Original Research

Inhibition of Mastermind-like 1 alleviates liver fibrosis induced by carbon tetrachloride in rats

Shaoping Zheng[1], Yixiong Chen[2], Shaojiang Zheng[3], Zhihui He[3] and Zhihong Weng[4]

[1]Department of Ultrasound, Tongji Medical College, Huazhong University of Science and Technology, Union Hospital, Wuhan 430022, China; [2]Division of Gastroenterology, Tongji Medical College, Huazhong University of Science and Technology, Union Hospital, Wuhan 430022, China; [3]Key Laboratory of Molecular Tumor Pathology, Hainan Cancer Hospital & The First Affiliated Hospital of Hainan Medical University, Haikou 571199, China; [4]Department of Infectious Diseases, Tongji Medical College, Huazhong University of Science and Technology, Union Hospital, Wuhan 430022, China

Corresponding author: Zhihong Weng. Email: wengzh@hust.edu.cn

Impact statement

Liver fibrosis is a common wound-healing response to all kinds of liver injuries. Hepatic stellate cells (HSCs) activation is the key event during liver fibrogenesis. Thus, the elucidation of mechanisms for regulating HSCs activation is helpful for identifying novel anti-fibrotic targets and strategies. MAML1, an important component of Notch signal, functions in critical transcriptional coactivation in the Notch and Wnt/β-catenin signal pathways. In the present study, we investigated the potential function of MAML1 during hepatic fibrogenesis in rats. Our results demonstrated that MAML1 participates in liver fibrosis through modulating HSCs activation via interrupting both the Notch and Wnt/β-catenin signal transductions. Additionally, the inhibition of MAML1 markedly attenuated CCl_4-induced hepatic fibrogenesis in rats. Our results shed a light for the exploitation of a new therapeutic strategy for hepatic fibrosis via targeting MAML1.

Abstract

Mastermind-like 1 (MAML1) functions in critical transcriptional coactivation in Notch and Wnt/β-catenin signal pathways, which participate in hepatic fibrosis. This study is aimed to reveal the potential role of MAML1 in liver fibrosis and identify its underlying mechanism. In present research, the enhanced expression of MAML1 was found in the fibrotic liver tissues in carbon tetrachloride (CCl_4)-induced hepatic fibrosis in rats, and MAML1 expression increased gradually during the activation of hepatic stellate cells (HSCs) isolated from the normal rat. Further studies showed that blocking MAML1 expression efficiently decreased the expression of α-SMA and collagen I (Col1a1) in HSCs. Interestingly, MAML1 may modulate HSCs activation via interrupting both Notch and Wnt/β-catenin signal transductions, and the inhibition of MAML1 by a recombinant adeno-associated virus type 1 vector carrying shRNA targeting MAML1 alleviated CCl_4-induced hepatic fibrosis in rats. These findings suggest that the selective regulation of MAML1 expression may be a feasible therapeutic approach to reverse liver fibrosis.

Keywords: Liver fibrosis, Mastermind-like 1, hepatic stellate cells, signal transduction, recombinant adeno-associated virus

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Introduction

Hepatic fibrosis is a common wound-healing response to all kinds of liver injuries. Hepatic stellate cells (HSCs) activation is the key event during liver fibrogenesis. HSCs dwell in the space of Disse in the liver. Upon liver injury, quiescent HSCs are activated and produce α-smooth muscle actin (α-SMA) and extracellular matrix (ECM). HSCs are pivotal targets for reversing fibrosis because they are the predominant source of ECM in liver fibrosis.[1,2] However, it is difficult to effectively inhibit HSCs activation in vivo.

Accumulating evidence has indicated that several signaling pathways participate in liver fibrogenesis.[3] The Wnt/β-catenin signal promotes liver fibrosis by enhancing HSCs activation.[4–6] Notch signal pathway is also essential for hepatic fibrogenesis.[7–10] Recently, studies demonstrated that the Mastermind-like (MAML) family functions in critical transcriptional coactivation in multiple signaling pathways. MAML1, a conserved transcriptional coactivator for Notch signal,[11,12] also takes part in the Wnt signal transduction via regulating β-catenin/TCF activity.[13] Moreover, MAML1 mediates cross-talk between Notch and

Wnt/β-catenin signal pathways.[14] Therefore, these findings provide a vital clue that MAML1 might be associated with liver fibrosis.

This study is aimed to investigate the potential role of MAML1 during hepatic fibrogenesis and elucidate its underlying mechanism. Here we show that MAML1 participates in liver fibrosis through modulating HSCs activation via interrupting both Notch and Wnt/β-catenin signal pathways. Furthermore, the inhibition of MAML1 by a recombinant adeno-associated virus type 1 vector carrying shRNA against MAML1 (rAAV1-MAML1-shRNA) could remit carbon tetrachloride (CCl_4)-induced hepatic fibrosis in rats. These findings imply that MAML1 plays a crucial role in liver fibrogenesis, and the regulation of MAML1 expression may be a feasible approach to reverse liver fibrosis.

Materials and methods

Reagents and antibodies

HSC-T6 cells were obtained from the Chinese Academy of Medical Sciences (China). Cell culture and molecular biology reagents were purchased from Life Technologies and Qiagen (CA, US). CCl_4 was obtained from Sigma Corporation (MO, US). Lipofectamine 2000 transfection reagent kit was purchased from Invitrogen (CA, US). As shown in Table 1, the antibodies used for immunofluorescence staining and Western blot are summarized.

Cell transfection experiment

HSC-T6 cells or activated primary HSCs were cultured in 6-well plates 12 h before transfection. The siRNA mixed with Lipofectamine 2000 in Opti-MEM I medium (Invitrogen, CA, US) was added to each well with fetal bovine serum (FBS) free Dulbecco's modified Eagle's medium (DMEM). After a 6-h incubation, the transfection mixture containing the siRNA was discarded. Then, 2 mL DMEM containing 10% FBS was added to incubate for 48 h. The siRNA sequence targeting rat MAML1 was 5′-UUGCCAUAAU CUAGCAUGGTT-3′, and the sequence of control siRNA was 5′-AACAGAGUCUGAGAGUUCCTT-3′. These siRNAs were designed and generated by Genepharma Company Limited (Shanghai, China).

Moreover, HSC-T6 cells were transfected with plasmid encoding FLAG-tagged full-length MAML1 (pCMV-FLAG-MAML1) or control vector (pCMV-FLAG), which were the gifts from Prof. Shaojiang Zheng. All cell transfection experiments were conducted using Lipofectamine 2000 according to the manufacturer's instructions.

Luciferase reporter assay

Wnt/β-catenin pathway (TCF/LEF) reporter kit, Notch pathway (CSL) reporter kit and Dual Luciferase (Firefly-Renilla) Assay System (BPS Bioscience) were used to monitor the activity of Wnt/β-catenin or Notch signal in HSC-T6 cells following the protocol provided by BPS Bioscience. Briefly, HSC-T6 cells were seeded into 96-well plates and cultured in complete DMEM. Lipofectamine 2000 was used to co-transfect with 60 ng of reporter vector (TCF/LEF or CSL luciferase and Renilla luciferase) or negative control reporter and 20 nM of synthetic MAML1-siRNA or negative control siRNA; 48 h post-transfection, luciferase activity was analyzed by Dual Luciferase Assay System (BPS Bioscience). Additionally, HSC-T6 cells were co-transfected with pCMV-MAML1 or empty control vector (pCMV), and with reporter vector (TCF/LEF or CSL luciferase and Renilla luciferase) or negative control reporter. Luciferase activities were also detected.

Preparation of rAAV1 vectors

The shRNA specific to rat MAML1 and the control shRNA were synthesized by Genepharma. The rAAV1-EGFP carrying MAML1-shRNA (rAAV1-MAML1-shRNA-EGFP) and control rAAV1 (rAAV1-NC) were produced as previously described.[10] Sequences used for the shRNAs were MAML1 shRNA forward oligo: 5′-CCGGCCATGCTA GATTATGGCAACTCGAGTTGCCATAAT

CTAGCATGGTTTTTG-3′; reverse oligo: 5′-AATTCAA AAATTGCCATAATCTA

GCATGGCTCGAGCCATGCTAGATTATGGCAA-3′,

and the control shRNA forward oligo: 5′-CCGGGGA ACTCTCAGACTCTGTTCTCGAGAACAGAGTCT

GAGAGTTCCTTTTTG-3′, reverse oligo: 5′-AATTCAA AAAAACAGAGTCTGA

GAGTTCCCTCGAGGGAACTCTCAGACTCTGTT-3′.

Animal experiment

Thirty-two male SD rats (200–240 g) purchased from the experimental animal center of Tongji Medical College (Wuhan, China) were divided randomly into four groups of eight rats each: normal (A), model (B), rAAV1-NC (C) and rAAV1-MAML1-shRNA (D). The rats in group A were subcutaneously injected olive oil (3 mL/kg) twice a week

Table 1. Primary antibodies used for immunofluorescence and Western blot.

Rats antigens	Poly/mono-clonal	Manufacturer	Dilution
MAML1	Polyclonal	Abcam, US	1:500 (1:2000 for Western blot)
α-SMA	Monoclonal	Abcam, US	1:500 (1:2000 for Western blot)
Collagen I	Monoclonal	Abcam, US	1:2000 for Western blot
c-Myc	Monoclonal	Santa Cruz Biotechnology, Inc., CA	1:200 (1:500 for Western blot)
Cyclin D1	Monoclonal	Cell Signaling Technology, Inc., US	1:1000 for Western blot
Hes1	Polyclonal	Santa Cruz Biotechnology, Inc., CA	1:200 (1:500 for Western blot)
GAPDH	Polyclonal	Santa Cruz Biotechnology, Inc., CA	1:1000 for Western blot
EGFP	Monoclonal	Abcam, US	1:100

for eight weeks, and the rats in groups B, C and D were administrated with CCl_4 (3 mL/kg) twice a week for eight weeks as previously described.[9,10] Four weeks after the initial treatment, rats in groups A and B were received injections of PBS via tail vein, and rats in groups C and D were administrated with a single dose of 3×10^{11}v.g. rAAV1-NC or rAAV1-MAML1-shRNA per rat. The rats in groups A and B were killed at initial, week 4 and week 8, and the rest of the animals were sacrificed eight weeks after the first administration. Partial liver tissue samples were fixed and embedded in paraffin, and other specimens were harvested and stored at −80°C.

All animals were fed in the animal house of the Experimental Animal Center of Tongji Medical College (Wuhan, China). The rats received humane care and this study was authorized by the Ethical Committee of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China).

Isolation of HSCs

Primary HSCs were isolated and purified from freshly perfused livers of healthy SD rats (400–450 g). Briefly, rat was anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). The liver was exposed and sequentially perfused with pronase and collagenase, and centrifuged with Nycodenz as described previously.[15] The cell purity of isolated HSCs was estimated by observing the autofluorescence of retinoids and was approximately greater than 90%. Isolated HSCs were cultured in DMEM supplemented with 10% FBS. Primary HSCs underwent spontaneous activation by culturing on plastic for seven days.[15]

Real-time PCR

The total RNA of isolated HSCs, HSC-T6 cells or liver tissues was extracted using Trizol reagent (Life technologies, US) and was used to synthesize cDNA through the SuperScript II First-strand Synthesis System (Invitrogen, US). After that, the cDNA underwent real-time PCR by the SYBR Green/Fluorescein qPCR Master Mix (2X) (Applied Biosystems, US). The primers used for real-time PCR are shown in Table 2.

Table 2. The primer sequences for real-time PCR.

Name	Primer	Sequence
MAML1	Forward	5′-GGGACTCTGATGTCAATGGG -3′
	Reverse	5′-TGGTCTGCCGTGGAATGTG -3′
c-Myc	Forward	5′-CGAGCTGAAGCGTAGCTTTT-′3
	Reverse	5′-CTCGCCGTTTCCTCAGTAAG-′3
CyclinD1	Forward	5′-GCGTACCCTGACACCAATCT-3′
	Reverse	5′-GCTCCAGAGACAAGAAACG-3′
Hes1	Forward	5′-GCGCCGGGCAAGAATAAATG-3′
	Reverse	5′-TCGGTGTTAACGCCCTCACAC-3′
α-SMA	Forward	5′-ACTGCTGCTTCCTCTTCTTC-3′
	Reverse	5′-TGCTGTTATAGGTGGTTTCG-3′
collagen I	Forward	5′-TGACTGGAAGAGCGGAGAGT-3′
	Reverse	5′-GAATCCATCGGTCATGCTCT-3′
β-actin	Forward	5′-CACGATGGAGGGGCCGGACTCATC-3′
	Reverse	5′-TAAAGACCTCTATGCCAACACAGT -3′

Histopathology and immunofluorescence

Hematoxylin-eosin (HE), Masson and Sirius Red staining were used to evaluate the histopathology and collagen deposition of liver tissues. HSCs were fixed in PBS containing 4% paraformaldehyde. The expression of α-SMA, MAML1, Hes1 and c-Myc in liver tissues of rat or in HSCs was examined using immunofluorescence assay. As shown in Table 1, the primary antibodies used for immunofluorescence are listed. The fluorescent secondary antibodies were conjugated with Alexa Fluor 488 (green) or Alexa Fluor 594 (red) (Invitrogen, US). Non-immune IgG instead of primary antibodies served as negative controls. All samples were detected using a laser confocal fluorescence microscope (Nikon, Japan).

Expression of rAAV1-MAML1-shRNA-EGFP in liver

To confirm the preferential binding of rAAV1-MAML1-shRNA-EGFP to fibrotic areas in liver, the co-expression of α-SMA and EGFP was detected by immunofluorescence staining in rat liver tissues embedded in paraffin. The primary antibodies used are shown in Table 1.

Western blot

Briefly, the proteins extracted from cultured HSCs were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA). Then, the PVDF membranes were incubated with the primary antibodies, followed by the incubation with horseradish peroxidase-conjugated secondary antibodies. Intensities of the protein bands visualized by BeyoECL Plus system (Beyotime, China) were analyzed with ImageJ 1.51b (NIH, US).

Flow cytometry analysis

The apoptosis of HSC-T6 cells was examined using Annexin V-APC/7AAD staining and flow cytometry analysis. HSC-T6 treated with or without siRNA targeting MAML1 were collected, washed, and resuspended in Annexin V binding buffer, followed by the addition of APC conjugated Annexin V and 7AAD successively. After incubation, cells were analyzed by flow cytometry.

Detection of hydroxyproline

Total hepatic hydroxyproline levels in the hydrolysates of liver specimens were assayed using the Hydroxyproline Testing Kit (Jiancheng, China). Briefly, liver samples or hydroxyproline standards were added to a 96-well plate. Then, a Chloramine T mixture was added to convert the hydroxyproline to a pyrrole. Finally, Ehrlich's Reagent was added to the well. The absorbance of the plate was read at 550 nm, and the content of hydroxyproline in liver samples was determined by comparison with a predetermined hydroxyproline standard curve.

Statistical analysis

Data are expressed as the mean ± SD from three independent experiments. Unpaired Student's *t*-test was performed

to compare the difference between two groups. The comparison among three groups was analyzed by ANOVA with Student–Newman–Keuls *post hoc* test. Statistical analyses were carried out using GraphPad Prism 5.0 (US) and SPSS 18.0 (IBM, US). $P < 0.05$ was considered significant statistically.

Results

Up-regulated MAML1 expression in fibrotic liver tissues in rats

We initially reveal the potential role of MAML1 in liver fibrogenesis. A rat model of CCl_4-induced hepatic fibrosis was generated and rats were sacrificed as described in Materials and Methods. We find that the expression of MAML1 and α-SMA was enhanced gradually on the mRNA and protein levels after CCl_4 administration, and significant high levels were observed in rats treated with eight week of CCl_4 (Figure 1(a)).

In addition, the expression of α-SMA and MAML1 in rat liver tissues was also analyzed by immunofluorescence. In Figure 1(b), positive staining for MAML1 and α-SMA was obviously strengthened in fibrotic liver tissues of rats in model group, whereas the normal control group did the opposite. These data indicated that the up-regulated expression of MAML1 might correlate with hepatic fibrogenesis.

MAML1 expression in HSCs isolated from rat livers

Moreover, HSCs were isolated from normal rat liver, followed by spontaneous activation by culturing on plastic for seven days. The expression of MAML1 and α-SMA increased gradually (Figure 2(a)), and MAML1 was confined in the cytoplasm and nucleus during HSCs activation (Figure 2(b)), which implies that MAML1 is required for HSCs activation.

Effects of inhibiting MAML1 expression on the activation of HSCs

To further reveal the character that MAML1 act as in the activation of HSCs, we assessed the functional activity of HSC-T6 cells after MAML1 knockdown. The results showed that MAML1-siRNA efficiently decreased the expression of MAML1, α-SMA and collagen I (Col1a1) on the mRNA and protein levels in HSC-T6 cells (Figure 3). We next isolated the primary HSCs from rat livers and cultured for seven days, then activated HSCs were administrated with MAML1-siRNA or control siRNA. In compared with the control group, the mRNA and protein levels of MAML1, α-SMA and collagen I were also sharply down-regulated in activated primary HSCs treated with MAML1-siRNA (Supplementary Figure S1).

Effects of blocking MAML1 expression on HSCs apoptosis

Moreover, the apoptosis of HSC-T6 cells treated with MAML1-siRNA was investigated using Annexin V-APC/7AAD staining and flow cytometry analysis. As shown in Supplementary Figure S2, the early and total apoptosis rates of HSC-T6 were not markedly changed in the MAML1-siRNA-treated group in compared with the normal or control group.

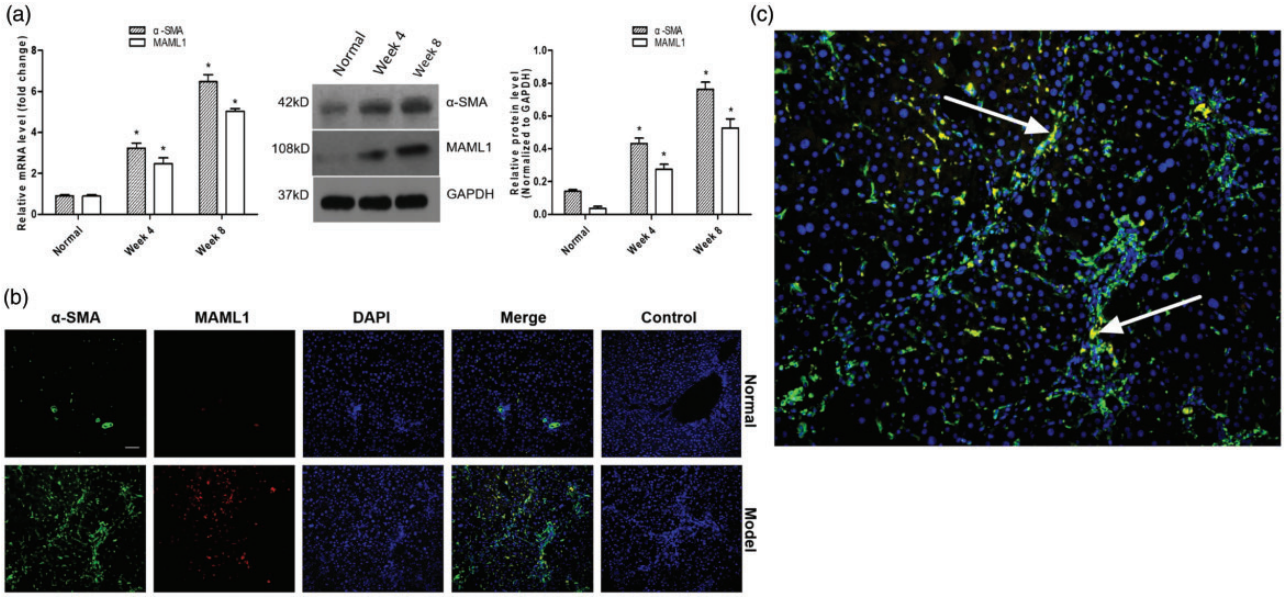

Figure 1. Expression of MAML1 in liver tissue in a rat fibrosis model induced by CCl4. Rats were administered CCl4 or olive oil for eight weeks. Real-time PCR and Western blot were performed to detect the expression of α-SMA and MAML1 in rats (a). Each value represents the mean for triplicate samples. *$P < 0.05$ versus rats at week 4 or normal control. The liver sections of rats were obtained and analyzed using immunofluorescence staining (b). Nuclei were stained with DAPI. Images were captured by confocal fluorescence microscopy. Arrows indicate α-SMA/MAML1 double positive cells (c), and the bar represents 20 μm. (A color version of this figure is available in the online journal.)

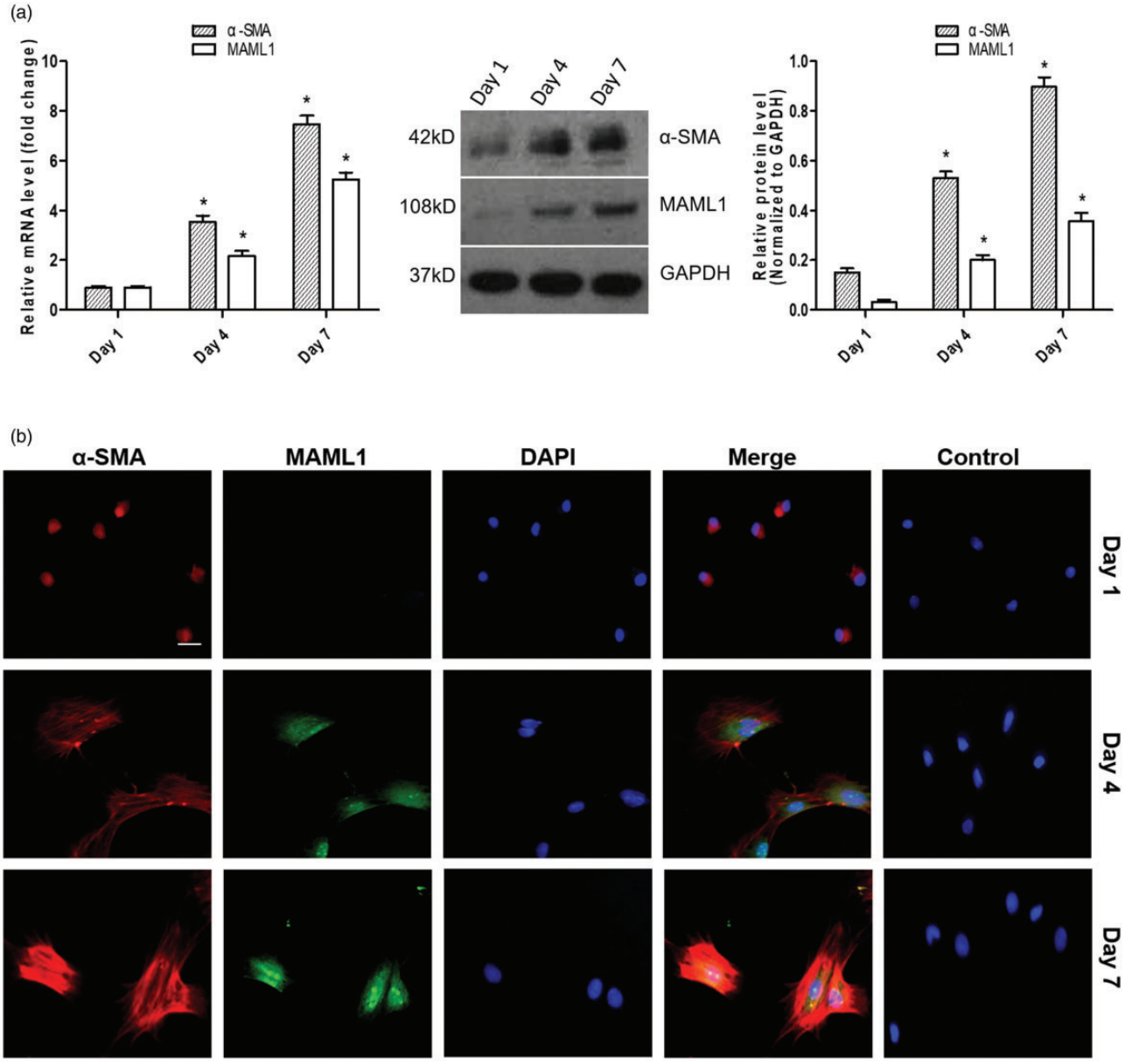

Figure 2. Expression of MAML1 in HSCs isolated from normal rat livers. HSCs were isolated from freshly perfused rat livers, and the expression of α-SMA and MAML1 in HSCs was detected using real-time PCR, Western blot (a) and immunofluorescence staining (b) for the indicated time. The bar represents 50 μm. Data represent the results of three independent experiment, $^*P < 0.05$ versus the HSCs in day 1 or day 4. (A color version of this figure is available in the online journal.)

Down-regulation of MAML1 inhibited both Notch and Wnt/β-catenin signal transductions in HSCs

We further examined the expression of down-stream target genes of the Wnt/β-catenin and Notch signals after MAML1 knockdown to identify whether it can dysregulate these two signaling pathways in HSC-T6 cells. Moreover, HSCs isolated from rat livers were cultured for seven days and subsequently administrated with MAML1-siRNA or control siRNA. We then detected the expression of Hes1, c-Myc and Cyclin D1 and found that these genes were markedly suppressed in HSC-T6 cells (Figure 4(a) to (c)) or activated primary HSCs (Supplementary Figure S1) treated with MAML1-siRNA compared to the normal or control group. These results consistently indicated that both these two signal transductions were blocked by MAML1 knockdown.

Also, we assessed the potential roles of MAML1 in the Wnt/β-catenin or Notch signal pathways using Wnt/β-catenin pathway reporter kit, Notch pathway reporter kit and Dual Luciferase Assay System to monitor the activity of Wnt/β-catenin or Notch signal in HSC-T6 cells. The result shows that MAML1-siRNA greatly inhibited the transduction of the Notch or Wnt/β-catenin signal in HSC-T6 cells (Figure 4(d) and (e)).

MAML1 overexpression induced HSCs activation and enhanced Notch or Wnt/β-catenin signal transduction

On the other hand, we investigated the functional activity of HSC-T6 and the target genes expression of the Notch and Wnt/β-catenin signals after MAML1 overexpression. It showed that the expression of MAML1, collagen I,

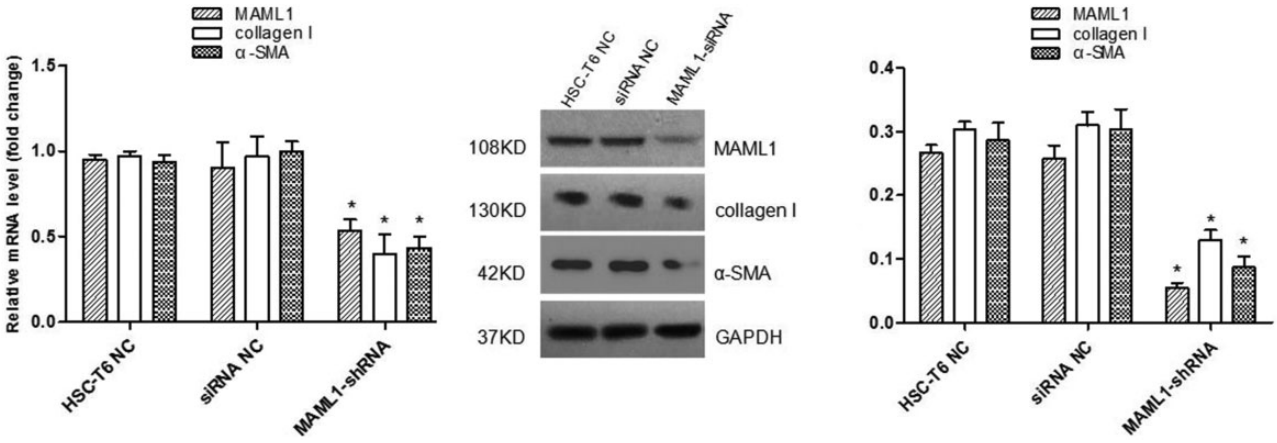

Figure 3. Effect of MAML1-siRNA on the activation of HSCs. HSC-T6 cells were treated with MAML1-siRNA or control siRNA. The expression of MAML1, α-SMA and collagen I in HSC-T6 cells was determined using real-time PCR and Western blot. Data shown are representative three independent experiments. $^*P < 0.05$ versus the normal HSC-T6 or control siRNA group.

α-SMA, Hes1, c-Myc and Cyclin D1 was markedly increased in HSC-T6 cells administrated with pCMV-MAML1 when compared with other groups. Meanwhile, the activity of Wnt/β-catenin or Notch signaling monitored by Dual Luciferase Assay System was also enhanced in HSC-T6 cells transfected with pCMV-MAML1 than that in control group (Supplementary Figure S3).

Binding of rAAV1 to fibrotic liver tissues

To show the specificity of rAAV1- MAML1-shRNA-EGFP infection in vivo, we detected the co-expression of α-SMA and EGFP by immunofluorescence staining in liver tissues from CCl_4-treated rats four weeks after rAAV1-MAML1-shRNA or rAAV1-NC infection. It showed that positive staining for EGFP and α-SMA was found predominantly in HSCs in the fibrotic areas of liver tissues (Figure 5).

rAAV1-MAML1-shRNA attenuated CCl4-induced hepatic fibrosis in rats

Furthermore, to investigate the effect of blocking MAML1 expression on CCl_4-induced liver fibrosis in rats, we evaluated the pathological alterations and collagen deposition in the liver tissues in rats, which were treated with CCl_4 and rAAV1-NC or rAAV1-MAML1-shRNA. As shown in Figure 6(a), the liver structure had been destructed and many fibrous septa were found in liver sections from the rats administrated with CCl_4 or rAAV1-NC. However, normal liver lobular structure was still observed and fewer fibrotic septa emerged in the liver sections from the rats treated with rAAV1-MAML1-shRNA than that in model or control group. Moreover, an evident decrease in collagen accumulation in liver tissue of the rat treated with rAAV1-MAML1-shRNA was demonstrated by Masson and Sirius red staining analysis (Figure 6(a) and (b)), and the level of hydroxyproline in liver of the rat administrated with rAAV1-MAML1-shRNA was much lower than that in model or control group (Figure 6(c)).

Inhibition of MAML1 ameliorated hepatic fibrosis via interrupting both Wnt/β-catenin and Notch signals

To reveal the potential mechanisms responsible for the role of MAML1 in liver fibrogenesis in vivo, the expression of MAML1, α-SMA, Hes1 and c-Myc in liver tissues in rats treated with CCl_4 and rAAV1-MAML1-shRNA or rAAV1-NC was tested using Western blot and immunofluorescence. Together, the results indicated that the expression of MAML1, α-SMA, Hes1 and c-Myc in the liver in rats received CCl_4 was markedly higher than those in normal group, and their expression was observably down-regulated after rAAV1-MAML1-shRNA treatment (Figure 7(a) and Supplementary Figure S4). Immunofluorescence analysis revealed that the expression of MAML1, α-SMA, Hes1 and c-Myc was significantly reduced in group treated with rAAV1-MAML1-shRNA than that in model or control group (Figure 7(b) and Supplementary Figure S4). These results strongly implied that the blockade of MAML1 attenuated CCl_4-induced hepatic fibrosis via interrupting the transductions of both Notch and Wnt/β-catenin signals in HSCs in rat liver.

Discussion

There is now overwhelming evidence indicating that the activation of HSCs represents a key step of liver fibrogenesis.[1–3] Thus, the elucidation of mechanisms for regulating HSCs activation is helpful for identifying novel anti-fibrotic targets and strategies. In the present study, MAML1 was found to participate in liver fibrogenesis through the regulation of HSCs activation via interrupting both the Notch and Wnt/β-catenin signal pathways. Interestingly, the inhibition of MAML1 by rAAV1-MAML1-shRNA could alleviate CCl_4-induced liver fibrosis in rats.

MAML1, an integral component of Notch signaling, regulates the Notch target gene expression through coordinating the assembly of the transcriptional activation complex.[11] MAML1 also acts as an essential co-activator of β-catenin/TCF and as a key integrator of Wnt/β-catenin signaling.[13] In the present study, we demonstrated that MAML1 participates in liver fibrogenesis. There are several

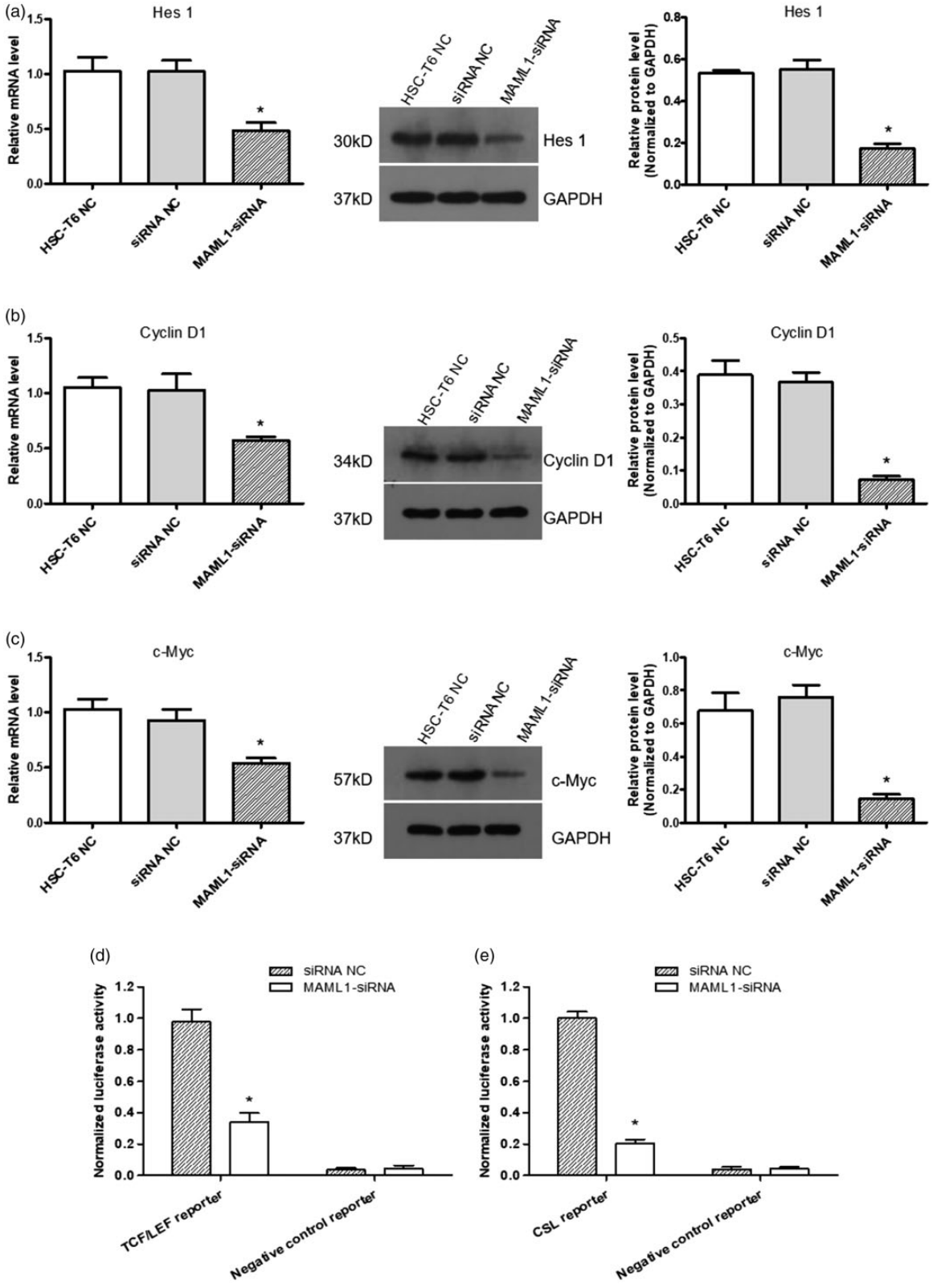

Figure 4. Effect of MAML1-siRNA on Notch and Wnt/β-catenin signal transduction in HSCs. HSC-T6 cells were treated with MAML1-siRNA or control siRNA. Then, the expression of Hes1 (a), Cyclin D1 (b) and c-Myc (c) was detected using real-time PCR and Western blot. Additionally, HSC-T6 cells were cultured in 96-well plates. Lipofectamine 2000 was used to co-transfect with 60 ng of reporter vector (TCF/LEF (d) or CSL (e) luciferase and Renilla luciferase) or negative control reporter and 20 nM of MAML1-siRNA or negative control siRNA; 48 h after transfection, luciferase activities were measured by the Dual Luciferase Assay System. Data represent the results of three independent experiments. *$P < 0.05$ versus the normal HSC-T6 or control siRNA group.

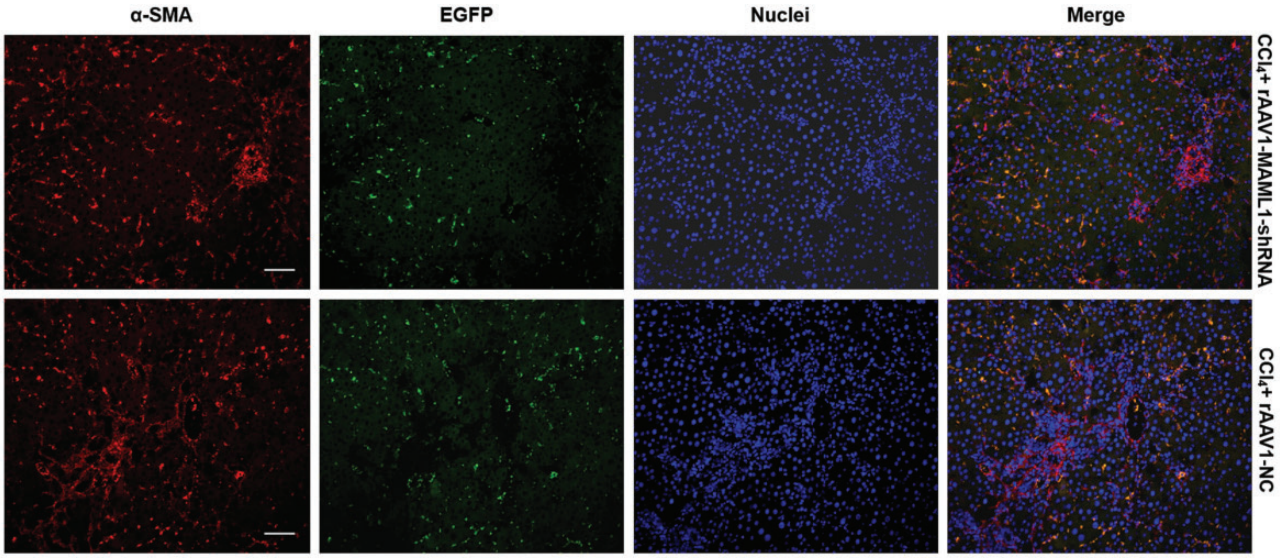

Figure 5. Preferential binding of rAAV1 to fibrotic areas in rat livers. The co-expression of α-SMA and EGFP was detected by immunofluorescence staining in liver tissues from CCl4-treated rats four weeks after rAAV1-MAML1-shRNA or rAAV1-NC infection. It was showed that approximately 30% of cells in the fibrotic areas of liver tissues co-expressed EGFP and α-SMA. The bar represents 20 μm. (A color version of this figure is available in the online journal.)

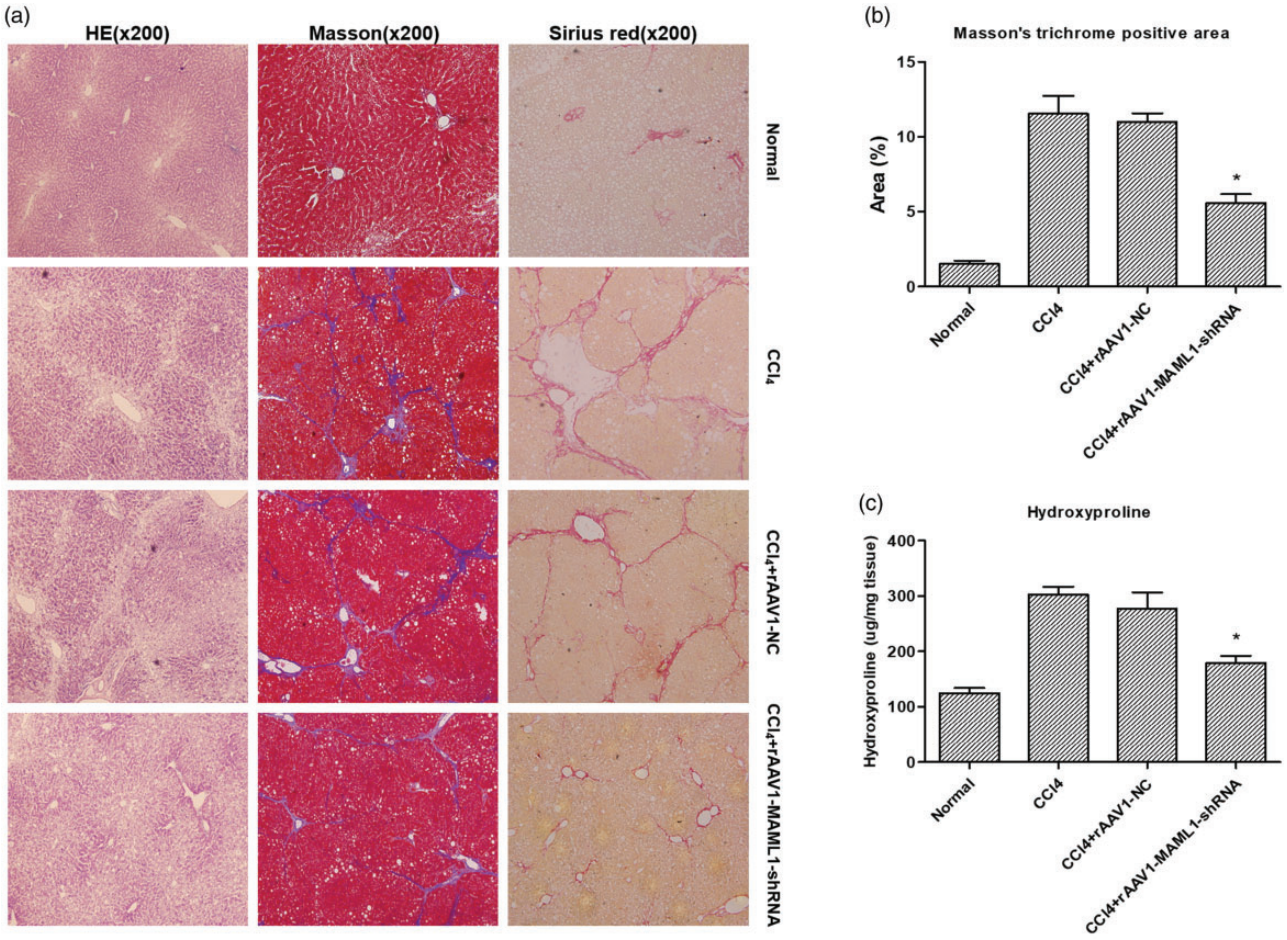

Figure 6. rAAV1-MAML1-shRNA ameliorated CCl4-induced liver fibrosis in rats. The pathological alterations and collagen deposition in liver tissues of rats in the normal, CCl4, rAAV1-NC and rAAV1-MAML1-shRNA groups were examined using hematoxylin-eosin, Masson's trichrome and Sirius red staining (a). The semi-quantitative analysis of the results of Masson's trichrome staining (b). The assay of hydroxyproline content (c). Data shown are representative three independent experiments. $^*P < 0.05$ versus the CCl4 or rAAV1-NC group. (A color version of this figure is available in the online journal.)

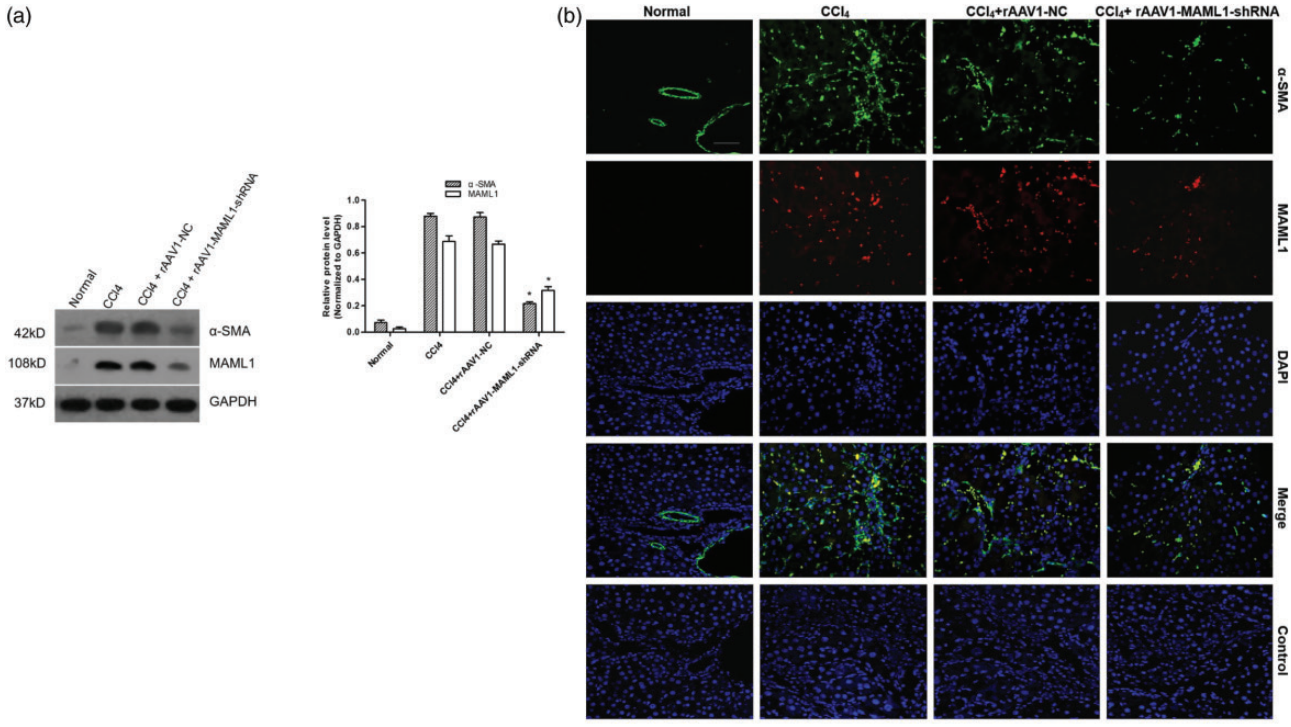

Figure 7. rAAV1-MAML1-shRNA administration downregulated MAML1 and α-SMA expression in rats. Rats were injected rAAV1-MAML1-shRNA or rAAV1-NC four weeks after CCl4 administration and were sacrificed eight weeks after the initial treatment. Western blot (a) and immunofluorescence (b) were performed to detect the expression of MAML1 and α-SMA in liver tissues of rats from normal, CCl4, rAAV1-NC and rAAV1-MAML1-shRNA groups. The bar represents 20 μm. Data represent the results of three independent experiments. $^*P < 0.05$ versus the CCl4 or rAAV1-NC group. (A color version of this figure is available in the online journal.)

findings that support this hypothesis. First, the expression of MAML1 in fibrotic liver tissues in CCl_4-induced hepatic fibrosis in rats was significantly up-regulated. Second, the expression of MAML1 increased gradually during the activation of HSCs isolated from normal rat livers. Moreover, blocking MAML1 expression had efficiently down-regulated α-SMA and collagen I expression in HSC-T6 cells. In addition, the inhibition of MAML1 markedly attenuated CCl_4-induced haptic fibrosis in rats, which was confirmed by the decrease in ECM accumulation. Collectively, these findings suggest that MAML1 involves in hepatic fibrosis through facilitating the activation of HSCs.

To reveal the potential mechanisms responsible for the activation of HSCs regulated by MAML1, we detected the expression of target genes of the Wnt/β-catenin and Notch signals after inhibiting MAML1 expression in HSCs. There are several studies which have demonstrated that Hes1, c-Myc and Cyclin D1 are the target genes of the Wnt/β-catenin and Notch signaling pathways.[16–18] Our study found that the expression of Hes1, c-Myc and Cyclin D1 was significantly down-regulated after MAML1 inhibition in HSCs. Furthermore, the detection using Dual Luciferase Assay System showed that the transductions of Notch and Wnt/β-catenin signals in HSC-T6 cells transfected with MAML1-siRNA were significantly blocked, which indicates that MAML1 may regulate HSCs activation by interrupting both the Notch and Wnt/β-catenin signals.

It was reported that the expression of Notch2, Notch3 and Hey2 significantly increases during the activation of quiescent HSCs.[7,19–21] Another study found that Hes1 stimulates the promoter activities of collagen I and α-SMA in HSCs.[16] Our previous studies demonstrated that Notch signal is significantly activated in HSCs in CCl_4-induced hepatic fibrosis in rats. Moreover, blocking Notch signaling activation significantly attenuated liver fibrosis.[8–10] In addition, Wnt/β-catenin signal functions in combination with TGF-β1 signaling during hepatic fibrogenesis by enhancing HSCs activation.[4–6,22] Therefore, the aberrant activation of Wnt/beta-catenin and Notch signals could drive liver fibrogenesis by regulating HSCs activation, suggesting that the modulation of both the Notch and Wnt signaling pathways simultaneously might be a feasible therapeutic approach to reverse hepatic fibrosis.

The rAAV coreceptor, fibroblast growth factor receptor 1α (FGFR-1α), has been demonstrated to express mainly in fibrotic liver tissues of CCl_4-treated rats, whereas FGFR-1α expression was not detected in hepatocytes,[23] and there was a preferential binding of rAAV to HSCs in fibrotic liver tissues of rat.[10,23,24] Our results indicated that the expression of EGFP was mainly confined to the activated HSCs in the fibrotic liver tissues in CCl_4-treated rats infected with rAAV1-MAML1-shRNA-EGFP. All these findings indicated that rAAV might be a feasible tool for treating liver fibrosis.

In summary, our present investigation indicated a key role for MAML1 in liver fibrogenesis. The potential mechanisms might be associated with the transductions of both Notch and Wnt/β-catenin signals. Importantly, our findings shed a light for the development of a novel therapeutic strategy for hepatic fibrosis by targeting MAML1.

Author contributions: ZHW and SPZ designed this study. SPZ and YXC conducted the experiments. ZHW, SJZ and ZHH analyzed the data. ZHW and SPZ wrote the paper. All authors approved the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article..

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