Original Research

Recombinant adeno-associated virus-mediated transfer of shRNA against Notch3 ameliorates hepatic fibrosis in rats

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Abstract

Liver fibrosis, a wound healing process following all kinds of liver injuries, is characterized by excessive deposition of extracellular matrix (ECM). Our previous study revealed that Notch3 might participate in liver fibrogenesis by regulating the activation of hepatic stellate cells (HSCs). The aim of this study was to assess the effects of Notch3 shRNA on hepatic fibrosis in a rat model induced by carbon tetrachloride (CCl_4) and to clarify the mechanisms underlying those effects. Recombinant adeno-associated virus type 1 (rAAV1) vector carrying Notch3 shRNA (rAAV1-Notch3-shRNA) was generated and transferred to rat livers via the tail vein. The expression of Notch3, Jagged1, Hes1 and α-SMA were detected by real-time RT-PCR and immunofluorescence. The effects of rAAV1-Notch3-shRNA on fibrosis was investigated by pathological and immunohistochemical examination. Our findings showed that Notch3, Jagged1, Hes1 and α-SMA were downregulated. This downregulation was accompanied by improved hepatic fibrosis after the inhibition of Notch3 *in vivo*. rAAV1-Notch3-shRNA treatment reversed the epithelial-mesenchymal transition (EMT) in fibrotic livers by decreasing the expression of transforming growth factor β1 (TGF-β1) and vimentin in a line with the increased expression of E-cadherin. The inhibition of Notch3 was not found to play a role in hepatocyte proliferation. Rather, it inhibited hepatocyte apoptosis *in vivo* to some extent. The results of the present study suggest that the inhibition of Notch3 can protect hepatocytes from undergoing apoptosis and attenuate liver fibrogenesis. This may be a viable therapeutic option for hepatic fibrosis.

Keywords: Hepatic fibrosis, recombinant adeno-associated virus, notch3, epithelial-mesenchymal transition

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Introduction

Hepatic fibrosis is initially a response of the liver to various types of chronic damage. After the injuries, hepatic stellate cells (HSCs) are activated and transdifferentiate into myofibroblast-like cells, which are responsible for excessive deposition of extracellular matrix (ECM) in the liver.[1] A rat model of hepatic fibrosis generated by carbon tetrachloride (CCl_4), which can produce hepatocyte necrosis and steatosis, has been used extensively to evaluate the molecular mechanism of hepatic fibrosis.[1] The most common anti-fibrotic therapies are engineered to inhibit the activation, proliferation and products of HSCs.[2,3] Multiple signalling pathways are implicated in HSC activation and proliferation. Transforming growth factor β1 (TGF-β1) is the main stimulating factor for profibrotic ECM synthesis, but the anti-fibrotic strategies targeting TGF-β1 are problematic because of the diversified nature of TGF-β1 action.[1]

Notch signalling plays an essential role in cellular specification, differentiation and proliferation in various organisms.[4] Notch signalling includes several receptors, Notch1–Notch4, and the related ligands, Delta1, Delta3, Delta4, Jagged1 and Jagged2.[5] When interacting with the ligand, Notch is cleaved by TNF-α converting enzyme and the γ-secretase complex to release the Notch intracellular domain (NICD). Then NICD translocates to the nucleus, where it activates the transcription of downstream genes such as Hes1 and Hey1.[4]

*The first three authors contributed equally to this work.

Increasing numbers of studies have demonstrated that Notch signalling is involved in human pulmonary, kidney and peritoneal fibrosis.[6–9] Our previous study found that the expression of Notch3 is significantly upregulated in fibrotic liver tissues of patients with hepatitis and that Notch signalling was activated in HSC-T6 cells.[10] The expression of α-SMA and collagen I induced by TGF-β1 in HSC-T6 was antagonized by Notch3 knockdown, which suggested that Notch3 might be implicated in liver fibrosis by regulating the activation of HSCs.[10]

In this study, we assessed the effects of Notch3 shRNA in a rat model of hepatic fibrosis and the associated molecular mechanisms. Our data provide evidence that inhibition of Notch3 by shRNA delivered via recombinant adeno-associated virus 1 (rAAV1) impeded the development of hepatic fibrosis, suggesting that selective suppression of Notch3 might have significant beneficial effects and therapeutic potential for hepatic fibrosis.

Table 1 The primer sequences for TaqMan real-time qPCR

Gene	Primer	Sequence
Notch3	Forward	5′-CCTGCCTGCCTCTATGACAAC-3′
	Reverse	5′-ACACTCCTCGGTGTTACAGCC-3′
	Probe	5′-ACTGCTACTCTGGTGGCCGCGAC-3′
Jagged1	Forward	5′-GTGGAAGAGGATGATATGGATAAGC-3′
	Reverse	5′-CTCCTCTCTGTCTACCAGCGTGTAC-3′
	Probe	5′-CCAGCAGAAAGTCCGGTTTGCCA-3′
Hes1	Forward	5′-TGCTACCCCAGCCAGTGTC-3′
	Reverse	5′-GCTTTGATGACTTTCTGTGCTCA-3′
	Probe	5′-CTGTCTTTGGTTTGTCCGGTGTCGT-3′
α-SMA	Forward	5′-TGGAAAAGATCTGGCACCACT-3′
	Reverse	5′-GGGACAGCACAGCCTGAATAG-3′
	Probe	5′-CTATAACGAGCTTCGCGTGGCCCC-3′
GAPDH	Forward	5′-GATGACATCAAGAAGGTGGTGAAG-3′
	Reverse	5′-ACCCTGTTGCTGTAGCCATATTC-3′
	Probe	5′-ACTTCAACAGCAACTCCCACTCTTCCACC-3′

Materials and methods

rAAV1 vectors preparation

The shRNAs targeting rat Notch3 mRNA and the control shRNA were designed using BLOCK-iT RNAi Designer (Invitrogen, USA) and produced by Shanghai Genepharma Co. Ltd. (China). The rAAV1-enhanced green fluorescent protein (EGFP) carrying Notch3 shRNA (rAAV1-Notch3-shRNA-EGFP) and rAAV1-NC were constructed as described previously.[11] The following sense shRNA sequences were used: Notch3 shRNA, 5′-GCTAG CTTCTCATGCGCTTGTTTCAAGAGAACAAGCGCATG AGAAGCTAGCTTTTT-3′; control, 5′-GAGTCTGTCTAC TCTACTTCATTCAAGAGATGAAGTAGAGTAGACAGA CTCTTTTT-3′.

Animal model

A model of experimental liver fibrosis was induced in rats using CCl_4 treatment. Forty male Sprague–Dawley rats weighing 180–220 g were obtained from the experimental animal center of Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China). They were randomly sorted into four groups of 10 rats each: normal, model, rAAV1-NC and rAAV1-Notch3-shRNA group. Rats in the model, rAAV1-NC and rAAV1-Notch3-shRNA groups received CCl_4 (3 mL/kg) dissolved in olive oil by subcutaneous injection twice a week for eight weeks and rats in normal group received olive oil (3 mL/kg) twice a week for eight weeks, as described previously.[12] Four weeks after the first administration, rats from the normal and model groups were injected via tail vein with PBS, and the rats in the rAAV1-Notch3-shRNA and rAAV1-NC groups were injected with a single dose of 3×10^{11}v.g. rAAV1-Notch3-shRNA or rAAV1-NC via tail vein per rat. Rats were killed eight weeks after the initial treatment.

This study was approved by the ethics Committee on Animal Experimentation of Huazhong University of Science and Technology and performed in compliance with the Guidelines for the Animal Care and Use. Permit numbers: 2011-237.

Real-time PCR

Total RNA was extracted from each liver tissue using Trizol Reagent (Invitrogen, USA) and converted into cDNA as described previously.[10] TaqMan RT-qPCR was performed using a StepOnePlus (ABI, USA.) and Premix Ex Taq™ (Takara, Japan). Primer sequences are shown in Table 1.

Histopathology and immunohistochemistry

Liver specimens were fixed in 4% paraformaldehyde and embedded with paraffin. The histopathological changes and collagen accumulation of liver tissues were evaluated by Hematoxylin-eosin (HE), Masson and Sirius Red staining. The expression of TGF-β1, vimentin, E-cadherin, and proliferating cell nuclear antigen (PCNA) in liver tissues was investigated using immunohistochemical examination. For negative control, the primary antibody was replaced with isotype control antibody. PCNA-positive hepatocytes (%) were quantified by ImageJ software (NIH, USA).

Immunofluorescence staining

The expression Notch3, Jagged1, Hes1 and α-SMA in liver tissues was detected using immunofluorescence staining. The antibodies used for immunohistochemistry and immunofluorescence are shown in Table 2. The fluorescent secondary antibodies were conjugated with Dylight488 (goat anti-rabbit, green), Dylight488 (goat anti-mouse, green) or Dylight594 (goat anti-rabbit, red) (Earthox LLC, USA). The staining of cell nuclei counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) was visualized under a laser scanning confocal fluorescent microscope (Nikon, Japan). Non-immune mouse and rabbit IgG served as negative controls, taking the place of primary antibodies.

The level of cell apoptosis was determined using a TUNEL Assay Kit (Roche, USA). TUNEL-positive hepatocytes (%) were quantified using ImageJ.

Western blot

Western blot was performed in accordance with the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Briefly, liver tissue was lysed in lysis buffer and protein was quantified using the BCA assay (Beyotime, China). The polyvinylidene fluoride (PVDF) membranes (Millipore, MA) were incubated with the indicated primary antibody followed by addition of horseradish peroxidase-conjugated second antibodies and detected by chemiluminescence using the BeyoECL Plus (Beyotime, China) with a digital luminescent image analyser Biospectrum 600 (UVP, USA). Western blot data were quantified using ImageJ. The primary antibodies are listed in Table 2.

Assay of hydroxyproline

Total hepatic hydroxyproline contents were detected in the hydrolysates of liver specimens using a Hydroxyproline Testing Kit (Jiancheng, China) in accordance with the manufacturer's protocol. Data are shown in terms of hydroxyproline (μg)/wet liver weight (mg).

Statistical analysis

Data are presented as mean ± SD. Statistical analysis was performed with SPSS13.0. P values < 0.05 were considered significant.

Results

Determination of rAAV1-Notch3-shRNA-EGFP expression *in vivo*

To investigate the effects of delivery of rAAV1-Notch3-shRNA-EGFP *in vivo*, we assessed the expression of EGFP in frozen liver sections four weeks after rAAV1-Notch3-shRNA-EGFP transfection. In rat livers delivered with rAAV1-Notch3-shRNA-EGFP, green fluorescence was observed predominantly in fibrotic liver tissues in which about 20–30% of cells expressed EGFP (Figure 1). However, there was no EGFP expression detected in the livers of rats not injected with rAAV1 (data not shown).

Inhibitory effects of rAAV1-Notch3-shRNA on Notch3 expression

The mRNA levels of Notch3, Jagged1, Hes1 and α-SMA in the liver were markedly higher in rats given CCl_4 than in control rats, and their mRNA levels were significantly downregulated after rAAV1-Notch3-shRNA treatment (Figure 2A). Immunofluorescence analysis revealed that Notch3, Hes1 and α-SMA were mostly expressed in the non-parenchymal cells in fibrotic areas in the model and rAAV1-NC groups. Weak Jagged1 expression of hepatocytes was observed in normal livers, but it was markedly increased in fibrotic

Table 2 Primary antibodies used for Western blot analysis and immunofluorescence or immunohistological staining

Rat antigens	Poly/mono-clonal	Manufacturer	Dilution
α-SMA	Monoclonal	Abcam, USA	1:50
Notch3	Polyclonal	Abcam, USA	1:250
Jagged1	Monoclonal	Epitomics, Burlingame, USA	1:250
Hes1	Polyclonal	Santa Cruz Biotechnology, Inc., Santa Cruz, CA	1:50
E-cadherin	Monoclonal	Santa Cruz Biotechnology, Inc., Santa Cruz, CA	1:100 (1:1000 for western blot)
vimentin	Monoclonal	Abcam, USA	1:500 (1:1000 for western blot)
TGF-β1	Monoclonal	Abcam, USA	1:100 (1:1000 for Western blot)
Mouse IgG1	Monoclonal	Abcam, USA	1:100
PCNA	Polyclonal	Santa Cruz Biotechnology, Inc., Santa Cruz, CA	1:100
β-actin	Polyclonal	Santa Cruz Biotechnology, Inc., Santa Cruz, CA	1:1000 for Western blot

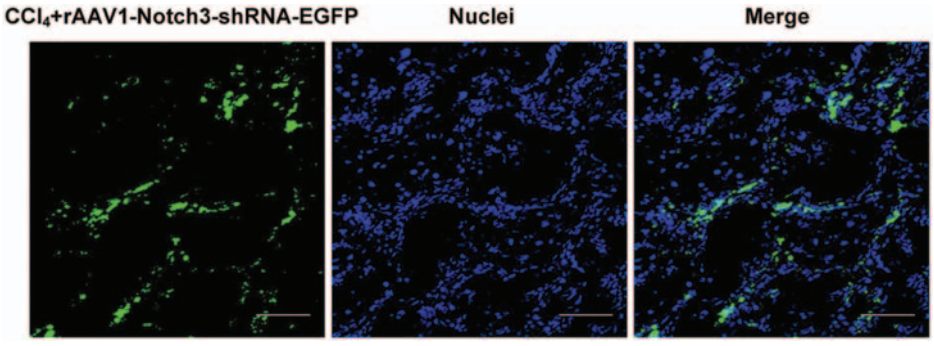

Figure 1 Determination of rAAV1-Notch3-shRNA-EGFP expression *in vivo*. EGFP expression in frozen sections was assessed by fluorescence microscopy four weeks after rAAV1-Notch3-shRNA-EGFP injection. In rat livers infused with rAAV1-Notch3-shRNA-EGFP, approximately 20–30% of cells in the fibrotic liver tissue expressing green fluorescence were observed. The white bars represent 50 μm. (A color version of this figure is available in the online journal)

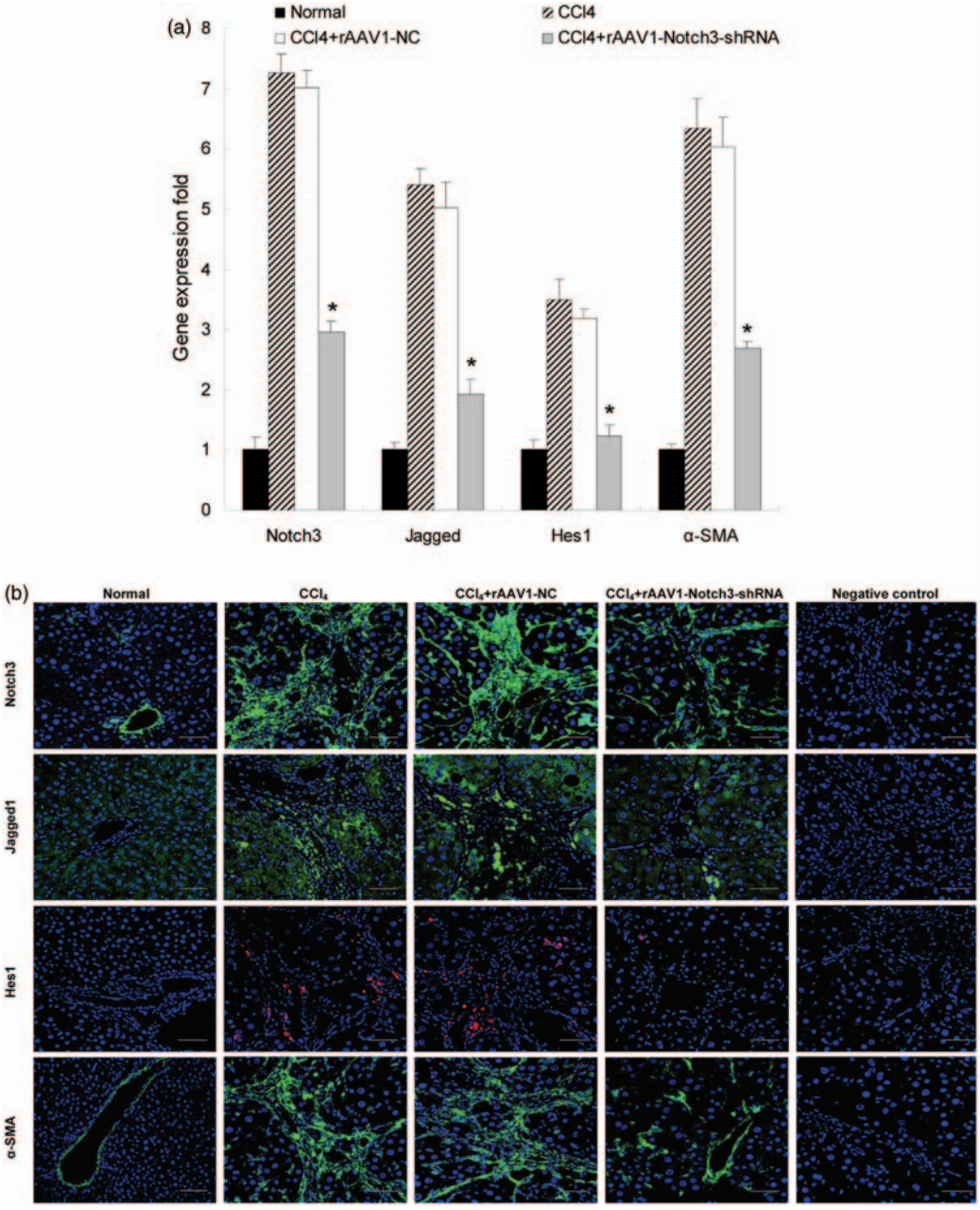

Figure 2 rAAV1-Notch3-shRNA administration downregulated Notch3 expression. (a and b) Rats were injected rAAV1-Notch3-shRNA or rAAV1-NC four weeks after CCl_4 and were sacrificed eight weeks after the initial treatment. Real-time RT-PCR and immunofluorescence were performed to detect the expression of Notch3, Jagged1, Hes1 and α-SMA in liver tissues of rats from normal, CCl_4, rAAV1-NC and rAAV1-Notch3-shRNA groups. mRNA expression was normalized against GAPDH. Each value represents the mean with the SD for triplicate samples. *Relative to CCl_4 or rAAV1-NC group, $P < 0.05$. (c) Liver sections of rats sacrificed eight weeks after CCl_4 were costained with antibodies against Hes1 and α-SMA. The white bars represent 50 μm. Arrows indicate Hes1/α-SMA double positive cells. (A color version of this figure is available in the online journal)

areas in model or rAAV1-NC groups. The expression of Notch3, Jagged1, Hes1 and α-SMA was markedly decreased in the rAAV1-Notch3-shRNA group than in model or rAAV1-NC groups (Figure 2B). These data indicated that rAAV1-Notch3-shRNA treatment could inhibit Notch signalling activation *in vivo*. Moreover, Hes1 expression was co-expressed with α-SMA in fibrotic liver of rats in the model group, as indicated by immunofluorescence (Figure 2C). This signified that the upregulation of Notch signalling participated in regulating the activation of HSCs *in vivo*.

Effects of rAAV1-Notch3-shRNA on CCl4-induced liver fibrosis

To evaluate the effects of rAAV1-Notch3-shRNA on hepatic fibrosis, we investigated the pathological alterations and collagen deposition in the liver tissues of rats using HE, Masson and Sirius red staining. The results showed that the liver sections from the normal group rats showed normal liver structures. Sections from the model group and rats treated with rAAV1-NC showed that the hepatic lobular structure had been largely destroyed and that many fibrotic septa were present. In the liver tissues of rats treated

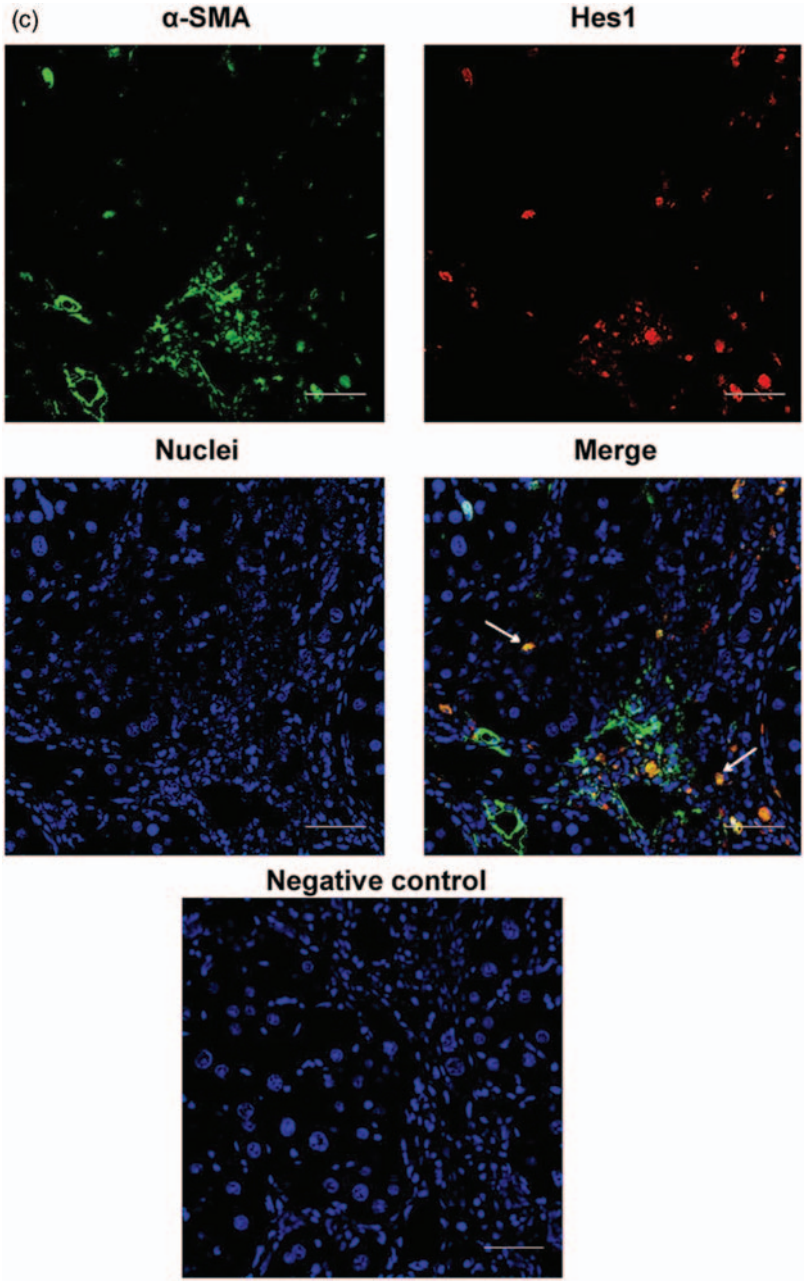

Figure 2 Continued

with rAAV1-Notch3-shRNA, normal hepatic lobule structures were still present and fewer fibrous septa and inflammatory infiltrates were seen than in model group rats or in rats treated with rAAV1-NC.

The results also indicated an obvious reduction in collagen deposition in the rAAV1-Notch3-shRNA-treated group (Figure 3A), as shown by Masson staining ($8.92 \pm 2.11\%$ in rAAV1-Notch3-shRNA group vs. $19.35 \pm 4.03\%$ in rAAV1-NC group, $P < 0.05$; Figure 3B). The results showed that the level of hydroxyproline in the rAAV1-Notch3-shRNA-treated group was $157.33 \pm 9.06\,\mu g/mg$, which was lower than in the rAAV1-NC group ($249.19 \pm 10.22\,\mu g/mg$, $P < 0.05$; Figure 3C).

rAAV1-Notch3-shRNA attenuates hepatic fibrosis via inhibition of epithelial-mesenchymal transition (EMT)

Furthermore, we investigated the levels of EMT markers using immunohistochemical staining and Western blot analysis. The expression of vimentin and TGF-β1 was significantly lower in the rAAV1-Notch3-shRNA-treated group than in the rAAV1-NC group, but the expression of E-cadherin was much higher in rAAV1-Notch3-shRNA-treated rats (Figure 4).

Immunohistochemistry showed that TGF-β1 expression was increased in regions around the fibrotic areas in CCl_4-treated liver tissues. Some hepatocytes near the fibrotic area showed high levels of TGF-β1 expression, which was

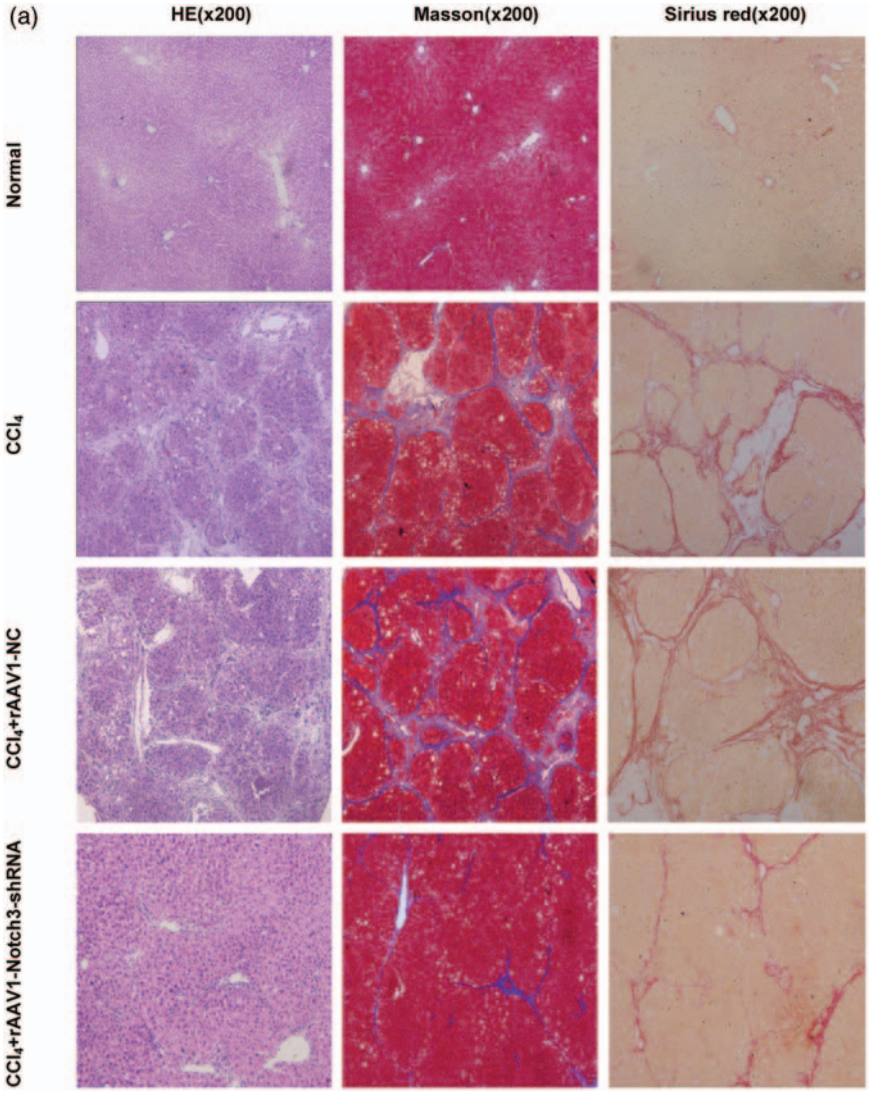

Figure 3 rAAV1-Notch3-shRNA ameliorated CCl_4-induced liver fibrosis. (a) Hematoxylin-eosin, Masson's trichrome and Sirius red staining were used to examine pathological alterations and collagen deposition in liver tissues of rats in the normal, CCl_4, rAAV1-NC and rAAV1-Notch3-shRNA groups. (b) Semiquantitative analysis of the results of Masson's trichrome staining. Each value represents the mean with the SD for triplicate samples. (c) Assay of hydroxyproline content. Each value represents the mean with the SD for triplicate samples. *Relative to the CCl_4 or rAAV1-NC groups, $P < 0.05$. (A color version of this figure is available in the online journal)

significantly reduced by the administration of rAAV1-Notch3-shRNA (Figure 4A,B).

Effects of rAAV1-Notch3-shRNA treatment on hepatocyte proliferation and apoptosis

Liver cell proliferation is an important adaptive response to the destruction of hepatocytes by inflammation or other injury. PCNA staining was carried out to determine whether rAAV1-Notch3-shRNA treatment could play any role in cell proliferation *in vivo*. Results showed many proliferating hepatocytes in the liver tissues of rats treated with CCl_4. However, there was no marked difference in hepatocyte proliferation between rats administrated with rAAV1-Notch3-shRNA and those in the rAAV1-NC or model groups ($P > 0.05$, Figure 5A,B). This suggests that rAAV1-Notch3-shRNA does not affect hepatocyte proliferation *in vivo*.

TUNEL staining results showed that there were many apoptotic hepatocytes in liver specimens from the rats in model group. However, rAAV1-Notch3-shRNA treatment was found to protect hepatocytes from apoptosis, as indicated by differences between the treated group, rAAV1-NC group, and model group (Figure 5A,C).

Discussion

Hepatic fibrosis involves excessive accumulation of ECM. This tends to develop in response to acute or chronic liver injury, ultimately resulting in cirrhosis. HSC activation, which is characterized by upregulation of α-SMA, plays a key role in fibrogenesis by facilitating the migration and deposition of ECM components.[13] In this way, better understanding of the regulation mechanisms that underlie HSCs activation is essential to the development of new therapeutic treatments for liver fibrosis.

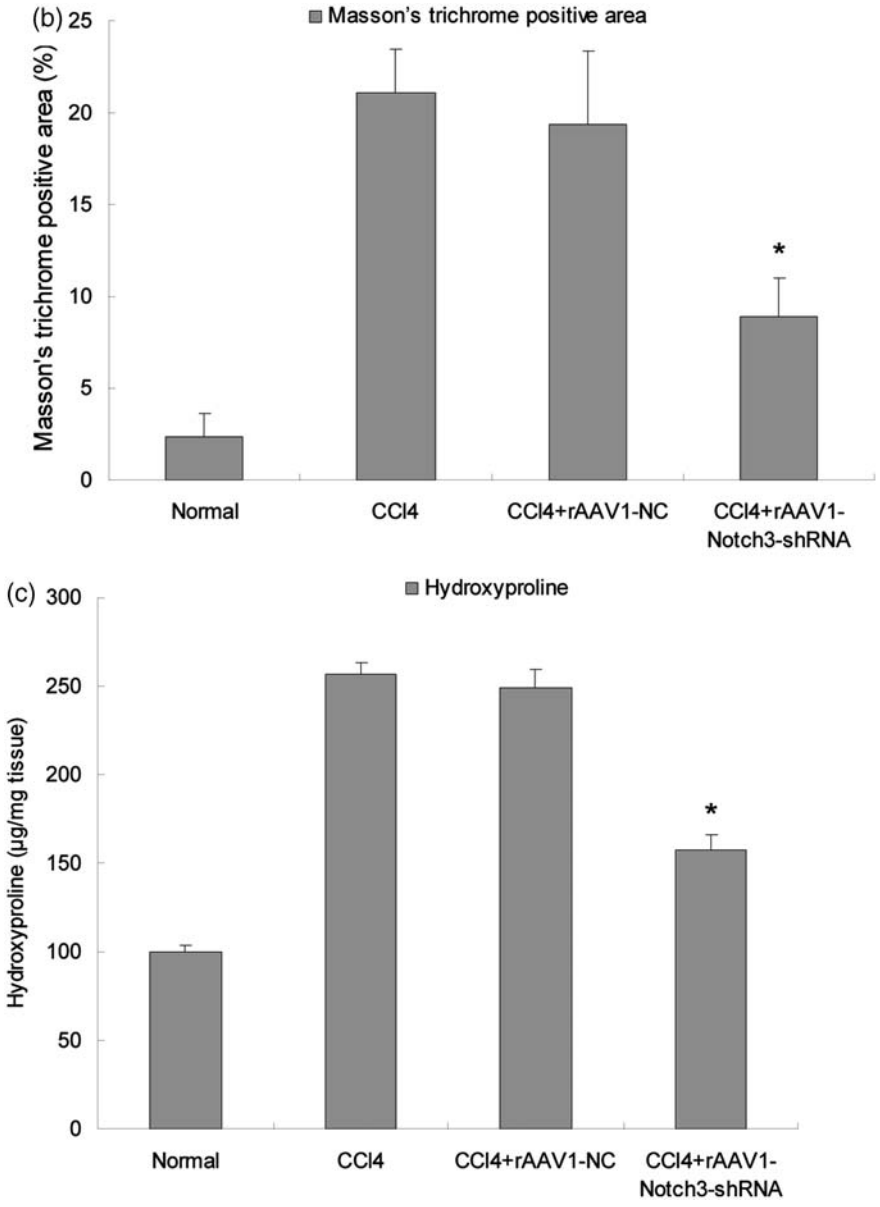

Figure 3 Continued

Notch signalling is a conserved local cell-signalling pathway. It is important to the regulation of cell differentiation. Aberrant activation of Notch signalling is involved in the formation and development of several malignancies.[14,15] Recent studies have indicated that Notch is implicated in human fibrotic diseases.[6–9] We have found that Notch3 may participate in liver fibrogenesis by regulating the activation of HSCs.[10] In the present study, Notch3, Hes1 and α-SMA were dramatically upregulated in fibrotic rat livers, and their expression levels were significantly downregulated after rAAV1-Notch3-shRNA treatment. Immunofluorescence analysis showed that Hes1-positive cells were coexpressed with α-SMA in fibrotic livers. These results reveal that activated Notch signalling takes part in the regulation of the activation of HSCs *in vivo*.

Many reports have demonstrated that myofibroblasts can be replenished through cholangiocytes and hepatocytes via EMT in liver fibrosis.[16–19] Notch signalling has been shown to be a critical regulator of the EMT process.[20–23] EMT induced by TGF-β1 was found to be blocked by RNAi targeting of Hey1 or Jagged1.[23] The current study reveals that rAAV1-Notch3-shRNA treatment can markedly attenuate CCl_4-induced liver fibrosis, as confirmed by the reduction in ECM deposition. It simultaneously inhibited the expression of TGF-β1 and vimentin in parallel with increased expression of E-cadherin in fibrotic livers. Recent studies support the point of view that vimentin acts as a positive modulator of the EMT process. Upregulation of vimentin appears to be necessary for EMT induction.[24] The downregulation of E-cadherin seems to be a key step during the EMT process.[25] Our findings suggest that rAAV1-Notch3-shRNA treatment can improve hepatic fibrosis through the reversion of EMT.

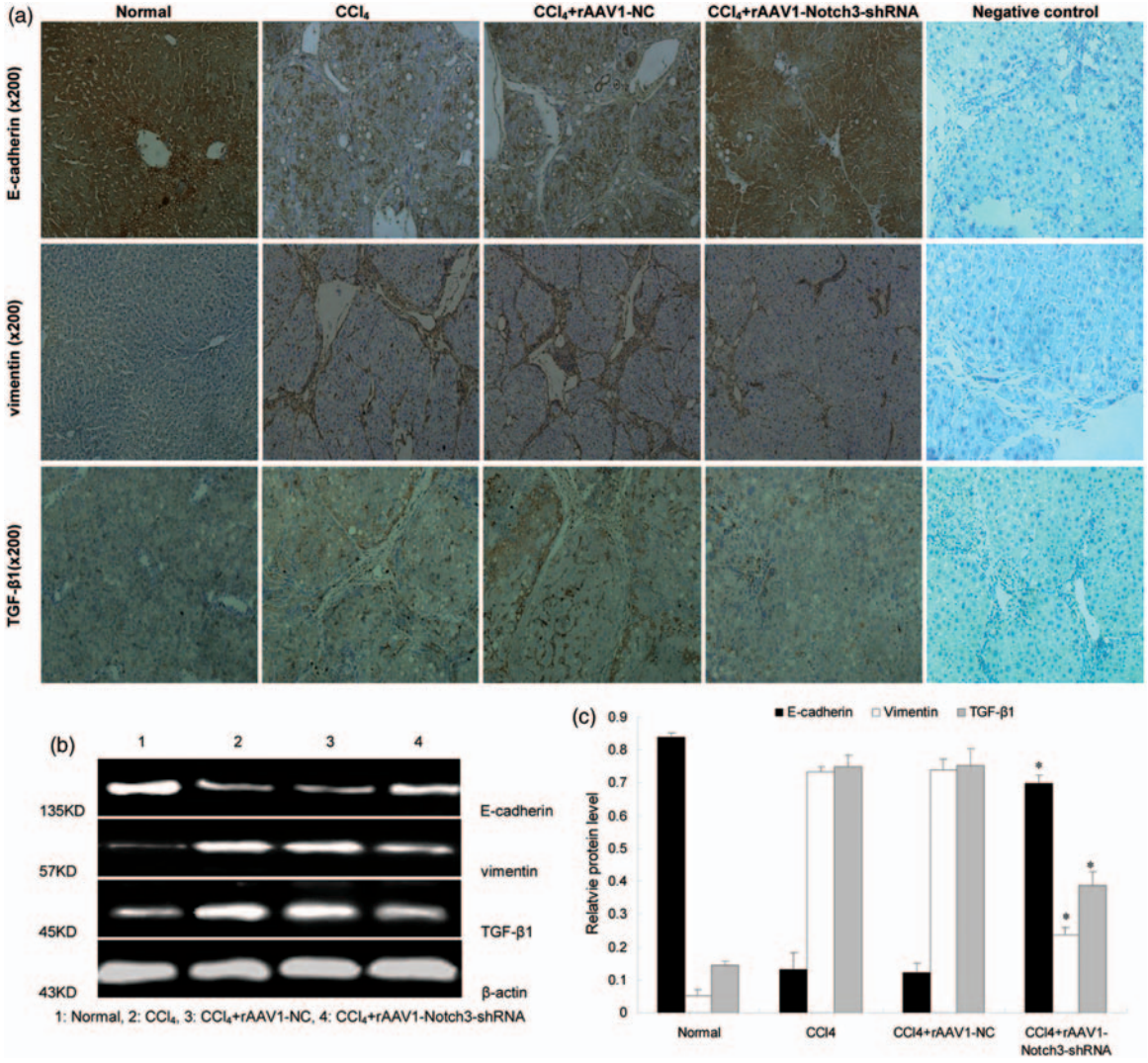

Figure 4 rAAV1-Notch3-shRNA treatment inhibited EMT in fibrotic livers in rats. Immunohistochemical staining and Western blot analysis were performed to detect the expression of E-cadherin, vimentin and TGF-β1 in liver tissues of rats from normal, CCl_4, rAAV1-NC and rAAV1-Notch3-shRNA groups. Protein expression was normalized against β-actin. Each value represents the mean with the SD for triplicate samples.*Relative to the CCl_4 or rAAV1-NC groups, $P < 0.05$. (A color version of this figure is available in the online journal)

Liver fibrosis, a result of chronic liver injury, is characterized by hepatic cell death and the replacement of liver parenchymal cells with fibrotic tissue, which eventually results in the loss of liver function. Stimulating the regeneration of liver cells and inhibiting apoptosis is important to the treatment of liver fibrosis.[26] In this study, rAAV1-Notch3-shRNA administration did not affect hepatocytes proliferation. However, the inhibition of Notch3 inhibited hepatocyte apoptosis to some extent *in vivo*. We also found that many hepatocytes near the fibrotic region showed high levels of TGF-β1 expression. TGF-β1 is a key cytokine that can promote fibrogenesis *in vivo* and *vitro*. However, direct targeting of TGF-β1as is not a feasible anti-fibrotic strategy are because of the pleiotropic functions of TGF-β1.[1] Our findings reveal that inhibition of Notch signalling by rAAV1-Notch3-shRNA attenuates hepatic fibrosis by suppressing the expression TGF-β1, which can cause hepatocyte apoptosis, stimulate ECM deposition, and induce EMT in fibrotic liver.[1,27]

Adeno-associated virus (AAV), a single-stranded DNA parvovirus, has shown potential as a gene therapy tool because of its ability to transduce both dividing and non-dividing cells and its capacity for achieving long-term transgene expression with no marked side effects.[28–30]

One recent study has shown that AAV1 vectors have resemble levels of liver tissue transduction in healthy animals and in rats with different degrees of hepatic fibrosis induced by CCl_4 or thioacetamide.[31] Our findings showed green fluorescence predominantly in the fibrotic tissues of livers infused with rAAV1-Notch3-shRNA-EGFP in a CCl_4-induced hepatic fibrosis rat model. Tsui *et al*.[32] reported that there was a preferred binding of AAV to HSCs in fibrotic parts of rat livers. All these data indicate that AAV might be an ideal tool for the delivery of therapeutic agents to fibrotic livers.

In summary, our findings support the notion that Notch3 may be a novel target suitable for use in hepatic fibrosis therapy. Inhibition of Notch3 by rAAV1-Notch3-shRNA

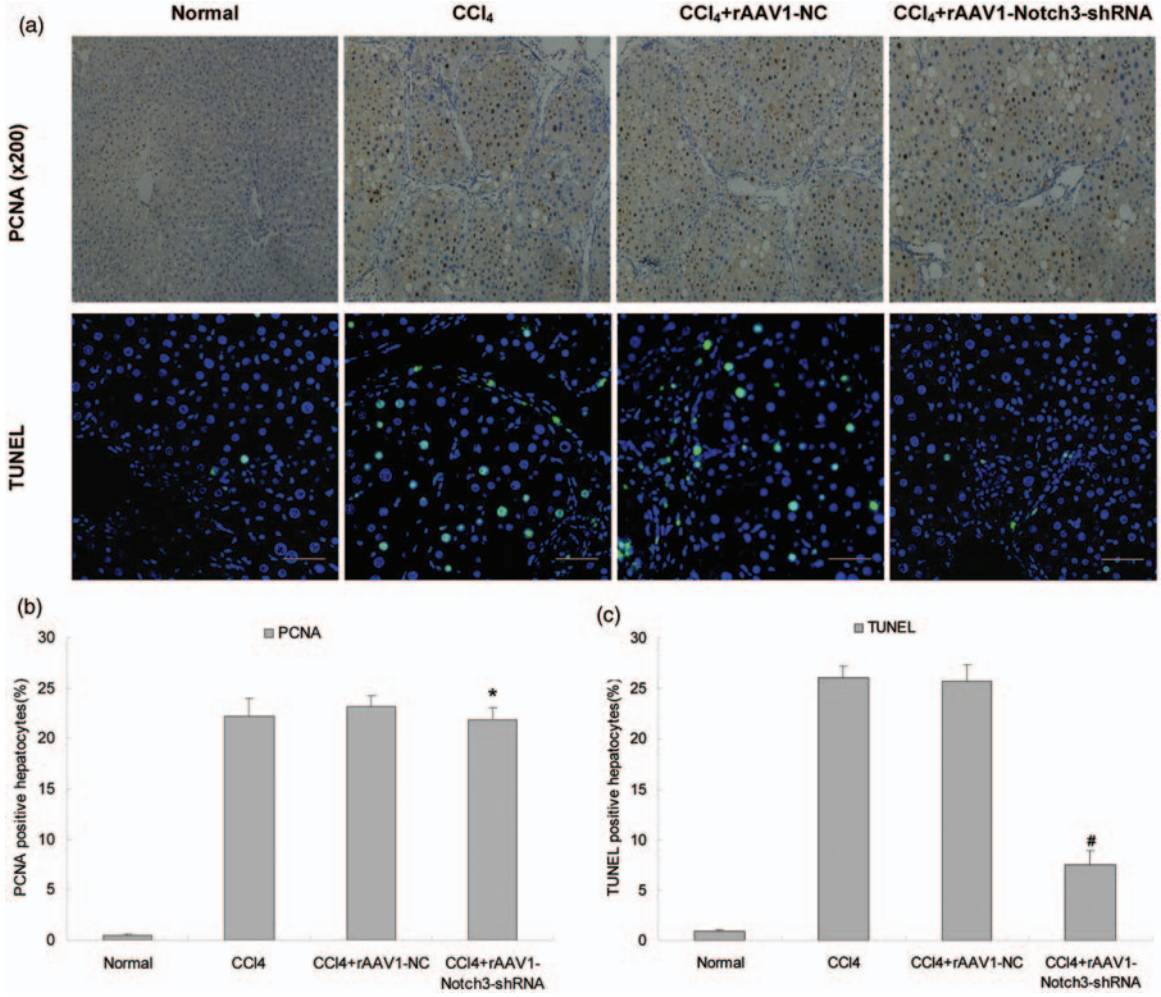

Figure 5 Effects of rAAV1-Notch3-shRNA treatment on cell proliferation and apoptosis. (a) Immunohistochemical and immunofluorescence staining were used to determine the expression of PCNA and TUNEL in liver tissues of rats from the normal, CCl_4, rAAV1-NC and rAAV1-Notch3-shRNA groups. (b and c) Cells subjected to positive PCNA and TUNEL staining were quantified using ImageJ as described in the materials and methods section. The white bars represent 50 μm. Each value represents the mean with the SD for triplicate samples. *Relative to the CCl_4 or rAAV1-NC groups, $P > 0.05$; #Relative to the CCl_4 or rAAV1-NC groups, $P < 0.05$. (A color version of this figure is available in the online journal)

was not only found to protect hepatocytes from apoptosis but also markedly repressed liver fibrogenesis in CCl_4-induced hepatic fibrosis.

Author contributions: ZHW and SPZ contributed to design the experiments; SPZ, YXC, JLG and DQ performed the experiments; ZHW, SPZ, JLG, SJZ and SLZ analysed the data; ZHW and JLG wrote and revised the paper.

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