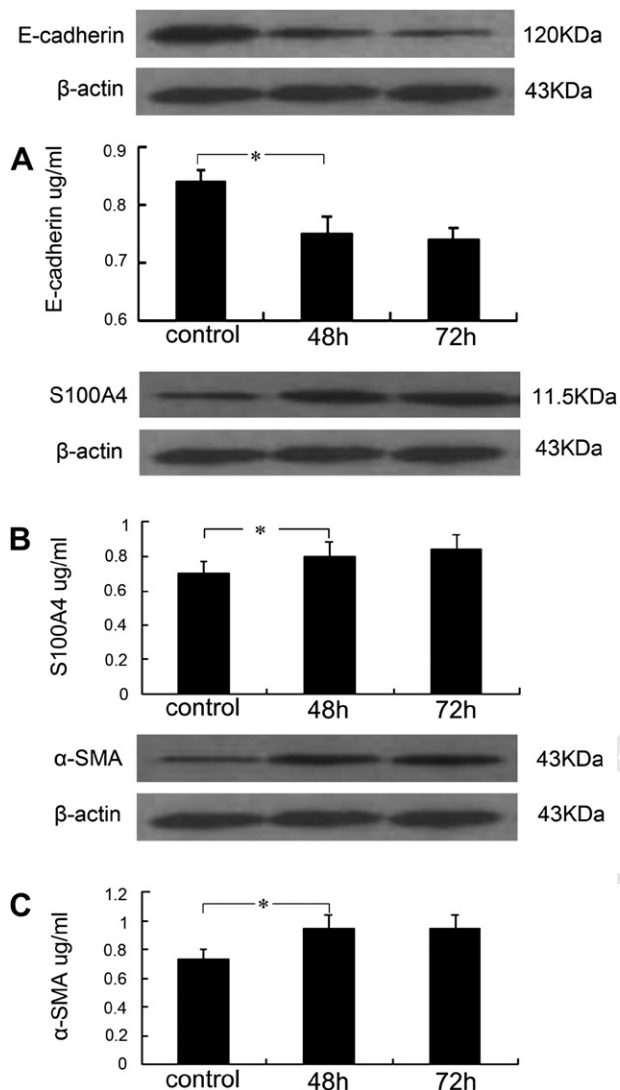


FIG. 3. LPS-induced protein expression of E-cadherin, S100A4, and α -SMA in HIBEpCs. Western blot analysis of epithelial marker E-cadherin (A), mesenchymal markers S100A4 (B), and α -SMA (C). With stimulation of LPS, the protein levels of epithelial markers E-cadherin decreased significantly, whereas the mesenchymal markers (S100A and α -SMA) increased dramatically. The data represent results of four independent experiments. * $P < 0.01$.

the mRNA of epithelial marker E-cadherin was significantly up-regulated. Both at 48 and at 72 h, E-cadherin mRNA increased by 64% and 20%, respectively (Fig. 5B). Expression of mesenchymal markers S100A4 and α -SMA was significantly reduced (Fig. 5B and C), suggesting that Smad 2/3 played a key role in the TGF- β /Smad signaling pathway.

Forty-eight to 72 h after the transfection of siRNA to Smad2/3, the level of E-cadherin protein in HIBEpCs increased by 40% and 20%, respectively (Fig. 6B). The level of S100A4 protein decreased by 21% and 41%, and α -SMA protein decreased by 35% and 44% (Fig. 6C and D). Inhibition of S100A4 and α -SMA at

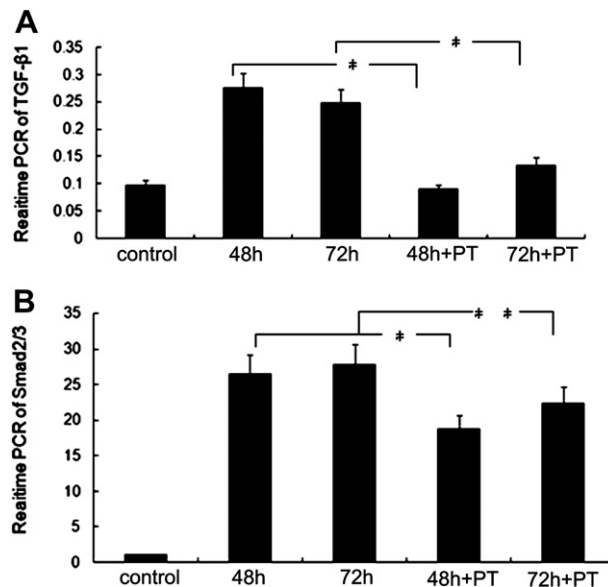


FIG. 4. mRNA expression of TGF- β 1 and Smad2/3 after PT treatment in HIBEpCs. It is shown that paclitaxel significantly inhibited the expression of the TGF- β 1 and Smad2/3 mRNA level in HIBEpCs co-cultured with LPS. The data represent results of four independent experiments. * $P < 0.01$; ** $P < 0.05$.

72 h was more efficient than that at 48 h ($P < 0.01$), but the expression of E-cadherin showed no significant difference between the two time points ($P > 0.05$).

DISCUSSION

In this study, stimulation with LPS causes a TGF β /Smads-mediated EMT of HIBEpCs. These results indicated that biliary infection played a potential role in inducing EMT of bile duct epithelial cells and subsequent peribiliary fibrosis. Biliary infection is an independent risk factor for liver injury and fibrosis. However, the exact molecular mechanism remains unclear. Bacteria, mainly gram-negative bacteria, retrograde into the biliary tract through cholangiopancreatic ampulla. LPS, endotoxin of gram-negative bacteria, interact with its membrane receptors and mediate pathologic changes in the infected cell through signal transduction. LPS not only activates endothelial cells, smooth muscle cells, fibroblasts, and mononuclear macrophages, but also induces synthesis and release of proinflammatory cytokines. Administration of LPS stimulates expression of fibroblast markers of gallbladder epithelial cells [8]. Meanwhile, the EMT is a common phenomenon in cultured IBECs and primary biliary cirrhosis, and is involved in the pathogenesis of biliary diseases and liver fibrosis. In this study, LPS was administrated to HIBEpCs to simulate conditions of biliary infections. Both the down-regulated expression of epithelial markers and the up-regulated expression of

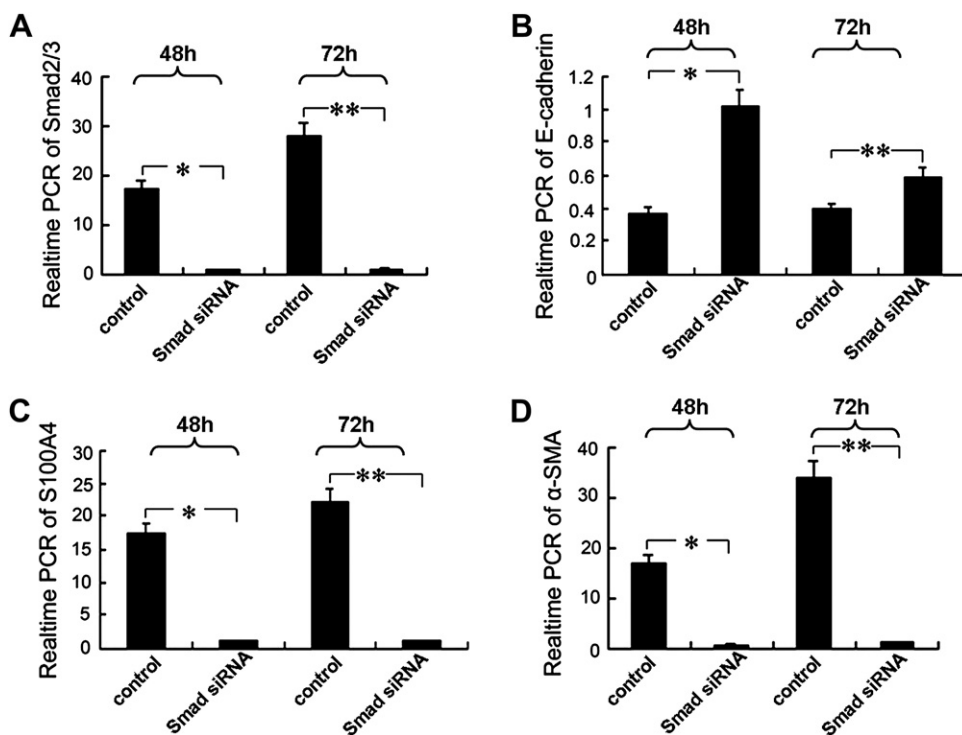


FIG. 5. Effects of Smad2/3 siRNA on mRNA expression of EMT markers. The results of real-time PCR showed that in HIBEpiCs transfected with Smad 2/3 siRNA the Smad 2/3 mRNA levels were significantly decreased, the mRNA levels of epithelial marker E-cadherin were significantly up-regulated, and the mRNA levels of mesenchymal markers S100A4 and α -SMA were significantly reduced. The data represent results of four independent experiments. * $P < 0.01$; ** $P < 0.05$.

mesenchymal markers indicate the existence of the EMT. These results suggest that when infected, the bile duct epithelial cells undergo EMT and may participate in the process of liver fibrosis.

TGF- β 1 is known to be the most potent inducer of EMT. Meanwhile, TGF- β 1 is recognized as one of the most important liver fibrosis inducers [9]. It is known that Smad2 and Smad3 are direct downstream molecules in TGF- β 1 transduction, and Smads play an important role in the TGF- β 1 signaling pathway [10, 11]. Activation of the TGF- β receptor induces Smad 2/3 phosphorylation. Then, the phosphorylated Smad 2/3 forms a heteromeric complex with Smad4, translocates into the nucleus, and regulates related gene expression. When TGF- β was added to the medium of human intrahepatic biliary cells, biliary cells started to express S100A4, α -SMA, fibrin, and developed to be fibroblasts. These studies suggest that the EMT of biliary epithelial cells occurs under certain conditions in the biliary system and that it is regulated by the TGF- β 1/Smad2/3 signaling pathway. In this study, we found that BECs regulated their response to LPS through autocrine TGF- β 1. As both autocrine and paracrine TGF- β 1 is secreted with a positive feedback in the progression of liver fibrosis [12], we speculate that both LPS and TGF- β 1 promote BEC to release TGF- β 1 and that there is a positive feed-

back between BEC and TGF- β 1. Increased TGF- β 1 induces the expression of Smad 2/3 and downstream fibrosis markers, leading to the accumulation of extracellular matrix and, ultimately, to fibrosis. At the same time, autocrine or paracrine plasminogen factor, which is released in response to TGF- β 1, promotes HIBEpiCs to secrete additional TGF- β 1 to start the transformation of epithelial cells.

An important feature of EMT is its potential for reversibility. A number of factors can reverse the EMT. In the renal fibrosis model, BMP-7, one of the TGF- β family members, blocks TGF- β 1 signal transduction and, thus, reverses renal EMT [13]. Hepatocyte growth factor can reverse the EMT in the ligation-induced liver fibrosis model and can alleviate liver fibrosis [2, 14, 15]. Some reagents can inhibit the occurrence of the EMT in biliary epithelial cells after orthotopic liver transplantation [16]. PT, first isolated from the bark of the Pacific yew tree, is a mitotic inhibitor used in cancer chemotherapy. It has been found that PT-related pathways overlap with LPS-related pathways and that TGF- β 1 is on the crossroad. Previous studies have shown that the anti-fibrosis effect of paclitaxel is mediated by inhibiting the release of TGF- β 1 of HIBEpiCs and gallbladder myofibroblasts (GBMFs) as well as by blocking the synthesis of type I collagen in GBMF and subepithelial myofibroblasts of the gallbladder wall. Consistent

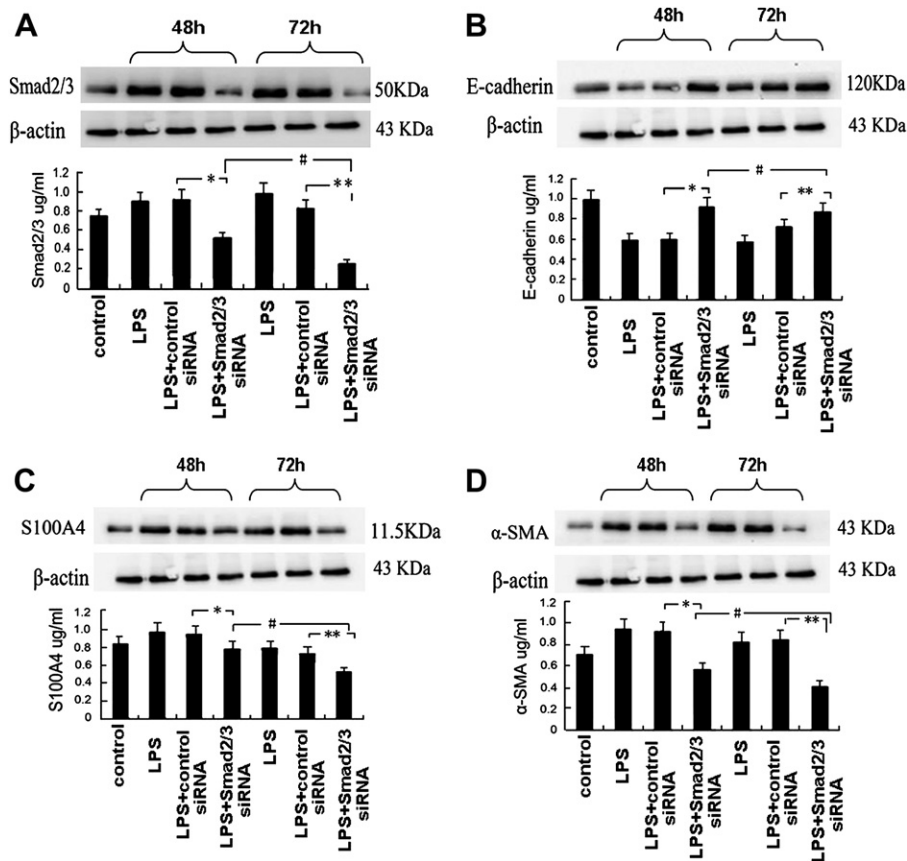


FIG. 6. Western blotting for EMT markers after knockdown of Smad2/3. Relative protein levels of Smad2/3 (A), E-cadherin (B), S100A4 (C), and α -SMA (D) were analyzed by Western blotting in HIBEpiCs. Forty-eight to 72 h after transfection with Smad 2/3 siRNA, the E-cadherin protein increased dramatically in HIBEpiCs co-cultured with LPS. After transfection with Smad 2/3 siRNA, protein levels of S100A4 and α -SMA decreased in HIBEpiCs co-cultured with LPS. The data represent results of four independent experiments. * $P < 0.01$; ** $P < 0.05$.

with these studies, we observed that administration of paclitaxel leads to up-regulation of epithelial markers and down-regulation of mesenchymal markers, resulting in a reversal of the EMT. These results indicate that PT is a potential agent for the treatment of fibrosis around the bile ducts in patients with HL.

However, there are some limitations in this study, which needs further investigation, such as the difference between *in vitro* and *in vivo* experiments, whether *in vitro* administration of LPS is a truly representative model for chronic biliary lithiasis, and its associated chronic inflammation remain to be elucidated. Additionally, the administration of LPS alone may not sufficiently reflect the complexity of the *in vivo* processes of biliary infection, since a number of other inciting factors could induce biliary inflammation.

In conclusion, we found that LPS induced the EMT of HIBEpiCs *in vitro*, probably through the TGF- β 1/Smad2/3 pathway. PT inhibited the expression of TGF- β 1 and Smad2/3 and, thus, reversed the EMT of HIBEpiCs. Our findings provide a potential therapeutic target and strategy for the treatment of fibrous biliary

diseases, such as cholecystolithiasis, primary hepatolithiasis, and primary sclerosing cholangitis.

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SUPPLEMENTARY DATA

Supplementary data associated with the article can be found in the online version, at doi:10.1016/j.jss.2010.04.059.

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