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Myofibroblast-specific YY1 promotes liver fibrosis

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Abstract

Liver fibrosis is a common consequence of various chronic hepatitis and liver injuries. The myofibroblasts, through the accumulation of extracellular matrix (ECM) proteins, are closely associated with the progression of liver fibrosis. However, the molecular mechanisms underlying transcriptional regulation of fibrogenic genes and ECM proteins in myofibroblasts remain largely unknown. Using tamoxifen inducible myofibroblast-specific Cre-expressing mouse lines with selective deletion of the transcription factor Yin Yang 1 (YY1), here we show that YY1 deletion in myofibroblasts mitigates carbon tetrachloride-induced liver fibrosis. This protective effect of YY1 ablation on liver fibrosis was accompanied with reduced expression of profibrogenic genes and ECM proteins, including TNF- α , TGF- β , PDGF, IL-6, α -SMA and Colla1 in liver tissues from YY1 mutant mice. Moreover, using the human hepatic stellate cell (HSC) line LX-2, we found that knockdown of YY1 in myofibroblasts by siRNA treatment diminished myofibroblast proliferation, α -SMA expression, and collagen deposition. Collectively, our findings reveal a specific role of YY1 in hepatic myofibroblasts and suggest a new therapeutic strategy for hepatic fibrosis-associated liver diseases.

Keywords

Liver fibrosis; Myofibroblast; Yin Yang 1 (YY1); Postn^{MCM} mouse line

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Author contributions

HL, SZ, and ZGJ wrote the manuscript with comments and contributions from all authors. HL, SZ, and MK performed experiments. SX proofread and revised the manuscript. EMS provided key reagents. ZGJ conceived the study. All authors approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2019.05.004>.

Conflicts of interest

The authors declare that they have no conflict of interest.

1. Introduction

Liver fibrosis is a critical process during chronic liver injury progresses into cirrhosis and liver cancer, a major cause of morbidity and mortality in the world. Chronic liver injury, resulting from autoimmune disease, chronic viral infection, and nonalcoholic steatohepatitis, are the fundamental causes of liver fibrosis [1]. Liver fibrosis can be reversed when spotted in the early stages and steps are taken to prevent further damage [2]. Thus, there is a tremendous need for finding new therapeutic approaches to prevent the progression from reversible liver fibrosis to irreversible cirrhosis and eventually liver cancer. Liver fibrosis is characterized by excessive accumulation of extracellular matrix (ECM), mainly produced by myofibroblasts, in the liver [3,4]. Myofibroblasts (also known as activated fibroblasts) in the liver are mainly transdifferentiated from hepatic stellate cells (HSCs) that are activated by cytokines such as profibrogenic transforming growth factor beta (TGF- β). Myofibroblasts are the primary cell type responsible for the excessive production of fibrillary type I collagen and α -SMA (the myofibroblast phenotype marker gene) [5]. Accelerated ECM production alters the hepatic architecture and leads to subsequent hepatocellular dysfunction, ultimately resulting in cirrhosis [6]. Thus, myofibroblast proliferation and synthesis of ECM serve as the primary causes of liver fibrosis and represent novel targets for antifibrotic treatments. However, the precise molecular mechanisms underlying the regulation of myofibroblast activation and ECM production remain largely unexplored.

The transcription factor Yin Yang 1 (YY1) plays a crucial role in various biological processes, including cellular proliferation, differentiation, and development [7]. However, it is unclear whether YY1 contributes to myofibroblast activation and ECM production during the development of liver fibrosis. By utilizing newly engineered tamoxifen-inducible Postn Cre recombinase gene-targeted (Postn^{MCM}) mice for genetic ablation of YY1 in myofibroblasts, here we report that myofibroblast-specific YY1 deletion inhibited myofibroblast formation and attenuated hepatic fibrosis in mice challenged with carbon tetrachloride (CCL4).

2. Materials and methods

2.1. Mice and treatments

To assess the potential effect of YY1 deficiency in myofibroblasts on the CCL4-treated fibrosis mice model, myofibroblast-specific YY1 deficient (YY1^{flox/flox}, Postn^{MCM+}) mice were generated by crossing YY1^{flox/flox} mice [8] with Postn^{MCM+} mice [9]. The conditional knockout YY1 (YY1^{flox/flox}) mice [8] were purchased from Jackson Laboratory; Postn^{MCM+} mice were gifted by Jeffery D Molkentin [9] (Cincinnati Children's Hospital). YY1^{flox/flox} mice were used as littermate wild-type (WT). 10-week-old YY1^{flox/flox}; Postn^{MCM+} mice were fed a tamoxifen food diet (Envigo, Cat No. TD.130857) for 8 weeks with the CCL4 (Sigma, Cat No.289116 2 μ l/g, diluted 1:4 in olive oil (Sigma, Cat No.O1514) injected once a day for five days. (Total 12 times). After eight weeks, the mice were sacrificed under anesthesia 48 h after the last dose of CCL4. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Rochester Medical Center.

2.2. Histology and immunohistochemistry

Paraffin-embedded liver sections were routinely stained with hematoxylin and eosin (H&E) (Darmstadt, Cat No.PS103-01). Picro Sirius red staining (Abcam, Cat No.Ab150681) was conducted to stain collagen fibers. Stained sections were imaged with a BX51 light microscope and analyzed using spot5.0 software. For immunofluorescent studies in liver tissues and LX-2 cells, fixed frozen sections or cells grown on 35 mm dishes were blocked with 10% goat serum albumin, and incubated with primary antibody overnight with the following antibodies: α -SMA (dilution 1:100; Cat No.M0851 Dako) and YY1 (dilution 1:100; Cat No.Ab109231, Abcam). For unconjugated antibodies, appropriate secondary antibodies (dilution 1:100; Sigma-Aldrich, Cat No.ab1507, Tauf-kirchen) were added on the next day, before mounting with Prolong Gold anti-fade mounting media with DAPI (Thermo Fisher, Cat No.D1306).

2.3. Cell culture experiments

Human hepatic stellate cell (HSC) line LX-2 [10] (MilliporeSigma, Cat.No. SCC064). LX-2 cells were cultured in DMEM High Glucose (Millipore Cat. No. SLM-021-B), 2% FBS (Millipore Cat. No. ES009-B), 1% Pen/Strep (Millipore Cat. No. TMS-AB2-C) and 1% Glutamine (Millipore Cat. No. TMS-002-C) media. LX-2 cells were subcultured with trypsin (Millipore Cat. No. SM-2003-C) and then passaged before use. LX-2 cells were stimulated by TGF- β (10 μ g/ml; Sigma, Cat No.SAB4502958) for 24 h to transform into myofibroblast.

2.4. siRNA transfection

Myofibroblasts at greater than 80% confluence in 60-mm dishes were used for transfection. In brief, RNAiMax transfecting agent (6 μ l; Invitrogen; Cat No.13-778-030) was mixed with Opti-MEM (250 μ l; Invitrogen; Cat No.11-058-021), and then siRNA human YY1 (25 nM, Invitrogen; Cat No.AM16708) or non-target control siRNA (25 nM, Invitrogen; Cat No.AM4065) diluted in 250 μ l Opti-MEM was added to the solution, mixed gently, and incubated at room temperature for 20 min. A total of 0.5 ml of this mixture was added to MF in 1.5 ml Opti-MEM and incubated for four hours. Then the media was replaced with DMEM complete medium and cells were treated after 48 h after transfection [11].

2.5. Quantitative real-time PCR

After treatment, total RNA was extracted using a QIAGEN RNeasy Mini kit (Qiagen, Cat No.74136) [11]. RNA concentration and purity were determined by Nanodrop2000 Spectrophotometer (Thermo Fischer Scientific). For reverse transcription, 0.5—1 μ g of total RNA was converted first to strand complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cat No. 4374966) following the manufacturer's instructions. Quantitative real-time PCR was then performed with a Bio-Rad iQ5 real-time PCR thermal cycler, using iQ SYBR Green Supermix (Bio-Rad, Cat No. 1708886) for relative mRNA quantification. All primer sequences were listed in Table S1. The comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$) was used to determine the relative mRNA expression of target genes after normalization to the housekeeping gene GAPDH or β -actin.

2.6. Western blot analysis

Frozen liver tissues and total cell lysates were harvested in freshly-prepared lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β -Glycerolphosphate, 50 mM NaF, 1 mM Na₃VO₄, and 1% protease inhibitor cocktail). After clarification at 4 °C, the cells were spun down at 12,000 g for 10 min; total cell lysate was collected for SDS-PAGE gel analysis. After a 1.5 h transfer at 250 mV, the membranes were blocked in LI-COR blocking buffer diluted 1:1 with PBS at room temperature for one hour. Then, the blots were incubated with primary antibodies (listed in Table S2) diluted in 3% BSA at 4 °C overnight or room temperature for one hour, followed by incubation with LI-COR IRDye[®] 680RD goat anti-mouse IgG (H + L) or IRDye[®] 800CW goat anti-rabbit IgG (H + L) or IRDye[®] 680RD donkey anti-goat IgG (H + L) (dilution at 1:10,000) at room temperature for 30 min. Images were visualized using an Odyssey Infrared Imaging System (LI-COR) [11]. Densitometry analysis of blots was performed using NIH Image J software (<http://imagej.nih.gov/ij/>).

2.7. Statistical analysis

Data are presented as means $\pm$ SEM. Statistical analysis was performed using GraphPad Prism Software Version 5.02 (GraphPad Software, La Jolla, CA). Results were evaluated by *t*-test or by one- or two-way analysis of variance (ANOVA) when appropriate. A P value $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Generation and characterization of myofibroblast-specific YY1 deficient mice

To investigate the potential role of YY1 in liver fibrosis, we first examined the expression of YY1 in the liver of C57BL/6J mice treated with CCL4 for the induction of liver fibrosis. We found that the level of YY1 and myofibroblast marker gene α -SMA protein expression was increased in the fibrotic liver tissue (Fig. 1A and B, C). To explore the function of myofibroblast-derived YY1 *in vivo*, we specifically ablated YY1 gene in murine myofibroblasts by generating myofibroblast-specific YY1 knockout mice. The *Postn* genetic locus was targeted with a tamoxifen-inducible MerCreMer (MCM) cDNA (*Postn*^{MCM}) [9], and these mice were crossed with a YY1^{flx/flx} targeted strain [8] (Fig. 1D). We generated *Postn*^{MCM+}; YY1^{flx/flx} mutant (*Postn*^{MCM+}; YY1^{flx/flx}) mice and littermate control YY1^{flx/flx} wild-type (WT) mice (Fig. 1E). To establish a mouse model of liver fibrosis, adult mutant and WT mice (8–10 weeks) were subjected to intraperitoneal injection of CCL4 over 8 weeks and simultaneously fed tamoxifen (TAM)-containing diet so that the Cre protein could facilitate recombination in activated fibroblasts/myofibroblasts to delete YY1 gene (Fig. 1F). Using dual immunofluorescence staining of YY1 and myofibroblast marker α -SMA, we observed an approximate 80% deletion of YY1 protein in myofibroblasts during liver fibrosis (Fig. 1G). These results indicate that the *Postn*^{MCM} allele effectively deletes loxP-targeted gene YY1 in liver myofibroblasts of mice exposed to CCL4.

3.2. YY1 deficiency in myofibroblasts alleviates liver fibrosis in mice

We established a mouse model of liver fibrosis by injecting CCL4 to adult Postn^{MCM/+}; YY1^{flox/flox} mice and WT mice (YY1^{flox/flox}) over 8 weeks in the presence of tamoxifen diet so that the MCM protein could intermediate recombination in myofibroblasts. Mouse liver was harvested, and the level of liver fibrosis was first analyzed by whole liver macroscopic images (Fig. 2A). The size of the liver from Postn^{MCM/+}; YY1^{flox/flox} mice was larger than that from WT mice. Fewer nodules in the liver surface of Postn^{MCM/+}; YY1^{flox/flox} mice were visible compared to WT mice (Fig. 2A). The liver weight was increased in Postn^{MCM/+}; YY1^{flox/flox} liver than in the WT liver (Fig. 2B). Furthermore, histology analysis of H&E staining revealed less necrosis and formation of regenerative nodules and fibrotic septa in Postn^{MCM/+}; YY1^{flox/flox} mice, while WT groups displayed severe steatosis and necrosis (Fig. 2C). Picro Sirius red staining showed that hepatic collagen deposition was decreased remarkably in Postn^{MCM/+}; YY1^{flox/flox} mice compared with that in WT mice (Fig. 2D). These results indicate that the deletion of YY1 from newly activated fibroblast (myofibroblasts) significantly reduces the liver fibrotic response and effectively inhibits liver fibrosis progression.

3.3. Myofibroblast-specific YY1 deletion inhibits fibrotic gene expression in the liver

To elucidate the cellular and molecular basis for the decrease of CCL4-induced liver fibrosis in myofibroblast-deficient YY1 mice, we examined the fate of activated fibroblasts/myofibroblasts in the liver following YY1 deletion. By using qPCR (Fig. 3A), Western blot (Fig. 3B), and immunofluorescence staining (Fig. 3C) analysis, we observed that the number of α -SMA-positive cells was significantly reduced in the liver from Postn^{MCM/+}; YY1^{flox/flox} mice compared with that in WT mice. To gain further understanding of the molecular origin for YY1-dependent regulation of fibrogenic activity and fibrosis, qPCR-based expression analysis of various profibrogenic factors was performed to substantiate the effects of YY1 deficiency on myofibroblasts. Transcript expression levels of transform growth factor beta (TGF- β), platelet-derived growth factor (PDGF), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF- α), all of which can serve as autocrine drivers of hepatic stellate cell (HSC) activation, invasion, proliferation, or transdifferentiation to myofibroblasts [18], were significantly decreased in the liver from Postn^{MCM/+}; YY1^{flox/flox} mice than that from WT mice after CCL4 and tamoxifen-diet treatment (Fig. 3A). Consequently, expression of the myofibroblast markers, α -SMA and collagen Ia (Fig. 3B and C) was significantly reduced in the liver from Postn^{MCM/+}; YY1^{flox/flox} mice than those in WT. Taken together, these results indicate that the deletion of YY1 in activated fibroblasts/myofibroblasts significantly diminishes profibrogenic gene expression and ECM deposition in CCL4-injured mouse livers.

3.4. YY1 depletion by siRNA attenuates profibrogenic property of human myofibroblasts

Myofibroblasts produce a significant amount of α -SMA and collagen Ia, which contributes to liver fibrogenesis [12]. To determine the functional relevance of YY1 in fibrogenesis triggered by myofibroblasts, we cultured human HSC cells (LX-2 cell line) and treated the cells with TGF- β for 24 h to induce HSC activation/differentiation into myofibroblasts, followed by the treatment of YY1 siRNA for 24 h to knockdown endogenous YY1

expression. Consistently, qPCR (Fig. 4A), Western blot (Fig. 4B), and immunocytochemistry (Fig. 4C) analysis showed that there was a significant reduction of α -SMA expression in YY1-depleted myofibroblasts than in cells treated with scrambled control siRNA. These results indicate that loss of YY1 attenuates myofibroblast α -SMA expression, suggesting that YY1 depletion may halt the phenotypes of myofibroblasts and prevent these cells from secreting ECM proteins (Fig. 4D).

4. Discussion

In the present study, we demonstrate that genetic ablation of YY1 in myofibroblasts protects against the progression of hepatic fibrosis in a mouse model of CCL4-induced liver fibrosis. Furthermore, we have also substantiated that knockdown of YY1 expression in cultured human myofibroblasts attenuates myofibroblast marker gene expression. Our findings reveal a crucial role of myofibroblast-specific YY1 in liver fibrosis and suggest that targeting YY1 in hepatic myofibroblasts might provide a new avenue to limit the progression of liver fibrosis.

Increasing evidence shows that fibrosis is a dynamic and reversible process. The understanding of the underlying sources and mediators of fibrosis progression has generated enthusiasm towards developing effective antifibrotic drugs. To date, however, no drug has been approved as an effective antifibrotic medicine. Thus, there is a tremendous need for novel therapeutic approaches to prevent the progression from reversible liver fibrosis to irreversible advanced liver fibrosis toward cirrhosis and eventually hepatocellular carcinoma. Myofibroblast proliferation and the synthesis of ECM, as the primary cause of liver fibrosis, have become targets of antifibrotic treatments. Therapeutic strategies that target myofibroblasts proliferation and collagen synthesis, and induce myofibroblasts apoptosis, could be an effective treatment of liver fibrosis. Of importance, it has also been shown that YY1 was abundantly expressed in mouse and human myofibroblasts [13]. Using newly-developed Postn^{MCM} mice, we generated myofibroblast-specific YY1 knockout mice to delineate the role of YY1 in liver fibrosis. Our results indicate that the Postn^{MCM} allele effectively erases loxP-targeted gene YY1 from activated fibroblasts in the liver. Postn^{MCM} Cre mice have recently been used in several studies of cardiac fibrosis and proved to be a very useful tool to dissect the signaling cascades underlying cardiac fibrosis and heart failure [14]. By utilizing a mouse model of CCL4-induced liver fibrosis, we found that genetic ablation of YY1 in myofibroblasts protects against liver fibrosis progression. Our findings reveal that specific inhibition of YY1 in hepatic myofibroblasts could limit liver fibrosis progression *in vivo*.

Myofibroblasts produce major fibrous scar materials in liver fibrosis [15]. Hepatic myofibroblasts are largely derived from the transdifferentiation of HSCs during liver injury [16]. It has been demonstrated that hepatocytes and activated Kupffer cells (tissue-residential macrophages in the liver) produce a large number of growth factors and cytokines (such as TGF- β) that promotes the transdifferentiation of HSCs into myofibroblasts [17]. In addition, cytokines can be secreted by myofibroblasts in an autocrine fashion to sustain profibrotic activation [2,18]. Interestingly, we also observed that YY1 depletion in myofibroblasts significantly attenuated the production of PDGF and TGF- β in the liver. To prove the direct

effect of YY1 in the regulation of fibrogenic gene expression in myofibroblasts, we used human HSC cell line LX-2. We showed that siRNA knockdown of YY1 significantly inhibited α -SMA expression in human myofibroblasts transdifferentiated from LX-2 cells stimulated with TGF- β . Our results indicate that YY1 directly contributes to α -SMA expression in hepatic myofibroblasts. It remains tempting to speculate on the molecular pathways behind these effects of YY1 deficiency on myofibroblasts. It has been reported that YY1 binds to the collagen I α promoter upstream of the TATA box in BALBc/3T3 fibroblasts [19]. Furthermore, YY1 can directly up-regulate α -SMA in lung fibroblasts via enhance α -SMA promoter activity [20]. Given the well-documented interplay between YY1 and α -SMA/collagen I α in fibroblasts, it is likely that YY1 may directly regulate α -SMA and collagen I α in liver myofibroblasts. Further studies are needed to clarify the molecular mechanisms underlying the specific effect of YY1 on gene expression in hepatic myofibroblasts during the progression of liver fibrosis.

Liver-resident cells including hepatocytes, HSCs, and Kupffer cells are implicated in the development and progression of liver fibrosis. Previous studies revealed that hepatocyte-specific YY1 deficiency alleviates the non-alcoholic fatty liver disease (NAFLD) progression in patients undergoing bariatric surgery [21,22]. Although it is likely that hepatocyte YY1 deficiency attenuates liver fibrosis [21], the present work unravels myofibroblasts as another important player, conveying protective effects of YY1 deficiency against liver fibrosis. Indeed, myofibroblasts give rise to an overwhelming majority of α -SMA and collagen-producing cells and play a key role in liver fibrosis [2,18,23]. In this study, attenuated liver fibrosis in $yy1^{flox/flox}$; $postn^{MCM}$ mice was accompanied by a significant reduction of α -SMA and collagen I α accumulation and, as a likely consequence, attenuated recruitment of myofibroblasts in the injured liver. This may open a promising avenue for therapeutic intervention when pharmacological small-molecule drugs targeting YY1 are available. Interestingly, it has been reported that nitric oxide [24] inhibits YY1 expression in human tumor cells and lung fibroblasts [25]; but it remains to be investigated whether nitric oxide could be applied to rather specifically target YY1 in myofibroblasts.

In summary, this study unravels a novel function of myofibroblast-specific YY1 in promoting liver fibrosis. Our findings implicate YY1 as a novel therapeutic target to combat the progression of liver fibrosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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Fig. 1.

Generation of myofibroblast-specific YY1 deficient mice. (A) Western-blot analysis showed protein expression of YY1 and α-SMA in the liver of C57BL/6J mice with CCL4 induced fibrosis. (B) Relative protein expression of YY1 in the liver from control and CCL4-treated mice. (C) Relative protein expression of α-SMA in the liver from control and CCL4-treated mice. (D) The schematic breeding strategy of different mouse lines. The *Postn* genetic locus containing a tamoxifen-regulated MCM cDNA cassette inserted into exon 1 (E1) was crossed with *YY1*^{flx/flx} containing gene-targeted lines. The mouse chromosome related to each allele is shown. (E) Representative image for genotyping PCR results of *Postn*^{MCM/+};

YY1^{flox/flox} mice and YY1^{flox/flox} mice. **(F)** An experimental scheme whereby mice were injected with CCL4 for 8 weeks and fed tamoxifen food 48 h before CCL4 injection and then maintained on tamoxifen diet until harvesting. **(G)** Representative immunofluorescence staining showing that YY1 expression on hepatic myofibroblasts (α -SMA positive cells) in the liver from WT mice and Postn^{MCM/+}; YY1^{flox/flox} mice after 8 weeks of CCL4 injection (n = 4). Scale bar: 20 μ m.

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Fig. 2.

Myofibroblast-specific deletion of YY1 reduces liver fibrosis in mice. (A) Representative macroscopic images of livers from Postn^{MCM/+}; YY^{flox/flox} mice and WT (flox/flox) mice after eight weeks of CCL4 injection and tamoxifen treatment. (B) Comparison of the liver weight of Postn^{MCM/+}; YY^{flox/flox} mice and WT mice. (C) Representative H&E staining of livers from Postn^{MCM/+}; YY^{flox/flox} mice and WT mice. (D) Histological photographs of Picro Sirius red staining of liver fibrosis in Postn^{MCM/+}; YY^{flox/flox} mice and WT mice.

Fig. 3.

Myofibroblast-specific YY1 deletion diminishes the expression of ECM genes and profibrogenic factors in the liver. (A) Quantitative real-time PCR showed fibrosis-related gene expression in the liver from WT and Postn^{MCM/+}; YY1^{flox/flox} mice after 8 weeks of CCL4 injection and tamoxifen treatment. Data were represented as mean $\pm$ SEM for six mice. * $p < 0.05$; ** $p < 0.01$ versus YY1^{flox/flox} WT mice. (B) Western-blot analysis showed protein expression of α -SMA in the liver of Postn^{MCM/+}; YY1^{flox/flox} mice compared to WT

mice. (C) Immunofluorescence detected α -SMA expression in Postn^{MCM/+}; yy^{flox/flox} mice liver compared to WT mice. The blue color is nuclei DAPI staining. Scale bar, 100 μ M.

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Fig. 4.

YY1 depletion by siRNA attenuates profibrogenic property of human myofibroblasts. (A). Quantitative real-time PCR detected the mRNA level of α -SMA and Collagen I α . Graphs showed quantification of α -SMA mRNA expression in LX-2 cells exposed to TGF- β for 24 h and then treated with YY1 siRNA (si-YY1) or control siRNA (si-Con) for 24 h. Data represent mean $\pm$ SEM for 3 independent experiments. * $p < 0.05$; ** $p < 0.01$ versus vehicle. (B). Western blot shows the expression of α -SMA and YY1 in LX-2 cells exposed to TGF- β for 24 h and then treated with YY1 siRNA and control siRNA for 24 h. (C). Immunofluorescent staining showed much less fluorescence intensity of α -SMA and YY1 in YY1-siRNA treated cells compared with control siRNA-treated cells. Representative images were presented, scale bar, 100 μ M. (D) Schematic diagram of myofibroblast-specific YY1 deficiency protects against liver fibrosis.