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## Coexpression of Smad7 and UPA attenuates carbon tetrachloride-induced rat liver fibrosis

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### Summary

**Background:**

There is a great need for developing novel therapies to treat liver fibrosis. Previous studies showed that both Smad7 and uPA were inhibitors of liver fibrosis. Therefore, we explored the therapeutic effects of combinational gene therapy with Smad7 and uPA on CCl<sub>4</sub>-induced liver fibrosis.

**Material/Methods:**

Smad7 and uPA genes were cloned into an adenovirus vector. To observe the therapeutic effects of coexpression of Smad7 and uPA genes, the recombinant adenovirus were delivered into CCl<sub>4</sub>-induced fibrosis models. Fibrillar collagen, hydroxyproline,  $\alpha$ -SMA, TGF- $\beta_1$ , MMP-13, TIMP-1, HGF and PCNA were detected to evaluate the fibrosis and to explore the mechanisms underlying the treatment with Smad7 and uPA.

**Results:**

The results showed that single Smad7 or uPA adenovirus reduced CCl<sub>4</sub> induced liver fibrosis significantly; while combination of Smad7 and uPA had more significant therapeutic effect on CCl<sub>4</sub> induced liver fibrosis. Then the markers underlying the therapeutic effect of combination of Smad7 and uPA were also explored. Over-expression of Smad7 and uPA inhibited the expression of  $\alpha$ -SMA and TGF- $\beta_1$  significantly. Combinational gene therapy also enhanced extracellular matrix degradation by increasing the expression of MMP-13, inhibiting TIMP-1 expression, and promoted hepatocyte proliferation, while single Smad7 or uPA only induced part of these changes.

**Conclusions:**

These results suggest that combinational gene therapy with Smad7 and uPA inhibited CCl<sub>4</sub>-induced rat liver fibrosis by simultaneously targeting multiple pathogenic pathways.

**key words:**

**liver fibrosis • gene therapy • Smad7 • urokinase plasminogen activator**

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## BACKGROUND

Liver fibrosis is a wound-healing response caused by reiterated liver tissue injury due to hepatitis B virus or hepatitis C virus infection, alcohol abuse, nonalcoholic steatohepatitis, toxin/drug-induced injury, and autoimmune damage. The main characteristic of liver fibrosis is the excessive accumulation of extracellular matrix (ECM) proteins, including collagens I and III, fibronectin, laminin, and proteoglycans. Advanced liver fibrosis leads to liver cirrhosis, which is a major cause of mortality and a worldwide public health challenge. Liver fibrosis involves a complex mechanism by which tissue injury factors activate fibrogenetic cells, especially hepatic stellate cells (HSC), to proliferate and secrete ECM proteins, cytokines, and enzymes. All of these alterations lead to increased ECM protein deposition, decreased ECM degradation, and impaired hepatocyte function [1]. Therefore, the ideal antifibrotic strategy to treat liver fibrosis should include inhibition of fibrogenesis, acceleration of fibrolysis, and stimulation of hepatocyte regeneration. Currently, no drugs are available to inhibit liver fibrosis completely; therefore, a gene therapy strategy represents a novel therapeutic approach to treat liver fibrosis. The majority of gene therapy strategies tested for the treatment of liver fibrosis used only a single gene to inhibit the fibrotic process; however, since the pathogenesis of liver fibrosis is multifaceted and involves multiple pathways and genes, it is likely that a combined 2-gene approach, which targets 2 separate pathways involved in liver fibrosis, will be more effective than a single-gene treatment. Thus, we investigated the antifibrotic effects of a 2-gene therapeutic approach in this study.

Many profibrogenic cytokines are involved in liver fibrosis, among which TGF- $\beta_1$  plays a pivotal role in initiating and sustaining the fibrogenesis of the liver [2]. Transgenic mice that overexpress active human TGF- $\beta_1$  develop severe fibrosis of the liver [3]. TGF- $\beta_1$  contributes to liver fibrosis in 2 ways: (1) by increasing ECM deposition, and (2) by protecting the matrix from degradation and inducing hepatocyte apoptosis [4]. TGF- $\beta_1$  downregulates the expression of several matrix metalloproteinases (MMPs), including MMP-1 [5] and MMP-9 [6], and upregulates the expression of both tissue inhibitor of metalloproteinases 1 (TIMP-1) [7] and plasminogen activator inhibitor-1 (PAI-1) [8]. The intracellular signal transduction of TGF- $\beta_1$  is mediated by Smad proteins. After being phosphorylated by the activated TGF- $\beta$  type I receptor (TGF $\beta$ RI), Smad2 and Smad3 form a complex with Smad4, and this complex translocates into the nucleus where it acts as a transcriptional activator of target genes. In contrast, Smad7 is a negative regulator of this signaling pathway [9]. However, in activated HSCs, TGF- $\beta_1$ -induced Smad7 expression is diminished [10]. Therefore, upregulating Smad7 expression may inhibit TGF- $\beta_1$ -induced fibrogenesis, which has been shown to occur in the liver, lung, and kidney.

Recently, the urokinase plasminogen activator (uPA) system, consisting of the serine protease uPA, its inhibitors (PAI-1 and PAI-2), and receptor [urokinase plasminogen activator receptor (uPAR)], has also been implicated in the inhibition of liver fibrosis. Originally, uPA was thought to be a serine protease that converted inactive plasminogen into active plasmin, which can degrade ECM components directly or indirectly by activating the MMPs. However, new data

suggest that uPA also participates in cell proliferation, adhesion, migration, and angiogenesis by interacting with the uPAR in a plasmin-independent manner [11,12]. Ligation of the uPAR activates growth factors, such as fibroblast growth factor-2 (FGF2), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and hepatocyte growth factor (HGF) [13,14]. Recently, studies of knockout mice with an inactive uPA system indicated that uPA and plasminogen play a critical role in the liver repair process after injury. uPA gene knockout (uPA $-/-$ ) mice had abnormally high fibrogenesis, developing fibrin deposits in the skin, gastrointestinal tract, and the hepatic sinusoids [15]. The roles of uPA and plasminogen have also been investigated in rats using carbon tetrachloride (CCl<sub>4</sub>) or Fas-induced acute liver injury. In CCl<sub>4</sub> induced acute liver injury, lack of uPA [16] or plasminogen [17] led to the accumulation of fibrin and fibronectin within injured areas, insufficient removal of necrotic cells, and delayed repair. Similarly, after Fas-induced liver injury, uPA $-/-$  mice show delayed HGF maturation and hepatic regeneration [18]. Plasminogen deficiency also leads to excessive matrix accumulation and prominent activation of HSCs after chronic liver injury [19]. Further, PAI-1-deficient mice show accelerated wound closure [20]. In cultured HSC [21] and cirrhotic liver [22], there is decreased uPA expression concomitant with an increase in PAI-1 expression, which leads to low uPA activity and failure to resolve the fibrotic scarring.

Since both Smad7 and uPA are important inhibitors in liver fibrosis, then simultaneously increasing Smad7 and uPA expression in fibrotic liver tissues may inhibit liver fibrosis by inhibiting 2 different pathways involved in fibrotic damage. Adenovirus based vectors are the most popular vectors for gene therapy to treat the liver diseases, including liver fibrosis; however, high doses of adenovirus can cause severe adverse effects, including hepatic injury [23]. Thus, development of a single adenovirus vector for the expression of both Smad7 and uPA is a superior strategy to the use 2 separate vectors. Recently, bicistronic adenoviral vectors, utilizing an internal ribosome entry site (IRES) to direct the expression of 2 heterologous genes, have gained broad use [24]. In this study, we investigated the therapeutic effects of a bicistronic adenovirus vector co-expressing Smad7 and uPA to treat liver fibrosis and the possible mechanisms targeted by these 2 genes to limit fibrogenesis.

## MATERIAL AND METHODS

### Construction of the recombinant adenovirus AdSmad7-uPA

The recombinant adenovirus was constructed with routine molecular cloning techniques. Briefly, the cDNA fragments of rat Smad7 and uPA were obtained from pT<sub>Smad7</sub> and kidney RNA, respectively. The Smad7 and uPA cDNA were inserted into multiple cloning site (MCS) A and MCS B of the pIRES plasmid, respectively (Clontech, CA, USA). The pIRES plasmid contains an IRES from the encephalomyocarditis virus (ECMV), which allows translation of 2 consecutive open reading frames from the same messenger RNA (mRNA) [25,26]. Then, the bicistronic Smad7 and uPA expression cassette (Smad7-IRES-uPA) was inserted into an adenoviral shuttle plasmid. Following co-transformation of BJ5138 *E. coli* with the backbone plasmid, Adeasy-1, and

the shuttle plasmid, the adenoviral plasmid carrying Smad7 and uPA was generated by homologous recombination and the adenovirus was packaged in AD-293 cells (Stratagene, CA, USA). The adenovirus carrying Smad7, uPA or Smad7 and uPA were named as AdSmad7, AduPA, or AdSmad7-uPA, respectively. The recombinant adenovirus was purified by CsCl gradient centrifugation, and dialyzed against phosphate-buffered saline (PBS) plus 10% glycerol. The viral titers were determined by an end-point dilution assay. The adenovirus vectors AdGFP was used as controls. Correct construction of the vectors were determined by restriction enzyme digest and sequencing.

### Cell culture and adenovirus transduction

The L02 human hepatocyte cell line was seeded at  $1 \times 10^5$ /cm<sup>2</sup> and cultured for 24 h, then transduced with AdSmad7-uPA, AdSmad7, AduPA, at multiplicity of infection (MOI) of 20 particles per cell in minimal DMEM with 5% fetal bovine serum (FBS) for 4 h. The cells were then maintained in serum-free DMEM for 48 h. Cell lysates and supernatant serum-free medium were collected and stored at  $-80^\circ\text{C}$  for later analysis.

### Immunoblot analysis

Smad7, TGF- $\beta_1$ ,  $\alpha$ -SMA and HGF- $\beta$  protein expression levels were examined by immunoblot as described previously [18]. Cells and liver tissues were lysed in RIPA buffer, which contained 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and protease inhibitors, at  $4^\circ\text{C}$ , followed by centrifugation at 1000g for 30 minutes. The supernatant was collected and the protein concentration determined using the BCA assay. Protein samples (20  $\mu\text{g}$ ) were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, USA) using a semi-dry transfer cell. The membrane was blocked in 5% nonfat milk in Tris-buffered saline plus 0.1% Tween 20 (TBST), and incubated with a Smad7-specific antibody (Santa Cruz Biotechnology, CA, USA) diluted 1:1,000, HGF- $\beta$ -specific antibody diluted 1:1,000 (Santa Cruz Biotechnology, CA, USA), TGF- $\beta_1$  antibody diluted 1:1000 (Cell signaling Technology, MA, USA),  $\alpha$ -SMA antibody diluted according to manual (SIGMA, MO, USA) or  $\beta$ -actin-specific antibody diluted 1:2,000 (Santa Cruz Biotechnology, USA) overnight at  $4^\circ\text{C}$ . The membranes were then incubated with the appropriate HRP-conjugated secondary antibodies and the proteins visualized using an enzyme chemiluminescence (ECL) detection system (Pierce, Rockford, IL, USA). Band intensities were quantified using Quantity One 4.6.2 analysis software.

### ELISA

uPA secretion in cell culture supernatants and liver tissues was measured using a rat uPA ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

### Zymography

Proteolytic activity of the recombinant uPA was shown by zymography as described previously [27]. The media samples were separated by SDS-PAGE on 12% gels containing

$\alpha$ -casein (7 mg/ml) and human glu-plasminogen (Sigma, St. Louis, MO, USA, 20 mg/ml). The gels were then washed in 1% Tween 80 for 1 h at  $37^\circ\text{C}$  and subsequently incubated in PBS containing 0.1% Tween 80 overnight at room temperature. Then the gels were stained with Coomassie blue and destained in a solution of 10% acetic acid and 50% methanol. The bands displaying proteolytic activity were determined by comparison with protein molecular weight marker.

### Animals and experimental design

Male Sprague-Dawley rats weighing 200 to 250 g were used in this study. All animal experiments were performed in accordance with our institutional guidelines. Liver fibrosis was induced by injecting CCl<sub>4</sub> subcutaneously (3 ml/kg as a 2:3 mixture with olive oil) every 3 days for a total of 8 weeks and mock-treated animals were injected with olive oil alone as a negative control. At 2 and 4 weeks after the first CCl<sub>4</sub> injection, 0.5 ml ( $5 \times 10^9$  pfu) AdSmad7-uPA, AdSmad7, AduPA, AdGFP, or saline (control group) was injected into the tail veins of 11 rats per group. To examine the efficiency of adenoviral gene transfer to the liver, 3 days after the first injection of adenovirus, liver tissue samples were obtained randomly from 3 rats injected with AdSmad7-uPA, AdSmad7, AduPA, or AdGFP. The remaining animals were euthanized at the ninth week, and liver tissues were collected. The liver tissue samples of the right median lobe were snap-frozen in liquid nitrogen or fixed in 10% buffered formalin.

### Histology and immunohistochemistry

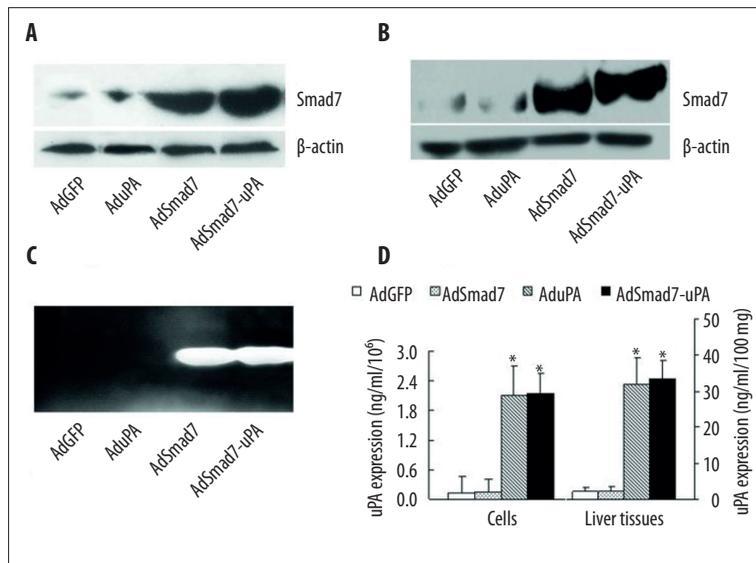
The liver tissues were fixed and embedded in paraffin. Five-micrometer sections were stained with hematoxylin-eosin and 0.1% Sirius red in saturated picric acid [28]. Immunohistochemical staining was performed by dewaxing slides in xylene and dehydrating in alcohol. Antigen retrieval was achieved by a 500-W microwave, heating the sections in citric saline for 15 min. After blocking endogenous peroxidases by 3% hydrogen peroxide for 10 minutes, sections were incubated with anti-Smad7 antibody diluted 1:400 (Santa Cruz Biotechnology, CA, USA),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) antibody diluted 1:500 (Santa Cruz Biotechnology, USA), TGF- $\beta_1$  antibody diluted 1:400 (Santa Cruz Biotechnology, USA), and proliferating cell nuclear antigen (PCNA) antibody diluted 1:300 (Boster, Wuhan, China) for 1 h at room temperature. After washing in PBS, sections were incubated with the appropriate peroxidase-conjugated secondary antibody for 20 min. Positive staining visualized using 3, 3'-diaminobenzidine tetrahydrochloride (DAB). Quantitative analysis of fibrosis and immunopositive cell area was carried out with the Image-Pro plus image software in 5 microscopic fields of specimens taken from 8 rats.

### Hydroxyproline assay

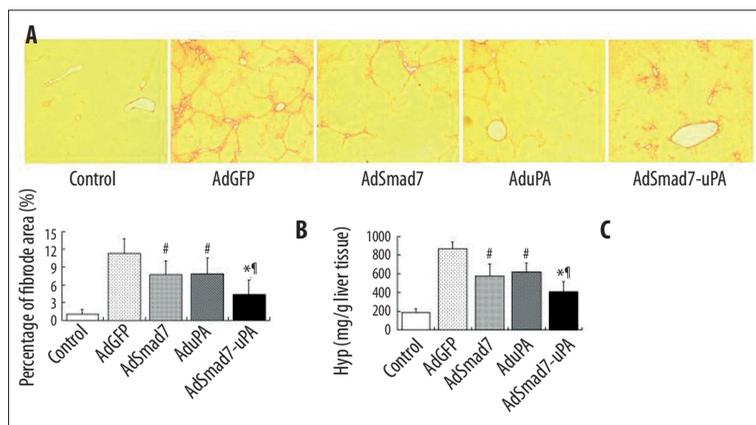
Liver tissue (100 mg) samples were subjected to acid hydrolysis to determine the amount of hydroxyproline, as previously described [29]. The hydroxyproline content was indicated as micrograms per gram of wet liver.

### Determination of mRNA level by real-time PCR

Total RNA was extracted from the liver with chloroform and Trizol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was



**Figure 1.** Expression of Smad7 and uPA *in vitro* and *in vivo*. Smad7 expressions were detected by western blot assay in (A) L02 cells and (B) liver tissues after AdSmad7 or AdSmad7-uPA transduction. The results showed smad7 was significantly over-expressed in both L02 cells and liver tissue after AdSmad7 or AdSmad7-uPA infection. (C) uPA enzymatic activity in L02 cells infected with AduPA or AdSmad7-uPA was analyzed by casin-plasminogen zymography assay. The result indicated good enzymatic activity of uPA in detected cells. (D) By ELISA assay, uPA was in a higher level both in L02 cells and liver tissue after AduPA or AdSmad7-uPA infection than after AdGFP infection. Liver sections were prepared 3 days after  $5 \times 10^9$  pfu adenovirus infection. Each experiment was performed in triplicate.



**Figure 2.** Hepatic fibrosis was suppressed by expression of Smad7 and uPA. (A) Collagen expression in the liver of fibrotic models was detected by sirius red staining ( $\times 40$ ). (B) Quantitative morphometric data showed that compared with the AdGFP, fibrosis was significantly reduced by 31.71%, 30.47% and 61.49% in the AdSmad7 ( $P < 0.05$ ), AduPA ( $P < 0.05$ ) or AdSmad7-uPA ( $P < 0.01$ ) treated group respectively. (C) Hydroxyproline was detected to evaluate liver fibrosis. The concentrations of hydroxyproline in AdSmad7 or AduPA treated rat liver tissues were  $577.43 \pm 123.3 \mu\text{g/g}$ ,  $617.41 \pm 97.24 \mu\text{g/g}$  respectively, which were significantly lower than  $868.85 \pm 67.69 \mu\text{g/g}$  in AdGFP treated group. And AdSmad7-uPA treatment led to a further reduction of hydroxyproline which was  $407.81 \pm 106.3 \mu\text{g/g}$ . Eight animals were examined in each experimental group. \*  $P < 0.01$  compared with AdGFP group; #  $P < 0.05$  compared with AdGFP group; \*\*  $P < 0.05$  compared with AdSmad7 or AduPA group.

generated by reverse-transcription using random primers. Real-time PCR was performed as described previously [30] using ABI prism 7100 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Sequences of the primers for MMP-13 and TIMP-1 were: MMP-13: forward- ACCCCAAAACACCAGAGAAGTGT, reverse- GGAAGTTCTGGCCAAAAGGACT; TIMP-1: forward- TCCTGGTTCCCTGTCATAATCT, reverse- AGCCCATGAGGATCTGATCTGTC. PCR was performed using SYBR green technology in a final volume of 25  $\mu\text{l}$ . Briefly, 100 ng (5  $\mu\text{l}$ ) of cDNA was mixed with 12.5  $\mu\text{l}$  of SYBR-green master mix (Roche Diagnostics, CA, USA) and 0.5  $\mu\text{l}$  of each primer (10 pmol/ $\mu\text{l}$ ). Condition was: 95°C for 5 min (initial denaturation), 95°C for 15 s, and 62°C for 20 s (45 cycles). For each amplification curve, the threshold cycle was determined for calculating the relative amount of target RNA using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

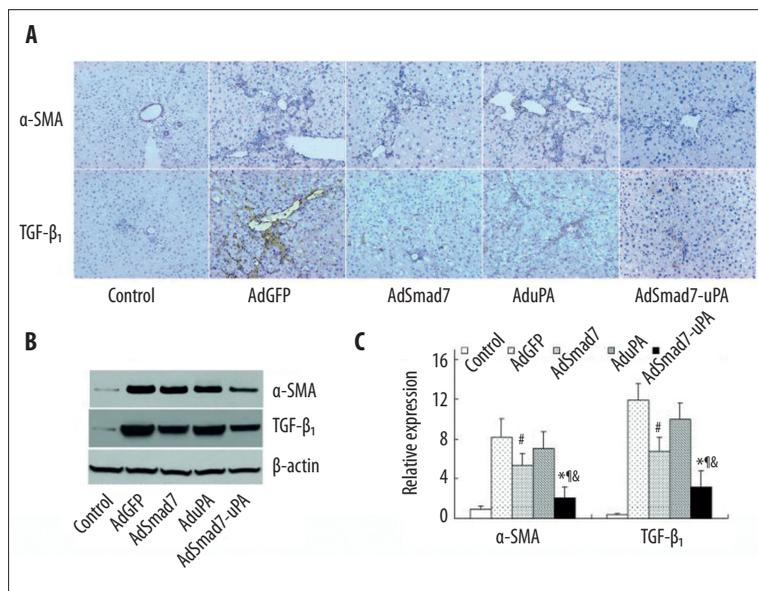
**Statistical analysis**

Experiments were performed in triplicate, and results are expressed as the means  $\pm$  standard deviation (SD). A 1-way analysis of variance (ANOVA) was used for statistical comparisons and P value less than 0.05 were considered statistically significant.

**RESULTS**

**High expression of Smad7 and uPA *in vitro* and *in vivo* was achieved by an adenoviral recombinant**

Adenoviral vectors encoding Smad7, uPA, or Smad7 and uPA were constructed as described in Material and Methods. Recombinant adenoviruses were generated after transfection of pAdSmad7, pAduPA, or pAdSmad7-uPA into AD-293 cells, and plaques from the first and final rounds of amplification were identified by PCR for the Smad7 and uPA genes (data not shown). After adenoviral infection, the level of



**Figure 3.** Immunohistochemical evaluation of  $\alpha$ -SMA and TGF- $\beta$ 1 expression in liver sections. (A)  $\alpha$ -SMA and TGF- $\beta$ 1 expression in liver tissues were analyzed by immunohistochemistry ( $\times 200$ ). (B) Western blot was used to detect the level of  $\alpha$ -SMA and TGF- $\beta$ 1 expression. (C) Quantity assay was performed based on the western blot results. As indicated, the expression of  $\alpha$ -SMA and TGF- $\beta$ 1 were increased to a high level in the fibrotic liver tissues induced by CCl<sub>4</sub>. And the expressions of  $\alpha$ -SMA and TGF- $\beta$ 1 were reduced partly after treatment with AdSmad7 or AduPA, while reduced further by AdSmad7-uPA treatment. \*  $P < 0.01$  compared with AdGFP group; #  $P < 0.05$  compared with AdGFP group; †  $P < 0.05$  compared with AdSmad7 group; &  $P < 0.01$  compared with AduPA group.

Smad7 protein in L02 cells infected with AdSmad7-uPA was similar to cells infected with the AdSmad7-positive control vector, and significantly higher than in cells infected with the AdGFP-negative control vector (Figure 1A).

The expression of recombinant uPA was determined by ELISA of the cell culture supernatants, and substantial amounts of uPA were secreted by AdSmad7-uPA or AduPA infected L02 cells, but uPA was not detected in the supernatants of AdGFP transduced cells (Figure 1D). Next, we tested the enzymatic activity of uPA expressed by AdSmad7-uPA using a zymography assay *in vitro*. As shown in Figure 1C, culture supernatants of L02 cells transduced with Adsmad7-uPA or AduPA yielded a 45-kDa band by zymography assay, indicating that the recombinant uPA expressed by the vector was active.

Further, the efficiency of intrahepatic expression of AdSmad7-uPA was analyzed by immunoblot (Figure 1B) and ELISA (Figure 1D). Three days after IV adenovirus injection, high level expression of Smad7 and uPA was detected in the livers of AdSmad7-uPA-treated animals; however, minimal detection of Smad7 and uPA was observed in the livers of AdGFP-treated animals. These results confirmed a sustained expression of Smad7 and uPA in liver tissues after IV injection of AdSmad7, AduPA, or AdSmad7-uPA.

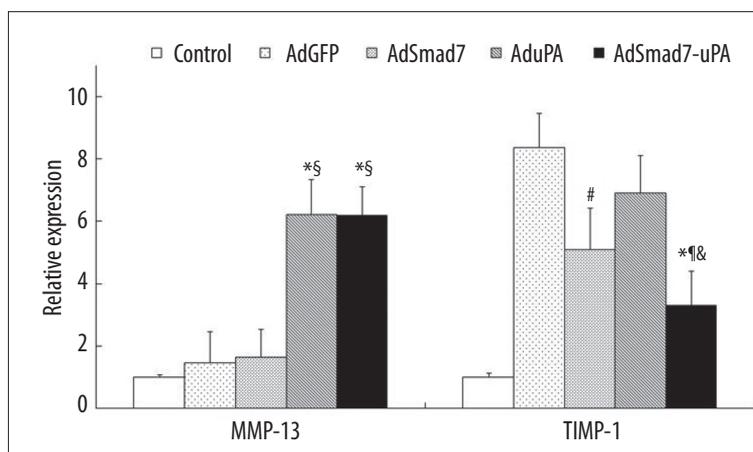
#### Smad7 and uPA co-expression suppressed the progression of liver fibrosis

In this study, advanced hepatic fibrosis and regenerating nodules were typically observed in rats treated with CCl<sub>4</sub> every third day for 8 weeks (Figure 2). AdSmad7 or AduPA treatment could inhibit the deposition of fibrillar collagen, and further inhibition of collagen deposition was observed in AdSmad7-uPA-treated animals. Quantitative morphometric data showed that compared with the AdGFP-treated rats, fibrosis was significantly reduced by 31.71%, 30.47% and 61.49% in the AdSmad7 ( $P < 0.05$ ), AduPA- ( $P < 0.05$ ) or AdSmad7-uPA- ( $P < 0.01$ ) treated group, respectively

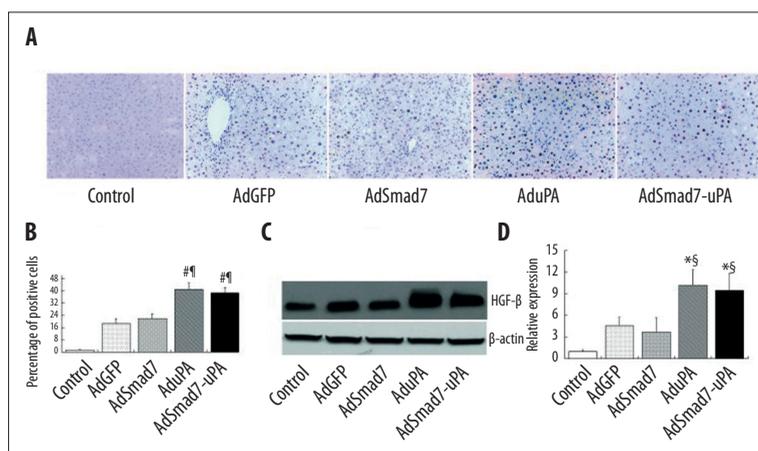
(Figure 2A, B). As the hydroxyproline level in the liver is known to parallel the extent of fibrosis, next we measured the liver hydroxyproline content in each group. Compared with the AdGFP-treated animals, over-expression of Smad7 or uPA alone inhibited the hydroxyproline accumulation induced by CCl<sub>4</sub> ( $P < 0.05$ ). Importantly, treatment with the combination of Smad7 and uPA led to a further statistically significant decrease of hydroxyproline accumulation ( $P < 0.01$ ). The concentrations of hydroxyproline in rat liver tissues treated with AdSmad7-uPA, AdSmad7, AduPA, and AdGFP were  $407.81 \pm 106.3$   $\mu\text{g/g}$ ,  $577.43 \pm 123.3$   $\mu\text{g/g}$ ,  $617.41 \pm 97.24$   $\mu\text{g/g}$  and  $868.85 \pm 67.69$   $\mu\text{g/g}$ , respectively (Figure 2C).

#### Smad7 and uPA expression inhibit the expression of SMA and TGF- $\beta$ 1

The activation of HSCs is the crucial event in liver fibrosis, and expression of SMA is commonly used to quantitate the number of activated HSCs [31]. Also, activated HSCs are the most important source of TGF- $\beta$ 1 [32], which plays a pivotal role in the deposition of ECM proteins. To further investigate the influences of Smad7 and uPA dual-gene therapy on activation of HSCs *in vivo*, we detected the expression of SMA and TGF- $\beta$ 1 in liver by immunohistochemical staining (Figure 3A) and Western immunoblot analysis (Figure 3B). Compared with the normal liver, the livers of CCl<sub>4</sub>-treated rats displayed markedly higher expression of  $\alpha$ -SMA and TGF- $\beta$ 1. Single Smad7 or uPA gene therapy significantly suppressed  $\alpha$ -SMA and TGF- $\beta$ 1 expression in the fibrotic liver of CCl<sub>4</sub> treated rats ( $P < 0.05$  for both); however, combinational treatment with both Smad7 and uPA further inhibited  $\alpha$ -SMA and TGF- $\beta$ 1 expression ( $P < 0.01$ ). As shown in Figure 3, compared with the AdGFP control vector, Smad7 treatment reduced the expression of  $\alpha$ -SMA and TGF- $\beta$ 1 by 35.48% and 43.38%, respectively, uPA treatment only slightly reduced the expression of  $\alpha$ -SMA and TGF- $\beta$ 1 expression ( $P > 0.05$  for both), and dual treatment with both Smad7 and uPA reduced the expression of  $\alpha$ -SMA and TGF- $\beta$ 1 by 75% and 73.77%, respectively (Figure 3C). Thus, combined therapy reduced fibrosis more significantly



**Figure 4.** MMP-13 and TIMP-1 mRNA levels in liver tissues. Real-time PCR was used to investigate the mRNA levels of MMP-13 and TIMP-1 in liver tissues. Results from each analysis were normalized to the amount of GAPDH mRNA. The data were mean  $\pm$  standard deviation of eight samples. Compared with AdGFP treatment, the MMP-13 mRNA level was elevated significantly after AdSmad7-uPA or AduPA treatment. As for the TIMP-1 mRNA level, it was higher in fibrotic liver tissues than in normal liver tissues, AdSmad7 could partly inhibit the elevation of TIMP-1 induced by CCL4, and AdSmad7-uPA further reduced the elevation of TIMP-1 mRNA. \*  $P < 0.01$  compared with AdGFP group;  $^{\S} P < 0.01$  compared with AdSmad7 group;  $^{\#} P < 0.05$  compared with AdGFP group;  $^{\P} P < 0.05$  compared with AdSmad7 group;  $^{\&} P < 0.01$  compared with AduPA group..



**Figure 5.** AdSmad7-uPA treatment promoted hepatocyte proliferation and HGF maturation. (A) Liver sections were subjected to immunohistochemical staining using an anti-PCNA antibody to determine hepatocyte proliferation activity ( $\times 200$ ). (B) Quantitation of the hepatic PCNA-labeling cells was shown. (C) Immunoblot analysis of mature HGF levels in livers using anti-HGF- $\beta$  antibody that recognized only active HGF. (D) Quantitation of the mature HGF levels after normalization to  $\beta$ -actin. Data represent the mean  $\pm$  standard deviation obtained from eight rats.  $^{\#} P < 0.05$  compared with AdGFP group;  $^{\P} P < 0.05$  compared with AdSmad7 group;  $^{\ast} P < 0.01$  compared with AdGFP group;  $^{\S} P < 0.01$  compared with AdSmad7 group.

than treatment with Smad7 or uPA alone. These results indicate that Smad7 and uPA treatment resulted in an additive effect to prevent the activation of the HSCs.

**Smad7 and uPA gene therapy enhanced ECM degradation**

ECM degradation is mainly catalyzed by the matrix metalloproteinases (MMPs), and the interstitial collagenase MMP-13 is an important enzyme that degrades collagen I and III in the rats [33]. The activities of MMPs are inhibited by tissue inhibitors of metalloproteinases-1 (TIMP-1). Liver fibrosis is characterized by downregulation of MMP activity and upregulation of TIMP-1 levels, which stabilizes the ECM components. In this study, we examined the MMP-13 and TIMP-1 mRNAs levels in the rat fibrotic livers induced by CCL4 by quantitative RT-PCR. The results indicate that TIMP-1 mRNA increased substantially, but MMP-13 mRNA levels only increased slightly, when liver injury was induced by CCL4. Although MMP-13 mRNA levels were not altered in AdSmad7-treated rat livers, there were 3.12-fold and 3.25-fold increases in AdSmad7-uPA-treated and AduPA-treated

rats livers, respectively ( $P < 0.01$  for both). In addition, in the AdSmad7-uPA-treated rats, the TIMP-1 mRNA level was significantly less than in the AdGFP- or AdSmad7-treated rats ( $P < 0.01$  and  $P < 0.05$ ) (Figure 4). In uPA-treated rats, TIMP-1 mRNA level had a decreasing trend compared to AdGFP-treated rats ( $P > 0.05$ ).

**Smad7 and uPA co-expression promoted hepatocyte proliferation**

It has been demonstrated that uPA is capable of converting the inactive form of pro-HGF into the active form, which stimulates hepatocyte proliferation. PCNA acts as an auxiliary protein of DNA polymerase-delta to initiate cell proliferation; therefore, the expression of PCNA is regarded as a marker for evaluating changes in cell proliferation [34]. In this study, immunohistostaining analysis was used

to detect the expression of PCNA to evaluate the proliferation of hepatocytes. Immunoblot analysis was performed to detect the expression of mature HGF in the livers using an antibody that recognizes only the active form of HGF- $\beta$ . As shown in Figure 5, immunohistological analysis of liver sections showed PCNA expression in the AdSmad7-uPA- and AduPA-treated rats was 2.07-fold and 2.2-fold greater than in AdGFP-treated rats ( $P < 0.01$  for both). Similarly, mature HGF expression was higher in the AdSmad7-uPA- or AduPA-treated rats than that in AdGFP- or AdSmad7-treated rats ( $P < 0.01$  for both). No significant increase in HGF- $\beta$  expression and PCNA-stained cells were observed in the AdSmad7-treated rats compared with AdGFP-treated rats.

## DISCUSSION

Liver fibrosis is characterized by excessive extracellular matrix (ECM) components deposition by the increase of synthesis and the decrease of degradation. Thus, liver fibrosis therapy may focus on promoting ECM degradation and inhibiting ECM synthesis. Until now, no drugs could inhibit liver fibrosis completely; therefore, more endeavors in developing new therapy strategy were needed. In this study we made a new attempt to deliver genes into CCL4-induced rat liver fibrosis models. The results showed single uPA or single smad7 gene therapy could inhibit CCL4-induced fibrosis significantly, while these 2 genes in combination could attenuate liver fibrosis more obviously than either single uPA or single smad7, and the reduction in liver fibrosis was mediated by inhibiting ECM deposition, accelerating ECM degradation, and promoting hepatocyte proliferation. To our knowledge, this is the first report demonstrating the use of a bicistronic adenoviral vector to mediate a dual-gene expression therapy for liver fibrosis.

It is currently thought that the TGF- $\beta_1$ /Smad signaling pathway acts as a central pathway leading to liver fibrosis, regardless of the initial pathogenic causes in disease conditions. Smad7, an inhibitory Smad, acts in a negative feedback loop to inhibit TGF- $\beta_1$  activity by preventing the phosphorylation of Smad2/3 [35]. In rats injected with CCL4 for 8 weeks, inhibition TGF- $\beta_1$  action by Smad7 alone led to partial reduction of liver ECM deposition, with no increase of metalloproteinase expression or hepatocyte proliferation. Similarly, inhibition of TGF- $\beta_1$  expression or signaling by different approaches, such as antisense oligonucleotides, truncated TGF- $\beta$  receptor, or ALK5 (the TGF- $\beta$  type I receptor) inhibitor [36–38], only partially relieves liver fibrosis. Taken together, these results indicate that blocking TGF- $\beta_1$  alone may miss other potentially major therapeutic targets for the treatment of liver fibrosis.

Extensive evidence supports the fact that the plasminogen activation system participates in the matrix remodeling process, and alternative expressions of the plasminogen activation system were found in fibrotic organs. Up to now, the antifibrotic activity of uPA was confirmed in animal models of liver and pulmonary fibrosis [27,39]. In this study we demonstrated that although single Smad7 gene therapy attenuates liver fibrosis, expression of both Smad7 and uPA significantly improves the therapeutic effects compared to single Smad7 or single uPA gene transfer. This is illustrated by the greater reduction in the extent of liver fibrosis, enhanced MMP-13 expression, and promotion of hepatocyte

proliferation. Beyond the beneficial effects of expressing Smad7 and uPA alone, several cross-talk mechanisms may explain the increased efficacy of the combined therapy. First, previous experiments demonstrated that TIMP-1, which is primarily secreted by HSC, may reduce MMP activity and suppress apoptosis of HSCs [29]. Thus, inhibition of HSC activation by Smad7 may enhance ECM degradation. Also, ECM degradation by uPA may alter ECM characters and cell-ECM interactions, which could facilitate HSC apoptosis and decrease ECM deposition [40]. Furthermore, HGF activated by uPA could antagonize TGF- $\beta_1$  directly, and inhibit ECM production [41]. While all of these ideas are consistent with published data, the mechanisms by which Smad7 and uPA inhibit liver fibrosis remain to be defined.

## CONCLUSIONS

In summary, combined gene therapy using Smad7 and uPA co-expression adenoviral vector inhibited CCL4-induced rat liver fibrosis by simultaneously targeting multiple pathogenic pathways. However, since overexpression of Smad7 and uPA in hepatocytes may lead to carcinogenesis [42] and can effect clotting, these concerns must be addressed before clinical application of this approach can be used to treat liver cirrhosis patients. It is possible that the use of cell- or tissue-specific promoters to restrict recombinant gene expression to HSCs may help to overcome these challenges [43]. Future studies on the safety and efficacy of the next generation of adenoviral vectors are also needed to optimize these approaches for clinical applications, but our current study may provide a foundation for designing future therapeutic regimens for inhibiting the progression of chronic liver diseases in clinical settings.

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## Conflict of interest statement

The authors declare that there is no conflict of interest in this work.

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