



Original Article

Investigation of HO-1 Regulation of Liver Fibrosis Related to Nonalcoholic Fatty Liver Disease Through the SIRT1/TGF- β /Smad3 Pathway

Mengjiao Sun[#], Xiaoqing Wu[#], Zhandong Lin, Congyue Zhang, Jiawei Cui, Yaoyao Mao, Yue Shi, Jiaming Zhang and Yuemin Nan^{*ID}

Department of Integrated Traditional Chinese and Western Medicine Hepatology, Hebei Medical University Third Hospital, Shijiazhuang, Hebei, China

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Abstract

Background and Aims: Heme oxygenase 1 (HO-1) has an influential yet insufficiently investigated effect on Sirtuin 1 (SIRT1), a histone deacetylase activated by nicotinamide adenine dinucleotide, which may impact the transforming growth factor- β (TGF- β)/Smad3 pathway in nonalcoholic fatty liver disease (NAFLD)-related liver fibrosis. This study aimed to elucidate the regulation of NAFLD-related liver fibrosis induced by HO-1 through the SIRT1/TGF- β /Smad3 pathway. **Methods:** HO-1 induction and inhibition were established in C57BL/6J mice fed a methionine- and choline-deficient (MCD) diet. Additionally, wild-type mice were fed either a normal diet or an MCD diet. Hematoxylin and eosin, Masson's trichrome, and Sirius Red staining were used to assess hepatic steatosis, inflammation, and fibrosis. *In vitro*, plasmid overexpression and small interfering RNA silencing of HO-1 were performed in LX-2 cells. Cell viability was assessed using the Cell Counting Kit-8, and apoptosis was evaluated via terminal deoxynucleotidyl transferase dUTP nick-end labeling and immunofluorescence. Flow cytometry was employed to assess apoptosis and reactive oxygen species production. Western blot and real-time quantitative reverse transcription polymerase chain reaction were used to analyze the mRNA and protein expression of genes related to HO-1, SIRT1, the TGF- β signaling pathway, and fibrosis. **Results:** MCD-fed mice developed significant liver damage, including steatosis, inflammatory infiltration, and pericellular fibrosis. Zinc protoporphyrin treatment exacerbated these conditions. Corroborating these findings, silencing HO-1 in LX-2 cells increased the expression of fibrosis-related genes. Furthermore, HO-1 overexpression not only increased SIRT1 expression but also reduced the activity of key regulatory factors in the TGF- β signaling pathway, suggesting a potential interaction between HO-1 and the SIRT1/TGF- β pathway. **Conclusions:**

HO-1 inhibits the activation of the TGF- β /Smad3 pathway in NAFLD-related liver fibrosis through SIRT1. These findings provide insights into new therapeutic strategies for treating NAFLD-associated liver fibrosis.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is emerging as one of the most prevalent causes of chronic liver disease worldwide.¹ NAFLD encompasses a range of conditions, from simple hepatic steatosis to more advanced diseases, such as nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and liver cancer. In NAFLD, lipid accumulation in the liver leads to NASH, which is characterized by progressive liver injury, inflammation, and oxidative stress. Over time, this condition can progress to severe fibrosis and cirrhosis.^{2,3} The progression of liver fibrosis in NASH is driven by sustained injury to the liver, which activates hepatic stellate cells. When activated by liver injury, these cells differentiate into myofibroblast-like cells, which produce extracellular matrix (ECM) components, including alpha-smooth muscle actin (α -SMA) and type I collagen (Col1a1/Col1a2).⁴ These cells contribute to the excessive buildup of ECM in liver tissue, promoting fibrogenesis.^{2,5,6} Another key factor in this process is transforming growth factor- β (TGF- β), which primarily mediates the activation of fibroblasts under fibrotic conditions.⁷ Given the critical role of TGF- β , targeting the TGF- β signaling pathway is a promising therapeutic strategy for managing liver fibrosis.⁴ NASH-related liver fibrosis has become a major global health concern, and managing its progression could prevent the subsequent development of liver cirrhosis and cancer.⁸

Heme oxygenase 1 (HO-1) is a key enzyme in the breakdown of heme. It catalyzes the conversion of heme into carbon monoxide, free iron, and biliverdin, which is further reduced to bilirubin by biliverdin reductase. In addition to its enzymatic function, HO-1 exhibits strong antioxidant and

Keywords: Non-alcoholic steatohepatitis-related liver fibrosis; Heme oxygenase-1; HO-1; Sirtuin 1; SIRT1; TGF- β pathway.

[#]Contributed equally to this work.

***Correspondence to:** Yuemin Nan, Department of Traditional and Western Medical Hepatology, Hebei Medical University Third Hospital, NO. 139 Ziqiang Road, Shijiazhuang, Hebei 050051, China. ORCID: <https://orcid.org/0000-0003-4192-0999>. Tel +86-18533112266, Fax +86-311-66781289, E-mail: nanyuemin@163.com or nanyuemin@hebmh.edu.cn.

anti-inflammatory properties.^{9,10} The induction or overexpression of HO-1 can help reduce liver damage in NAFLD mouse models.¹¹ We have previously established a link between HO-1 and NAFLD, demonstrating that high HO-1 levels can alleviate hepatic steatosis and inhibit fibrosis progression. These findings underscore the important role of HO-1 in mitigating liver fibrosis associated with NASH.^{12,13}

Sirtuin 1 (SIRT1) is a histone deacetylase activated by nicotinamide adenine dinucleotide (NAD⁺). It is essential in various cellular processes, including transcription, apoptosis, stress responses, and metabolism.^{14,15} Notably, SIRT1 is also a known protective factor against liver fibrosis.¹⁶ Furthermore, sirtuins, including SIRT1, exert protective effects against acute liver injuries.¹⁷ One of the factors involved in regulating SIRT1's protective effects on cells is HO-1.¹⁸ Moreover, growing evidence suggests that the overexpression or activation of SIRT1 can help slow fibrosis progression.¹⁹ Increased SIRT1 expression in the kidneys can reduce the levels of fibrosis markers, such as α -SMA and Colla1, to inhibit renal fibrosis.²⁰ Similarly, activating SIRT1 can enhance survival and reduce pulmonary fibrosis in a bleomycin-induced lung fibrosis mouse model.²¹ Thus, SIRT1 may inhibit fibrosis in different organs by modulating the TGF- β /Smad signaling pathway.⁴ Given its potential therapeutic benefits, SIRT1 presents a promising target for addressing metabolic syndrome, diabetes, and other diseases associated with insulin resistance. However, despite these insights, the precise biological functions of SIRT1 in the liver remain unclear and require further investigation.¹⁵

Methods

Animals and treatments

Eight-week-old male C57BL/6J wild-type mice were housed in an environmentally controlled facility under a 12-h light/dark cycle with ad libitum access to food and water. The mice were randomly divided into four groups ($n = 6$) and received group-specific diets for eight weeks: (1) Control group: Mice were fed a normal diet; (2) Methionine- and choline-deficient (MCD) group: Mice were fed an MCD diet (Research Diets, Inc., NJ, New Brunswick, USA); (3) Hemin group: Mice were fed an MCD diet and treated with the HO-1 chemical inducer Hemin three times per week; (4) Zinc protoporphyrin (Znpp) group: Mice were fed an MCD diet and treated with the HO-1 inhibitor Znpp three times per week. At the end of the eight-week period, all animals were fasted overnight and euthanized.²² Blood and liver samples were then collected. Livers were weighed and either fixed in 10% neutral-buffered formalin for histological analysis or rapidly frozen in liquid nitrogen and stored at -80°C for RNA and protein extraction. All procedures were conducted in accordance with the guidelines of the Animal Care and Use Committee of Hebei Province and were approved by the Animal Experiment Ethics Committee of Hebei Medical University.

Histological and biochemical analysis

Liver tissues were fixed in 4% neutral formalin and embedded in paraffin blocks. Hematoxylin-eosin, Masson's trichrome staining, and Sirius Red staining were used to stain 5 μm thick liver sections. The sum of steatosis, lobular inflammation, and fibrosis was assessed by two expert liver pathologists for NASH assessment using the NAFLD activity score algorithm.²³

Western blot analysis

Using RIPA buffer supplemented with PMSF, 20 mg of liver tissue was used for protein extraction. PVDF membranes

were used to transfer the proteins separated by SDS-PAGE. After blocking for 1 h in Tris-buffered saline with Tween 20 (TBST) with 5% skim milk or 3% BSA, membranes were incubated with antibodies against reduced glyceraldehyde-3-phosphate dehydrogenase (1:2,000, AB0036, Abways Technology, Shanghai, China); HO-1 (1:2,000, ab189491, Abcam, Cambridge, USA); SIRT1 (1:1,000, ab189494, Abcam, Cambridge, USA); TGF- β (1:1,000, ab179695, Abcam, Cambridge, USA); phosphorylated Smad2/3 (1:1,000, ab202445, Abcam, Cambridge, USA); Smad2/Smad3 (1:1,000, ab202445, Abcam, Cambridge, USA); α -SMA (1:2,000, CY1132, Abways Technology, Shanghai, China); and Colla1 (1:800, AF7001, Affinity, Jiangsu, China). After incubation, the membrane was washed three times with TBST for 10 min each time. The membrane was then incubated with the secondary antibody DyLight 800 goat IgG (1:7,000, A23920, Abbkine Scientific, Wuhan, China) at 37°C for 1 h. Following incubation, the membrane was washed with TBST three times for 10 min each time. Finally, protein bands were detected using the Odyssey Fluorescence Imaging System (LI-COR, USA) and analyzed for density using ImageJ software (National Institutes of Health, Bethesda, MD, USA).²⁴

Biochemical analysis

The mice were fasted for at least 8 h overnight and then euthanized. Blood samples were collected for serum analysis of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which were measured using appropriate enzyme assay kits on a dedicated automatic analyzer (Chemray 240, Shanghai, China).

Cell culture

LX-2 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin (both from Life Technologies, Gaithersburg, MD, USA), and 10% fetal bovine serum (Wuhan ProNova Life Science, Wuhan, China). Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 .

Cell transfection

The six-well plates were applied to incubate LX-2 cells overnight, which were transiently transfected utilizing Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA).²⁴ Small interfering RNAs (siRNAs) designed against HO-1 (hereinafter referred to as siHO-1), the siRNA control, HO-1 overexpression vector (OE HO-1), and overexpression control were synthesized by GenePharma (Shanghai, China). The siRNA sequences are shown in Supplementary Table 1 and Supplementary Figure 3.

Cell treatment

To establish an *in vitro* liver fibrosis model, LX-2 cells were stimulated with TGF- β 1 (10 ng/mL, Abways Biotechnology, Shanghai, China) for 48 h.²⁵ As previously described,^{26,27} the cells were treated with the SIRT1 activator SRT1720 (1 μM , HY-10532, MedChemExpress, Shanghai, China) or the inhibitor EX527 (10 μM , HY-15452, MedChemExpress), as shown in Supplementary Figure 1. An HO-1 knockdown model was established by transfecting cells with siRNA (GenePharma), and HO-1 overexpression was induced using the pcDNA3.1 HO-1 plasmid (GenePharma).

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was isolated from the liver tissue using TRIzol reagent (Invitrogen). First-strand cDNA synthesis was per-

Table 1. The primer sequences (mus)

Gene name	Primer sequences (5'-3')
GAPDH	F: CAAGAAGGTGGTGAAGCAGG R: AAAGGTGGAGGAGTGGGTGT
HO-1	F: AAGCCGAGAATGCTGAGTTCA R: GCCGTGTAGATATGGTACAAGGA
SIRT1	F: TGATTGGCACCAGATCCTCG R: CCACAGCGTCATATCATCCAG
TGFβ1	F: CTCCCGTGGCTTCTAGTGC R: GCCTTAGTTGGACAGGATCTG
Smad3	F: CATTCCATTCCCAGAACTAA R: GCTGTGGTTCATCTGGTGGT
α-SMA	F: GTACCACCATGTACCCAGGC R: GCTGGAAGGTAGACAGCGAA
COL1A1	F: TTCTCTGGCAAAGACGGAC R: CGGCCACCATCTTGAGACTT

formed using the High-Capacity cDNA Reverse Transcription Kit (Takara Bio, RR036A), according to the manufacturer's instructions. RT-qPCR was performed on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster, CA, USA), using TBGreen™ Premix Ex Taq™ II (Tli RNaseH Plus; Takara Bio, RR820A), as previously described.²⁸ The primers used for RT-PCR are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase expression was used as an internal control. Relative RNA expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method.

Immunofluorescence

As previously described,²⁹ LX-2 cells subjected to various treatments were fixed with cold methanol for 5 m. Subsequently, the cells were washed three times with PBS, followed by permeabilization with 0.1% Triton for 10 m. After culturing with 3% bovine serum albumin for 30 m, the cells were immediately incubated with the primary antibody at 4°C overnight. Then, the cells were incubated with the secondary antibody at room temperature for 1 h and washed with PBS three times. 4',6-Diamidino-2-phenylindole staining was performed, and the slide was sealed. Images were captured under a fluorescence microscope. Statistical analysis was performed using ImageJ image analysis software.

Flow cytometry

The cells were first treated with 1× Annexin V Binding Buffer (100 μL) for 10 m, then 2.5 μL of Annexin V-FITC Reagent and 2.5 μL of Phosphorus Incorporation Reagent (50 μg/mL) were added. The samples were gently vortexed and incubated at room temperature in the dark for 15–20 m. 1× Annexin V Binding Buffer (400 μL) was added to the samples and mixed well. The cell-cycle phase distribution and apoptosis were determined using a BD Accuri™ C6 flow cytometer (BD Biosciences, USA).³⁰

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining assay

The TUNEL apoptosis assay was performed using a TUNEL Apoptosis Assay Kit (Biyuntian, China), according to the manufacturer's instructions, as previously described.²⁸ As per the experimental requirements, the adherent cells were fixed with 4% paraformaldehyde for 30 m, followed by incubation with TUNEL solution for 1 h. The cells were then stained with 4',6-Diamidino-2-phenylindole in the dark for

10 min. Fluorescent images were captured using a fluorescence microscope (Olympus, Japan) from at least three randomly selected fields. Apoptosis was analyzed using ImageJ software.

Cell Counting Kit-8 (CCK-8) Assay

For each treatment group, 9×10^5 cells were suspended in 3 mL of Complete Cell Culture Medium. Each group of cells was seeded at a density of 100 μL cell suspension per well in a 96-well plate. The cells were cultured for 0, 12, 24, 36, 48, and 72 h. 10 μL of CCK-8 reagent (Beijing Solarbio Technology Co., Ltd.) was added to each well, and the plate was incubated for 1.5 h. The absorbance was measured at 450 nm using an enzyme-linked immunosorbent assay kit (Infinite F50, Swiss TECAN Company).³¹

Reactive Oxygen Species (ROS) Assay Kit

The ROS in the cells were measured using a ROS detection kit (Beijing Solarbio Technology Co., Ltd.). 2 μL of the red fluorescent probe stock solution was added to the cell suspension, and the cells were incubated in the dark at room temperature for 30 m. Then, the cells were centrifuged at 250–500 *g* for 5 m to remove the staining solution and washed twice with PBS. The analysis was performed using a flow cytometer (Beckman Coulter, USA).³²

Statistical analysis

The statistics were analyzed utilizing GraphPad Prism v8.0 software (GraphPad, San Diego, CA, USA). Intergroup comparison was conducted using one-way analysis of variance with Student's *t*-test. Data are presented as mean ± standard deviation. Statistical significance was indicated by a *p*-value of <0.05.

Results

HO-1 alleviates inflammation and fibrosis in MCD diet-induced NAFLD mice

The results of Hematoxylin-eosin, Masson's trichrome staining, and Sirius Red staining indicated that mice fed the MCD diet for eight weeks displayed signs of fatty degeneration, disruption of lobular architecture, inflammatory infiltration, and pericellular fibrosis. Treatment with hemin significantly reduced the severity of these liver abnormalities, whereas treatment with the HO-1 inhibitor Znpp exacerbated these conditions (Fig. 1E–H). Moreover, biochemical analysis showed that, compared with control mice, mice treated with hemin had significantly lower serum ALT and AST levels. In contrast, treatment with Znpp further elevated serum transaminase levels (Fig. 1C and D). Mice on the MCD diet also exhibited reduced body weight compared with the control group. Znpp treatment further decreased both body weight and the liver-to-body weight ratio. However, HO-1 induction increased both parameters (Fig. 1A and B). Overall, these findings indicate that HO-1 induction mitigated liver injury and fibrosis associated with MCD diet-induced steatohepatitis.

HO-1 regulates the SIRT1/TGF-β/Smad3 pathway in NAFLD-associated liver fibrosis

Findings from RT-qPCR analysis demonstrated that, compared with the control group, *SIRT1* mRNA expression was significantly lower in MCD mice. Treatment with Znpp further reduced *SIRT1* expression, whereas hemin increased it (Fig. 2A–F). To explore the relationship between HO-1 and

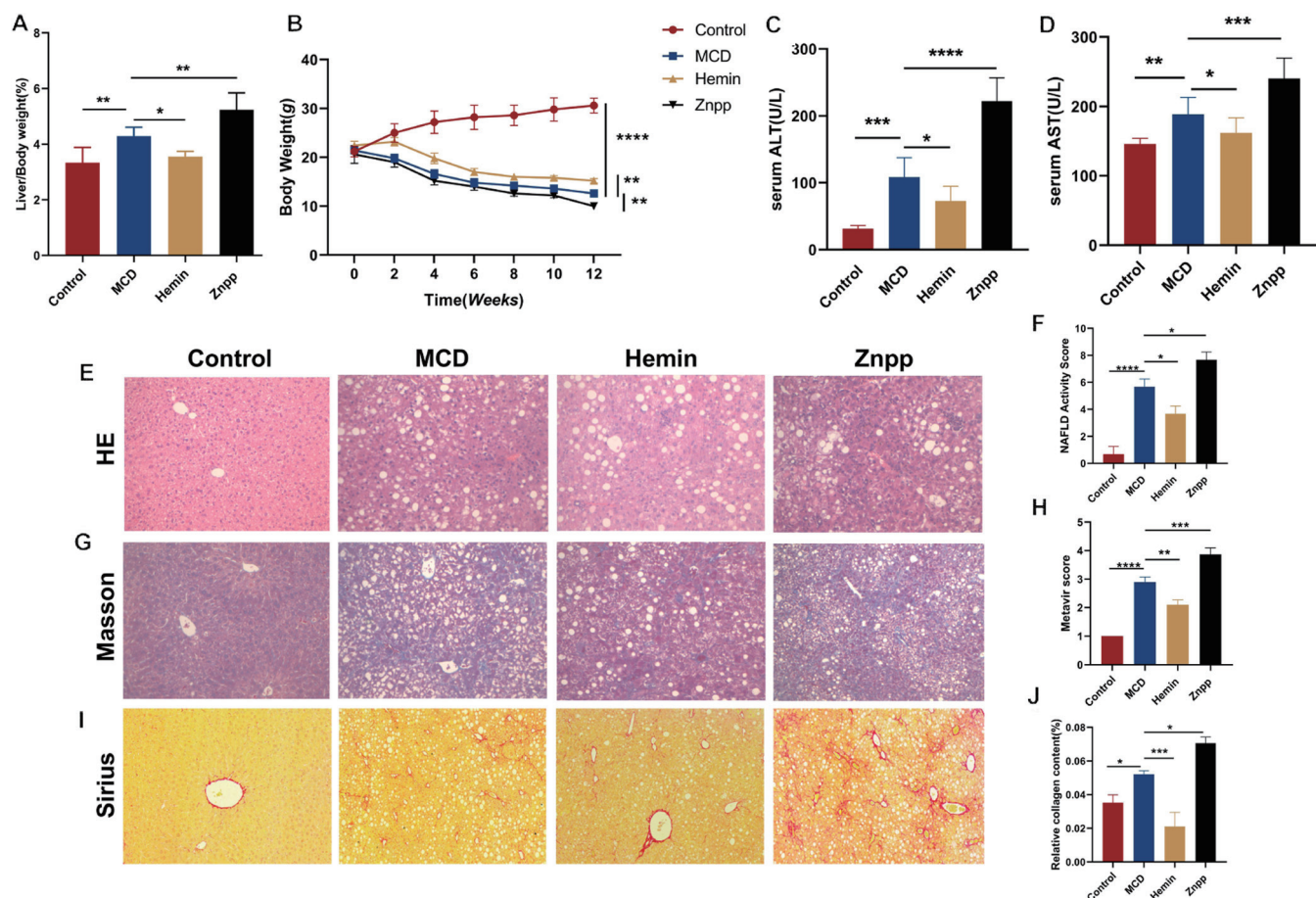


Fig. 1. Mice fed with MCD diet developed steatohepatitis-related liver fibrosis. (A) liver/body weight. (B) Body weight. Serum (C) ALT and (D) AST in each group. (E) Images of H&E ($\times 200$ magnification) staining of representative liver sections and (F) the NAFLD activity score. (G) Images of Masson ($\times 200$ magnification) staining of representative liver sections and (H) the Metavir score. (I) Images of Sirius ($\times 200$ magnification) red staining of representative liver sections and (J) Relative collagen content (%). Values are the mean $\pm$ SD ($n = 6$ per group). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. MCD, methionine-and-choline-deficient diet; NAFLD, nonalcoholic fatty liver disease; H&E, hematoxylin-eosin. ALT, alanine aminotransferase; AST, aspartate transaminase; Znpp, zincprotoporphyrin.

SIRT1, we performed immunofluorescence double-labeling, which revealed the targeted interaction between these two proteins (Fig. 2G). In the MCD diet-fed mice, this interaction was weaker than that in the control group, while inducing HO-1 enhanced the binding between HO-1 and SIRT1 (Fig. 2H-K). To further investigate the effects of HO-1 on NAFLD-related liver fibrosis via the SIRT1/TGF- β /Smad3 signaling pathway, we manipulated HO-1 levels (induction or inhibition), and Western blot analysis measured the protein expression of key pathway components (Fig. 3A-I). As expected, in the MCD diet-fed mice, SIRT1 protein levels were lower than those in the control group. This decrease was also observed in the group treated with Znpp. Conversely, treatment with hemin significantly increased SIRT1 protein levels compared with the MCD diet group. In contrast to the changes observed in SIRT1 expression, TGF- β and P-Smad2/3 protein levels increased in MCD diet-fed mice, which were further increased by Znpp treatment. Overall, these results demonstrate that HO-1 regulates NAFLD-related liver fibrosis by modulating the SIRT1/TGF- β /Smad3 pathway.

HO-1 mediates liver inflammation and fibrosis in vitro
To further explore how HO-1 regulates NAFLD-related liver

fibrosis through the SIRT1/TGF- β /Smad3 pathway, we developed an *in vitro* hepatic fibrosis model by stimulating LX-2 cells with TGF- β . Confirmation of HO-1 knockdown and HO-1 overexpression in LX-2 cells by both qPCR and Western blotting is shown in Supplementary Figure 2, and the siRNA sequences are shown in Supplementary Table 2. The successful knockdown and overexpression of HO-1 in LX-2 cells were verified by Western blot analysis (Fig. 4A-B). As shown in the figure, the expression levels of α -SMA and Colla1 were significantly elevated in the TGF- β -treated group compared with the control. Notably, in the SiHO-1 group, these markers were further increased relative to the TGF- β group, whereas in the OE HO-1 group, a decrease in α -SMA and Colla1 expression was observed in LX-2 cells (Fig. 4K-L). Immunofluorescence was also used to validate these results, and we found that overexpression of HO-1 or treatment with SRT1720 both inhibited fibrosis progression. Furthermore, we observed that SIRT1 activation counteracted the anti-fibrotic effect of HO-1 silencing (Fig. 5D-E).

Downregulation of SIRT1 expression in TGF- β -stimulated LX-2 cells

Given that sustained TGF- β activation is a key feature of fibrosis, we explored its potential role in regulating SIRT1

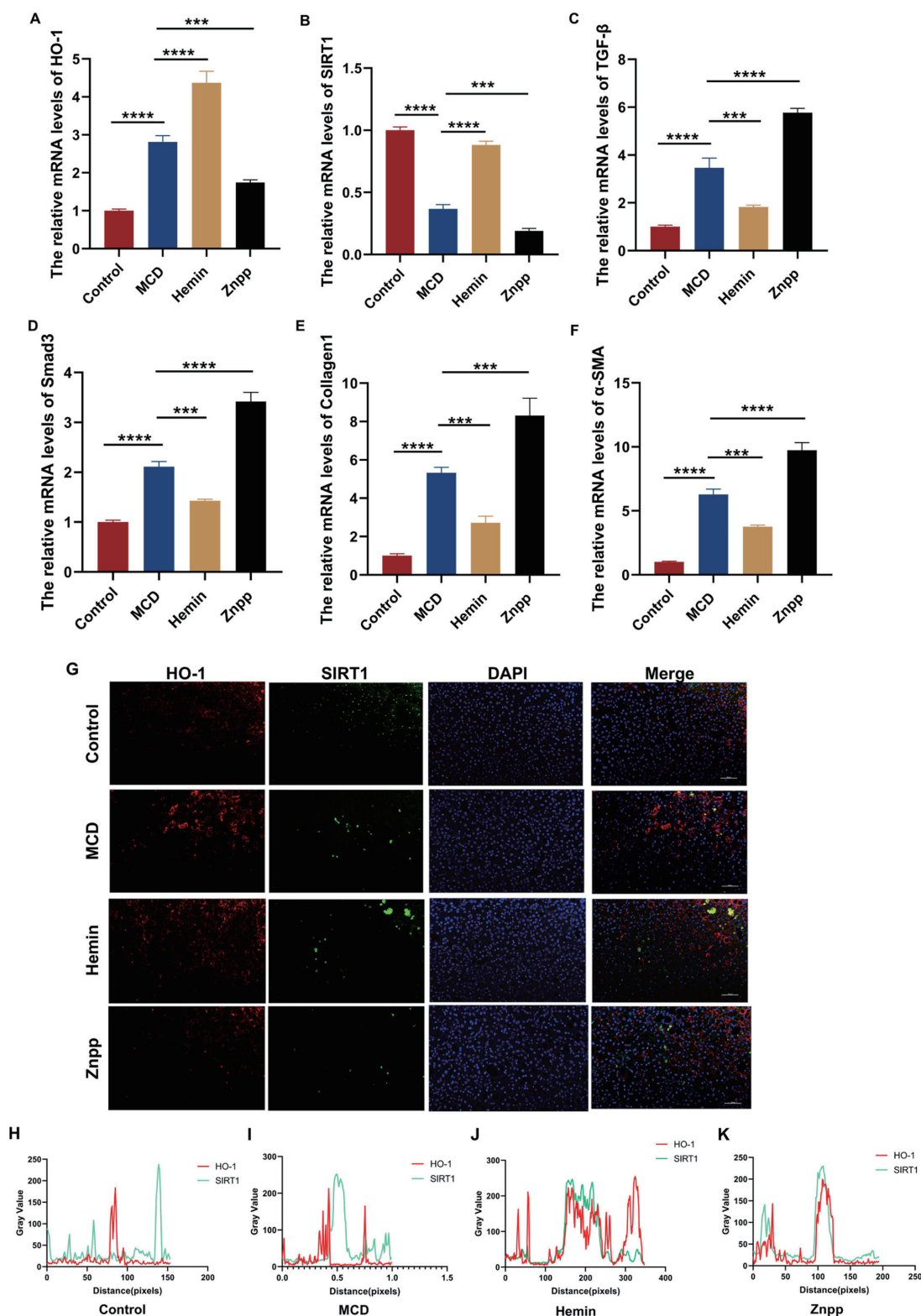


Fig. 2. HO-1 agonist ameliorates steatohepatitis-related liver fibrosis in MCD diet-fed mice. (A-F) The expression levels of HO-1, SIRT1, TGF-β, Smad3, Collagen1 and α-SMA were measured by RT-qPCR. (G) Immunofluorescence (×200 magnification) double staining between of HO-1 and SIRT1 in liver tissue. (H-K) Immunofluorescence double staining semi-quantitative analysis. GAPDH served as the loading control. Data are presented as representative results of three independent experiments. Values are the mean ± SD (n = 6 per group). ****p* < 0.001, *****p* < 0.0001. MCD, methionine-and-choline-deficient diet; HO-1, heme oxygenase 1; SIRT1, Sirtuin 1; TGF-β, transforming growth factor beta; α-SMA, alpha-smooth muscle actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Znpp, zincprotoporphyrin.

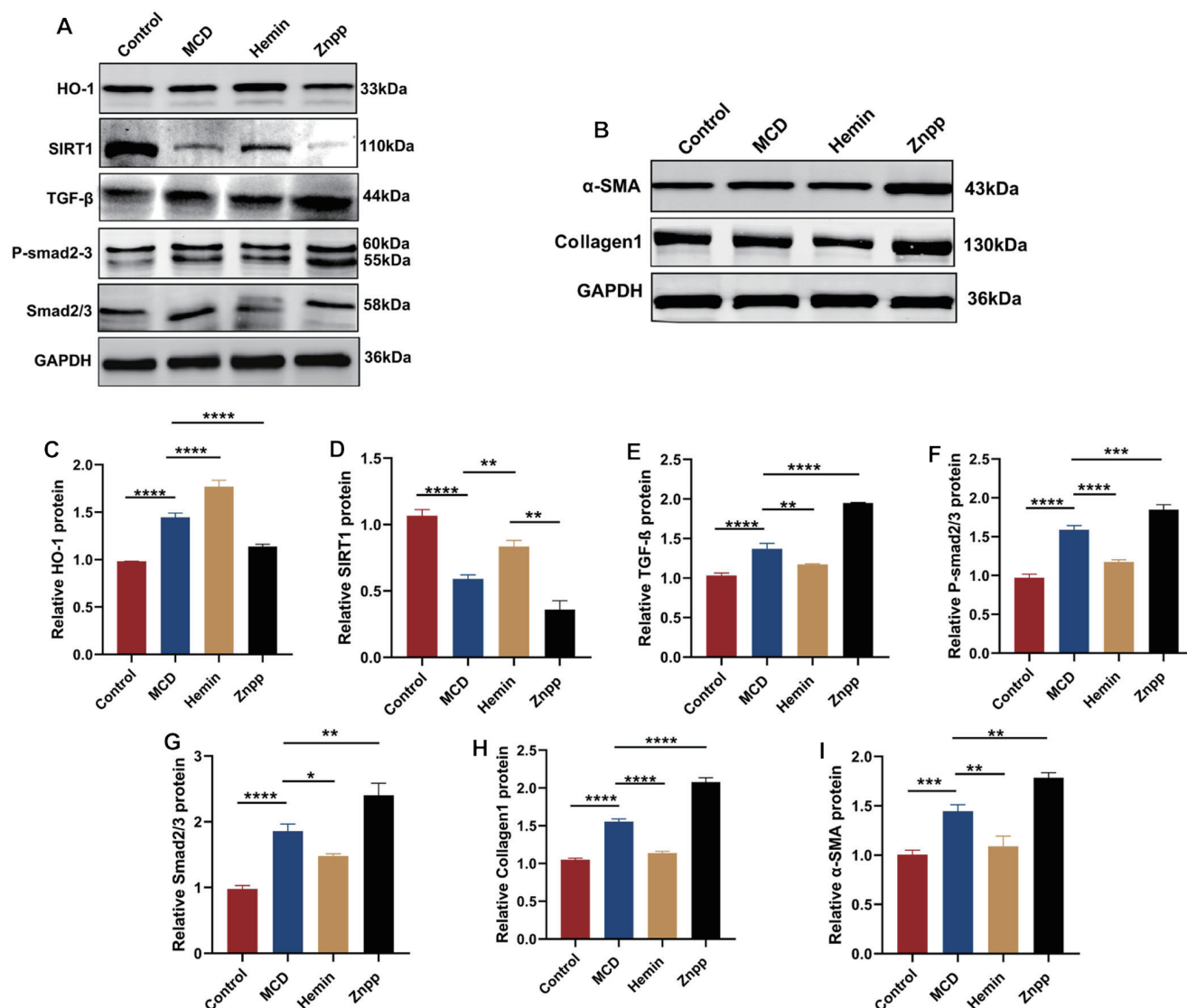


Fig. 3. HO-1 overexpression upregulates SIRT1 expression and exacerbates liver fibrosis in MCD mice. (A, C–G) Western blot analysis of HO-1, SIRT1, TGF-β, P-Smad2/3, and Smad2/3 in liver tissue. (B, H, I) Western blot analysis of α-SMA and Collagen1 in liver tissue. Values are the mean ± SD (n = 6 per group). **p < 0.01, ***p < 0.001, ****p < 0.0001. MCD, methionine-and-choline-deficient diet; HO-1, heme oxygenase 1; SIRT1, Sirtuin 1; TGF-β, transforming growth factor beta; α-SMA, alpha-smooth muscle actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Znpp, zincprotoporphyrin.

expression. As shown in the figure, Western blot analysis revealed that exposure of LX-2 cells to TGF-β reduced SIRT1 levels. In contrast, treatment with the SIRT1 activator SRT1720 in these TGF-β-stimulated cells increased SIRT1 expression. In contrast, inhibiting SIRT1 further decreased its expression in TGF-β-stimulated LX-2 cells (Fig. 4C–D). Moreover, immunofluorescence analysis confirmed that activation of SIRT1 inhibited the pro-fibrotic effects of TGF-β, including the differentiation of quiescent fibroblasts into myofibroblasts (Fig. 5A–C).

HO-1 regulates NAFLD-related liver fibrosis in vitro through the SIRT1/TGF-β/ Smad3 pathway

To further investigate the regulation by HO-1 of NAFLD-related liver fibrosis, we analyzed the expression of proteins involved in the SIRT1 signaling pathway by Western blot (Fig. 4E–I). Our results revealed that HO-1 knockdown re-

duced SIRT1 expression in TGF-β-stimulated LX-2 cells while simultaneously increasing TGF-β and P-Smad2/3 levels. Compared with the control group, the TGF-β group exhibited significantly lower SIRT1 expression and higher P-Smad2/3 levels. Conversely, the OE HO-1 increased SIRT1 expression and reduced TGF-β and P-Smad2/3 levels. To determine whether SIRT1 influences the antifibrotic effects of HO-1, we treated cells with the SIRT1 inhibitor EX527. This treatment significantly reversed the inhibitory effects of HO-1 on the TGF-β/Smad3 signaling pathway and liver fibrosis *in vitro*. Taken together, these findings demonstrate that HO-1 regulates NAFLD-related liver fibrosis through the SIRT1/TGF-β/Smad3 pathway.

Effects of HO-1 and SIRT1 on the biological behavior of LX-2 cells

We further explored the potential impact of HO-1 and SIRT1

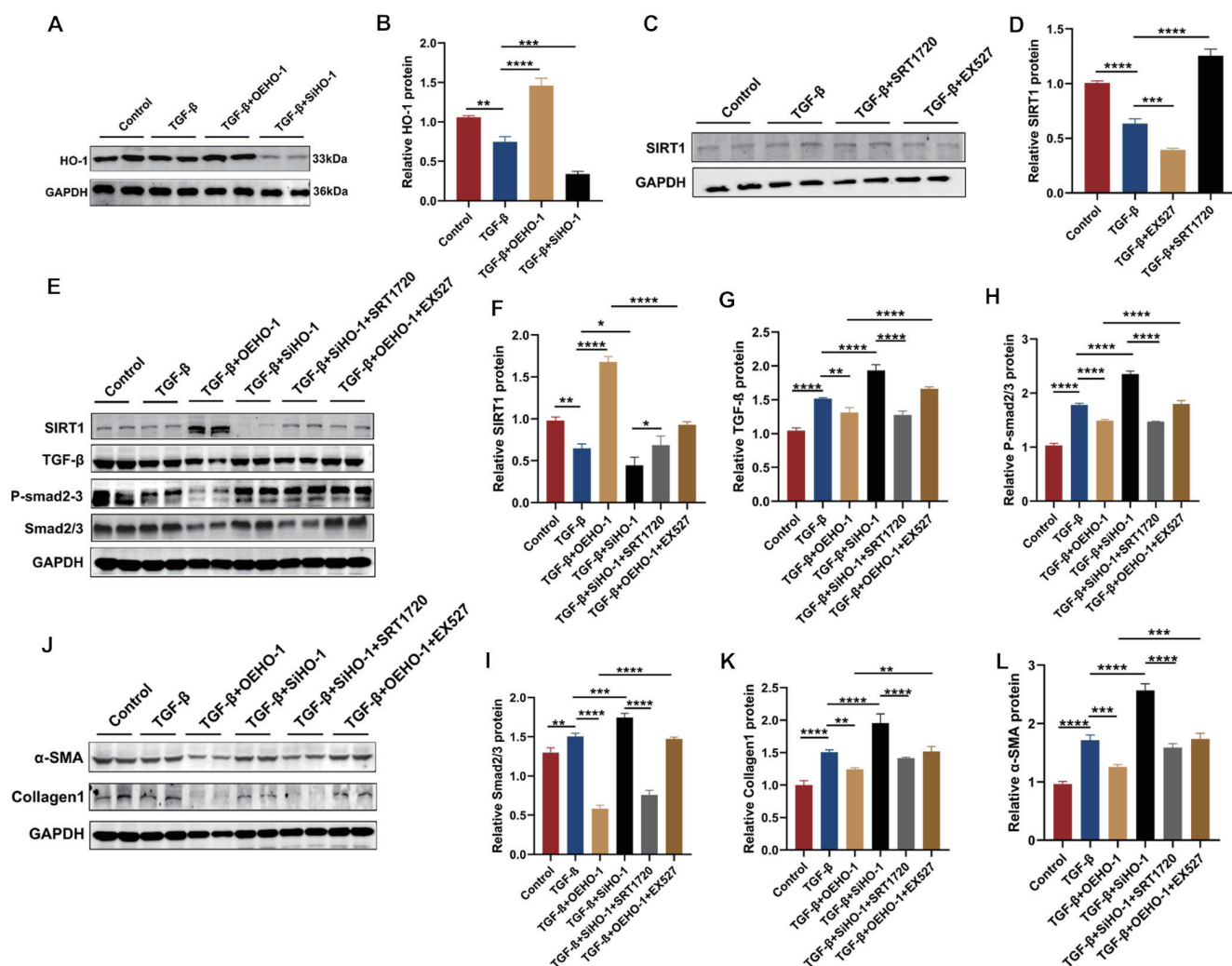


Fig. 4. HO-1 mediates NAFLD-related liver fibrosis *in vitro* by regulating the SIRT1/TGF-β/Smad3 signaling pathway. (A, B) Western blot analysis of HO-1 overexpression and silencing in LX2 cells. (C, D) Western blot analysis of LX2 cells treated with SIRT1 activators and inhibitors. (E–I) Western blot analysis of SIRT1, TGF-β, P-Smad2/3, Smad2/3 in LX2 cells. (J–L) Western blot analysis of α-SMA and Collagen I in LX2 cells. Values are the mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. MCD, methionine-and-choline-deficient diet; HO-1, heme oxygenase 1; SIRT1, Sirtuin 1; TGF-β, transforming growth factor beta; α-SMA, alpha-smooth muscle actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OE HO-1, overexpressing HO-1; SiHO-1, small interfering RNA silencing of HO-1.

on the biological behavior of LX-2 cells through a series of experiments. Cell proliferation was assessed using the CCK-8 assay (Fig. 6C). TGF-β stimulation significantly promoted LX-2 cell proliferation compared with the control group. Overexpression of HO-1 or treatment with SRT1720 suppressed LX-2 cell proliferation. In contrast, silencing HO-1 or inhibiting SIRT1 notably enhanced cell proliferation. Furthermore, we observed that SIRT1 activation counteracted the proliferative effect induced by HO-1 silencing. These findings are consistent with previous studies on the roles of HO-1 and SIRT1 in cell growth. We also assessed apoptosis using flow cytometry in the same experimental groups. The results revealed that, similar to the proliferation assay, TGF-β stimulation reduced LX-2 cell apoptosis compared with the control group. Overexpression of HO-1 or treatment with SRT1720 enhanced LX-2 apoptosis, whereas silencing HO-1 or inhibiting SIRT1 significantly decreased apoptosis (Fig. 6A–B). Additionally, TUNEL staining was performed to further validate these apoptosis findings (Fig. 7A–D). These observations

confirm that both HO-1 and SIRT1 are integral in regulating LX-2 cell behavior, and their modulation affects both cell proliferation and apoptosis.

Effects of induced HO-1 and SIRT1 on extracellular ROS in the liver *in vitro*

In the progression of NAFLD, sustained oxidative stress disrupts the balance between ROS and the antioxidant capacity of hepatocytes, resulting in lipid peroxidation. Our previous studies¹⁵ have shown that HO-1 plays a crucial role in NASH-related liver fibrosis and may serve as an antioxidant mediator under stress conditions in NASH. In damaged hepatocytes, the induction of HO-1 may trigger an adaptive stress response, potentially acting as a defense mechanism against oxidative stress and protecting hepatocytes from oxidative damage. Our previous studies also showed that overexpression of HO-1, achieved by injecting hemin into mice, significantly attenuates the severity of steatohepatitis and alleviates the overproduction of ROS.¹⁴ Further evidence

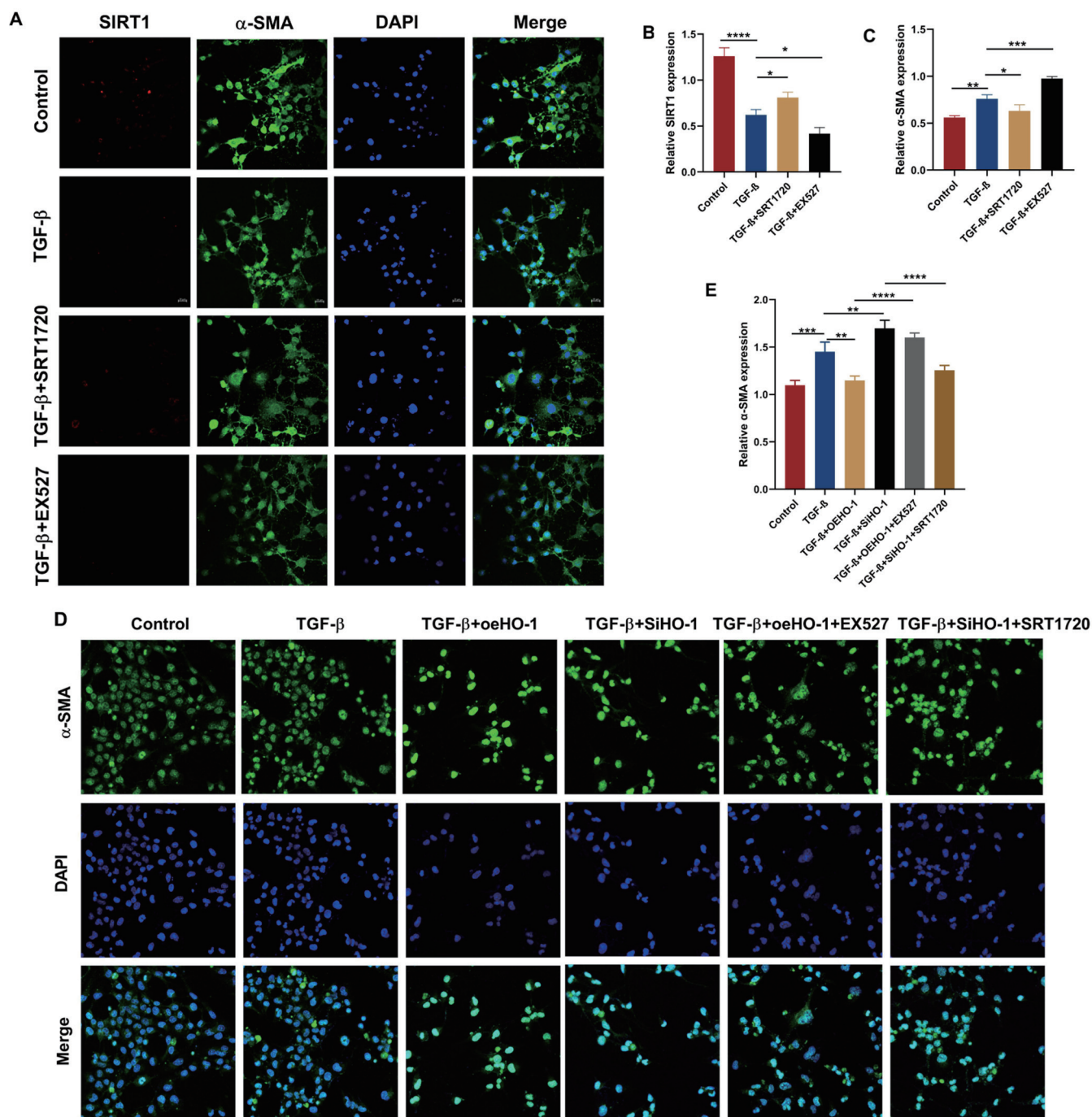


Fig. 5. SIRT1 inhibits the activation of hepatic stellate cells. (A–C) Immunofluorescence (×200 magnification) double staining of SIRT1 and α-SMA in LX2 cells. (D–E) Immunofluorescence (×200 magnification) showing α-SMA expression in LX2 cells. Values are the mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. SIRT1, Sirtuin 1; TGF-β, transforming growth factor beta; α-SMA, alpha-smooth muscle actin; OE HO-1, overexpressing HO-1; SiHO-1, small interfering RNA silencing of HO-1; DAPI, 4',6-diamidino-2-phenylindole.

from other studies demonstrated that knockdown of HO-1 increases ROS levels.³³ Similarly, SIRT1, a NAD⁺-dependent deacetylase, regulates gene expression through histone deacetylation. Elevated ROS levels can suppress SIRT1 activity through oxidative modification of its cysteine residues. SIRT1 is a NAD⁺-dependent protein deacetylase that reduces ROS levels and is involved in cell survival under oxidative stress

conditions. Studies have shown that SIRT1 deficiency in tissues such as the liver, pancreas, and brain significantly increases ROS levels and inflammatory responses.³⁴ Mitochondrial ROS significantly affect the development of NAFLD. To explore the influence of HO-1 and SIRT1 on ROS production in NAFLD-related liver fibrosis, we measured ROS levels in LX-2 cells by flow cytometry. As illustrated

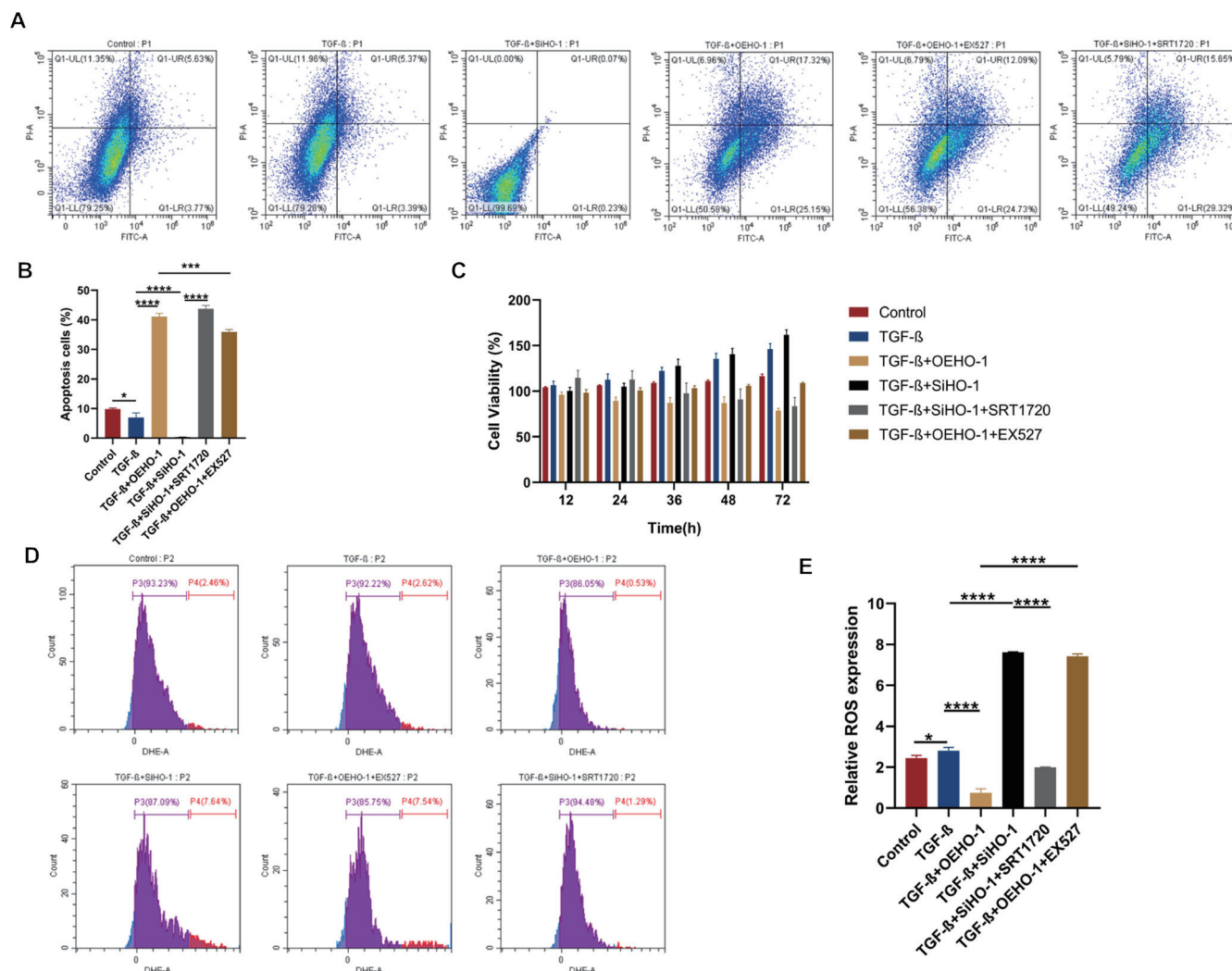


Fig. 6. The effects of HO-1 knockdown and overexpression, SIRT1 activation, and inhibition on LX2 cell proliferation, apoptosis, and ROS production. (A, B) The proportion of apoptotic cells was evaluated by flow cytometry. (C) The CCK-8 assay was used to detect cell viability. (D,E) The ROS production was analyzed using flow cytometry. * $p < 0.05$, **** $p < 0.0001$. ROS, reactive oxygen species; SIRT1, Sirtuin 1; TGF-β, transforming growth factor beta; α-SMA, alpha-smooth muscle actin; OE HO-1, overexpressing HO-1; SiHO-1, small interfering RNA silencing of HO-1.

in the figure, ROS levels were significantly elevated in the TGF-β stimulation group compared with the control group. However, overexpression of HO-1 or treatment with SIRT1 activators effectively reduced ROS levels. In contrast, silencing HO-1 or inhibiting SIRT1 significantly promoted ROS production (Fig. 6D–E).

Discussion

As NAFLD progresses, persistent oxidative stress disrupts the balance of oxidative processes, ultimately damaging cellular organelles.³⁵ HO-1 acts as an antioxidant mediator, protecting against NAFLD-associated liver fibrosis. In this study, we demonstrated that inhibiting HO-1 in LX-2 cells, when stimulated with TGF-β, led to enhanced ROS production, as revealed by flow cytometry assays, compared with cells treated with TGF-β alone. Mechanistically, our findings show that HO-1 mitigates NAFLD-related liver fibrosis through the SIRT1/TGF-β/Smad3 signaling pathway. RT-qPCR and Western blot analysis revealed that HO-1 expression was upregu-

lated in mice fed an MCD diet. Biochemical analysis revealed an elevation in ALT and AST levels in this group. However, in mice treated with hemin, ALT and AST levels were significantly reduced, indicating improved liver function. In contrast, treatment with Znpp increased ALT and AST levels, indicating intensified liver injury compared with the MCD diet alone. Consistent with this, compounds such as hemin (which induces HO-1 expression) and Znpp (which inhibits it) can modulate heme metabolism and influence liver injury and fibrosis progression.³⁶ Based on these findings, we further explored the role of HO-1 in liver fibrosis associated with NAFLD and its potential targets.

SIRT1 activators can mitigate fibrosis by inhibiting the TGF-β/Smad3 signaling pathway.³⁷ However, the potential role of HO-1 in modulating the SIRT1/TGF-β/Smad3 axis to alleviate NAFLD-related liver fibrosis, both *in vitro* and *in vivo*, is a novel area of research. SIRT1 plays a crucial role in various liver diseases, including alcoholic liver disease, NAFLD, and hepatocellular carcinoma.^{37–40} Intervening in SIRT1 expression and activity presents a promising

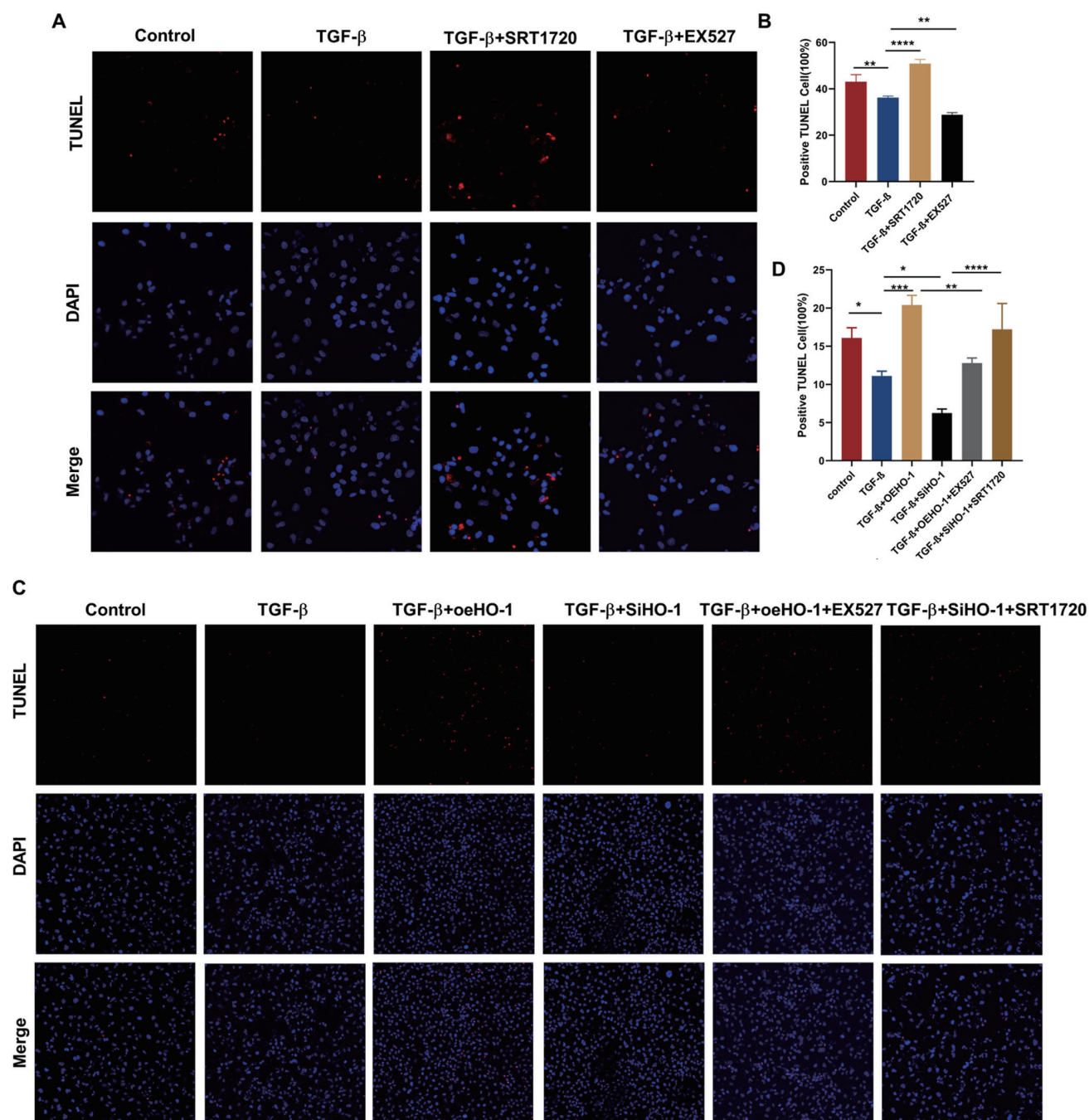


Fig. 7. SIRT1 promotes apoptosis in LX2 cells. (A, B) Detection of apoptotic cells by TUNEL assay ($\times 200$ magnification) following treatment with SIRT1 agonist and inhibitor. (C, D) Apoptotic cells were detected by TUNEL assay ($\times 200$ magnification). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. SIRT1, Sirtuin 1; TGF- β , transforming growth factor beta; α -SMA, alpha-smooth muscle actin; OE HO-1, overexpressing HO-1; SiHO-1, small interfering RNA silencing of HO-1; DAPI, 4',6-diamidino-2-phenylindole.

therapeutic approach for managing liver fibrosis associated with NAFLD. In our animal model, we used immunofluorescence double-labeling to show that HO-1 targets SIRT1. We observed that the interaction between HO-1 and SIRT1 was enhanced in the hemin-treated group but significantly reduced by Znpp treatment. Western blotting further confirmed that hepatic SIRT1 expression is closely linked to HO-1 activity. Specifically, SIRT1 protein levels increased

when HO-1 was overexpressed in the hemin-treated group. Other studies have supported the SIRT1-dependent beneficial effects of HO-1, as demonstrated by experiments involving the overexpression of the SIRT1 plasmid and siRNA silencing.¹² In line with these findings, Boily *et al.*⁴¹ reported that SIRT1 knockout mice exhibit increased metabolic activity, impaired hepatic mitochondrial function, and enhanced lipid oxidation rates. Moreover, SIRT1

not only slows the progression of liver fibrosis by promoting cell apoptosis and reversing the activation of stellate cells but also delays the aging process by regulating inflammation-related pathways. Furthermore, SIRT1 can inhibit TGF- β -induced collagen production and myofibroblast differentiation in pulmonary fibrosis.⁴² TGF- β is a potent fibrotic stimulant that activates stellate cells through the TGF- β /Smad3 signaling pathway.^{43–45} When TGF- β binds to its receptor, TGF- β R, it induces the phosphorylation of downstream Smad2 and Smad3. These activated Smads form a complex with Smad4, which then translocates to the nucleus to promote the transcription of ECM-related components.⁴⁶ This process leads to the upregulation of genes associated with ECM formation, including *TGF β R*, *SMAD2/3*, *COL1A1*, and *ACTA2*.⁴⁵ Our study confirmed the activation of the TGF- β /Smad3 pathway in liver fibrosis, which aligns with previous findings. Furthermore, we performed RT-qPCR, Western blot, and immunofluorescence experiments and observed that SIRT1 inhibited liver fibrosis onset and progression. Importantly, an inverse relationship was observed between SIRT1, TGF- β , and Smad3, indicating that SIRT1 alleviated liver fibrosis by modulating the TGF- β /Smad3 signaling pathway. We conducted *in vitro* experiments to further investigate the molecular mechanisms through which HO-1 influences the TGF- β /Smad3 pathway via SIRT1. In our study, we investigated the effects of SIRT1 modulation in LX-2 cells. After treatment with EX527 at a concentration of 10 μ M, RT-qPCR and Western blot analysis revealed that both SIRT1 mRNA and protein levels were significantly reduced in LX-2 cells. Immunofluorescence analysis revealed a notable increase in α -SMA expression, a marker of fibrosis. In contrast, when we treated LX-2 cells with 1 μ M of SIRT1720, the lowest nontoxic concentration, RT-qPCR and Western blot analysis revealed that both SIRT1 mRNA and protein levels significantly increased. Building on this, we then inhibited SIRT1 using EX527 in cells overexpressing HO-1. This intervention reversed the reduction in gene expression in the TGF- β /Smad3 signaling pathway previously observed with HO-1 overexpression, thereby worsening liver fibrosis. Conversely, when we silenced HO-1 and activated SIRT1 with SIRT1720, the downregulation of genes in the TGF- β /Smad3 pathway induced by HO-1 silencing was mitigated. Our results indicate that HO-1 regulates the TGF- β /Smad3 pathway through SIRT1, thereby mitigating liver fibrosis. Furthermore, we evaluated the effects of HO-1 and SIRT1 levels on apoptosis in LX-2 cells through flow cytometry analysis. We found that both HO-1 and SIRT1 expression promoted cell apoptosis, suggesting a protective role in the pathogenesis of liver fibrosis related to NAFLD, primarily by enhancing the apoptosis of hepatic stellate cells. Our previous clinical investigations suggested that HO-1 might serve as a potential diagnostic biomarker, and the activation of HO-1 could be a therapeutic approach for NAFLD or fibrotic NASH. Studies have shown that serum levels of HO-1 are significantly higher in NAFLD patients compared to healthy controls. Further analysis using ROC curves demonstrated that HO-1 is a potential diagnostic biomarker for NAFLD, with an exceptionally high sensitivity of 98.82%. Thus, HO-1 could serve as an important complementary biomarker in the molecular diagnostic framework for NAFLD.⁴⁷ Furthermore, in another study involving 30 patients with liver biopsy-proven NASH and 15 healthy controls, we confirmed by IHC and RT-qPCR that HO-1 can effectively inhibit the onset of endoplasmic reticulum stress. These results indicated that HO-1 may serve as a therapeutic target for personalized treatment strategies in

NASH.⁴⁸ Our experiments support these findings, as overexpression of HO-1 via plasmid transfection reduced hepatic fat accumulation and inflammation, thereby alleviating the progression of NAFLD.

SIRT1, a multifunctional protein involved in regulating liver lipid metabolism, plays a pivotal role in the development of NAFLD by deacetylating various target proteins. It has been reported that SIRT1 expression is decreased in the livers of NAFLD patients compared to healthy individuals. Additionally, hepatic SIRT1 mRNA levels have been found to be inversely correlated with the severity of hepatic steatosis and portal fibrosis. SIRT1 levels also exhibit a negative correlation with body mass index, the HOMA index, serum glucose, and ALT levels.¹⁷ Our experimental findings, both *in vitro* and *in vivo*, show a similar downregulation of SIRT1 expression in NAFLD. These findings highlight its potential as a therapeutic target for the treatment of non-alcoholic fatty liver disease.

Therefore, targeting HO-1 and SIRT1 for therapeutic intervention in NAFLD may be a promising strategy. While current research primarily focuses on animal and *in vitro* studies, ongoing advancements suggest that HO-1 and SIRT1 activators could emerge as therapeutic options for the treatment of NAFLD and its complications. These findings pave the way for potential clinical applications.

Conclusions

The results of this study demonstrate that HO-1 helps reduce hepatic fibrosis associated with NAFLD by modulating the SIRT1/TGF- β /Smad3 signaling pathway. Insights into the interactions between HO-1 and SIRT1 in NAFLD-associated liver fibrosis could facilitate the development of new biomarkers and treatment strategies for liver dysfunction. Ultimately, this deeper understanding could lead to improved quality of life and life expectancy for patients suffering from NAFLD-related hepatic fibrosis.

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Conflict of interest

YN has been an Editorial Board Member of *Journal of Clinical and Translational Hepatology* since 2022. The other authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (MS), analysis and interpretation of data (XW, ZL, CZ), performing the experiments (MS, JC, YM), manuscript preparation (MS, YS, JZ), and critical revision of the manuscript for important intellectual content (YN). All authors made significant contributions to this study and approved the final manuscript.

Ethical statement

The research protocol was approved and supervised by the Ethics Committee of the Third Hospital of Hebei Medical Uni-

versity (approval number: 2021-0851). All animals received human care.

Data sharing statement

The data used to support the findings of this study are included within the supplementary information file(s).

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