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Hesperetin derivative attenuates CCl_4-induced hepatic fibrosis and inflammation by Gli-1-dependent mechanisms

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ABSTRACT

Hepatic fibrosis, a common pathological feature and leading cause of various chronic liver diseases, still lacks effective therapy. Hesperetin derivative (HD) is a derivative of Traditional Chinese Medicine monomer isolated from the fruit peel of *Citrusaurantium L.* (*Rutaceae*). In the present study, we revealed the anti-fibrotic effects of HD in CCl_4-induced mouse hepatic fibrosis model and in TGF-β1-activated LX-2 cells, in vivo and in vitro. Results showed that HD prevented CCl_4-induced liver injury and histological damage. Consistently, HD inhibited the up-regulation of liver fibrogenesis markers α-SMA, Col1α1, Col3α1 and TIMP-1 in primary hepatic stellate cells (HSCs) and suppressed inflammatory responses in primary liver macrophages from hepatic fibrosis mice. Furthermore, HD promoted the apoptosis of activated HSCs, a key step in the onset of fibrosis regression. Mechanistically, the Hedgehog pathway was involved in HD-treated hepatic fibrosis, and HD specifically contributed to attenuate the aberrant expression of Glioma associated oncogene-1 (Gli-1). Interestingly, blockade of Gli-1 removed the inhibitory effect of HD on activated HSCs, indicating that Gli-1 may play a pivotal role in mediating the anti-fibrotic effect of HD in hepatic fibrosis. Collectively, our results suggest that HD may be a potential anti-fibrotic Traditional Chinese Medicine monomer for the treatment of hepatic fibrosis.

1. Introduction

Hepatic fibrosis, a wound-healing response, is characterized by the accumulation of extracellular matrix (ECM) following chronic liver injury. Persisting liver fibrogenesis often results in liver cirrhosis or hepatic failure [1]. Importantly, α-SMA-positive hepatic myofibroblasts, a subset of activated hepatic stellate cells (aHSCs) are the principal cell type responsible for the increased deposition of ECM during hepatic fibrosis [2,3]. Additionally, aHSCs play a key role in link liver inflammation to fibrogenesis [4]. Notably, a crucial step in the initiation of hepatic fibrosis is strong inflammatory responses [5,6], ongoing chronic inflammation and accumulation of ECM result in massive fibrogenesis and progressive substitution of liver parenchyma by scar tissue [7,8]. When liver resident macrophages are induced, they release proinflammatory and profibrotic mediators that activate quiescent HSCs into myofibroblasts [9,10]. The molecular mechanism of hepatic fibrosis is poorly understood, therefore, identifying effective therapies for hepatic fibrosis are urgently needed. Hesperetin, a flavanone glycoside compound extracted from the fruit peel of *Citrusaurantium L.* (*Rutaceae*), has a variety of pharmacological effects in renal fibrosis [11], liver fibrosis [12] and lung fibrosis [13]. Hesperetin derivative (HD), synthesized via Mannich bases in our laboratory to improve the water solubility and bioavailability of hesperetin (Fig. 1A), was utilized as a potential anti-inflammation monomeric compound for the treatment of acute liver injury [14]. Interestingly, in a recently study, we further found that HD exhibited anti-fibrotic effects on chronic hepatic fibrosis.

Another study provides evidence that hesperetin inhibited renal fibrosis by antagonizing the hedgehog signaling pathway [15]. Notably, hedgehog pathway is potentially important for biomarkers development and therapeutic targets development for hepatic fibrosis, because the activation of hedgehog signaling promotes the transition of quiescent HSCs to fibrogenic myofibroblasts [16,17]. In addition, hedgehog signaling is relates to liver injury and inflammatory responses, which substantially contribute to fibrogenesis occurrence [18]. Glioma associated oncogene 1 (Gli-1) protein is a member of the Kruppel family of

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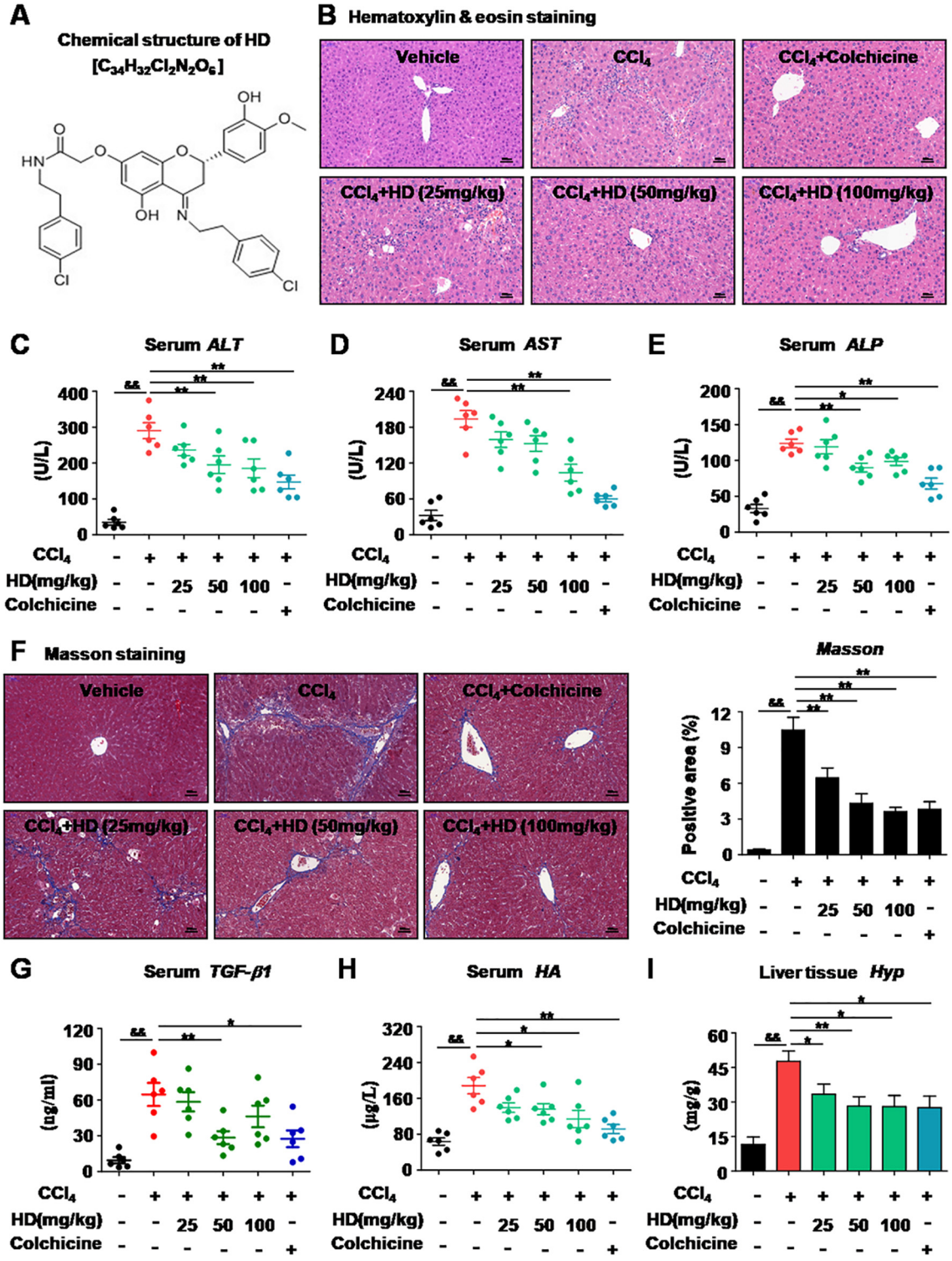

Fig. 1. Hepato-protective effect and anti-fibrotic effect of HD in CCl_4-induced hepatic fibrosis mice. (A) Molecular structural formula of Hesperetin derivative (HD). (B) Paraffin-embedded sections of liver tissue from six group mice stained with H&E staining. Representative views were presented, scale bar, 100 μM; magnification, 10×. (C, D, E) Live function was assessed by serum levels of ALT, AST and ALP in mice. (F) Paraffin-embedded sections of liver tissue from six group mice were stained with masson staining, and the positive staining areas were measured by Ipwin32 software. Representative views were presented, scale bar, 100 μM; magnification, 10×. (G, H, I) Test of serum TGF-β1 and HA levels and liver tissue Hyp level. Bars represent mean ± SEM for six mice. $^{\&\&}p < 0.01$ versus vehicle; $^{*}p < 0.05$, $^{**}p < 0.01$ versus CCl_4.

transcription factors [19], it have five conserved zinc-finger DNA binding domains and bind to the promoter of their target genes [20]. Hedgehog-Gli signaling relates to various cellular responses such as proliferation and differentiation and cell survival, Gli-1 acts as a positive feedback to reinforce Gli activity. Aberrant activation of Hedgehog-Gli pathway, usually manifested by up-regulation of Gli-1, involved in a multitude of human cancer types including liver cancer and renal interstitial fibrosis [11,21]. Based on these observations, we

hypothesized that HD may has a therapeutic effect on hepatic fibrosis by relating to liver fibrogenesis and inflammatory responses. We assessed the functions and molecular mechanisms of HD in hepatic fibrosis. In this regard, to our knowledge, this is the first study to detect the anti-fibrotic effect of HD in CCl_4-induced mouse chronic hepatic fibrosis model and TGF-β1-stimulated LX-2 cells, in vivo and in vitro.

2. Materials and methods

2.1. Mouse hepatic fibrosis model establishment and HD treatment

Experiments were approved by the Animal Experimentation Ethics Committee of Anhui Medical University. Littermate male C57BL/6J mice (8–10 weeks of age, 22–25 g body wt) were used in this study. Mice were divided into six groups (vehicle, CCl_4, CCl_4 + HD 25 mg/kg, CCl_4 + HD 50 mg/kg, CCl_4 + HD 100 mg/kg, CCl_4 + Colchicine 0.1 mg/kg) by random digits table. CCl_4-induce mouse model of hepatic fibrosis was established as described previously [22]. Hepatic fibrosis mice were exposed to biweekly intraperitoneal injection of 10% (*v/v*) CCl_4 at a dose of 0.001 ml/g for four weeks. Vehicle group of mice exposed to the same volume of olive oil. Three HD treated groups mice were exposed to biweekly intraperitoneal injection of CCl_4, and received different doses of HD (25 mg/kg, 50 mg/kg, 100 mg/kg) [14] by daily intragastric administration, respectively. Positive control group mice were exposed to biweekly intraperitoneal injection of CCl_4, and received Colchicine (0.1 mg/kg) [23] by daily intragastric administration. The vehicle and CCl_4 group mice received equal volume of normal saline. Mice were sacrificed three days after the final CCl_4 injection. Liver tissues were paraformaldehyde-fixed and paraffin-embedded from six mice of each group, and primary liver cells isolation from eight mice of each group.

2.2. Isolation of primary liver cells

Primary HSCs and macrophages were isolated by Collagenase-Pronase (Sigma-Aldrich, USA) perfusion of mouse livers as previously described [24]. Suspension of dispersed cells was layered by gradient centrifugation with 11.5% and 20% OptiPrep (Axis-shield, Norway), according to manufacture protocols, respectively. Liver sinusoidal endothelial cells (LSECs) were removed from Macrophages fraction by selective adherence [5].

2.3. Flow cytometry

Primary liver macrophages were isolated from mouse livers and analyzed by flow cytometry as previously described [25]. Cells were incubated with PE conjugated Rat Anti-Mouse F4/80 (BD, USA) and FITC conjugated Rat Anti-CD11b (BD, USA). Besides, cells incubated with isotype-matched irrelevant control antibodies and unstained cells were used as negative controls. Cells were detected by a CytoFLEX flow cytometer (Beckman Coulter, USA) and analyzed using CytExpert software (Beckman Coulter, USA).

2.4. Elisa

Serum levels of TNF-α and IL-1β were measured using RayBio® Mouse ELISA Kit (RayBiotech, USA). Assays were performed using the protocols recommended by the manufacturer. The absorbance of each well at 450 nm was measured by using a Thermomax microplate reader (bio-tekEL, USA). At least six independent experiments were performed.

2.5. Liver histological and immunohistological staining

To ascertain hepatic morphology and determine liver fibrosis in mice, Hematoxylin & eosin staining and Masson's trichrome staining were performed, respectively. Paraformaldehyde-fixed, paraffinembedded liver tissues were sectioned (4 μm) for hydrated and stained by standard methods [26]. Immunohistochemistry was performed in paraffin sectioned using a microwave-based antigen retrieval technique [27]. The areas of staining were photographed and converted using an automatic digital slide scanner (Pannoramic MIDI-3DHISTECH, Hungary), representative views were presented.

2.6. Cell culture

LX-2 cells, human immortalized HSC line, were cultured in DMEM (supplemented with 10% FBS and 1% P/S) (Gibco, USA) [5]. Recombinant human TGF-β1 (R&D Systems, MN) at concentration of 2 ng/ml was added to the cell culture for 48 h [28]. In addition, GANT61 (an inhibitor of Gli) at dosages of 5, 10 and 20 μM [29,30] was applied for a dosage-dependent assay in LX-2 cells according to the manufacturer's instructions. At least three independent experiments were performed throughout the study.

2.7. Gli-1 knockdown

Small interfering RNA (siRNA) of Gli-1 and negative control was synthesized by Hanbio Biotechnology (Shanghai, China). LX-2 cells were transfected with siRNA-Gli-1 or negative control using Lipofectamine RNAiMax (Life Technologies, USA) according to the manufacturer's protocol. After 6 h transfection, the culture medium was replaced with fresh medium for additional 48 h incubation. Silencing efficiency was confirmed by Real-time PCR after transfection. Sequences of siRNA-Gli-1 and negative control used in this study were listed in Table 2.

2.8. RNA extraction and real-time PCR

Total RNA was isolated from primary HSCs, macrophages and LX-2 cells by TRIZOL reagent (Invitrogen, CA) and quantified by NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Real-time PCR analysis was performed using CFX96 real-time PCR system (Bio-Rad, USA) as previously described [5]. Primers sequences used in this study were listed in Table 1. The ratio for the mRNA interested was normalized with β-actin.

2.9. Western blot

Protein was extracted with RIPA and PMSF lysis buffer, western blot procedure was performed as previously described [14]. Blotted membranes were blocked and incubated with the primary antibodies of interest. Antibodies used in this study included primary antibodies specific for α-SMA, Bax, Bcl-2 (1:2000, Abcam, UK); Col1α1, Gli-1, Shh, Smo, P65, p-P65 (1:500, Bioss, China); TNF-α, IL-1β, β-actin (1:1000, Bioworld, USA); pro-caspase3, cle-caspase3 (1:1000, Cell Signaling, MA). Bands were visualized by enhanced chemiluminescence system (Bio-Rad, CA). Signal intensities of each western blot were quantified by using the Image J software (NIH, Bethesda, MD, USA) and normalized to β-actin as internal control.

2.10. Statistical analysis

Data collected from this study were expressed as mean ± SEM and analyzed using one-way analysis of variance (ANOVA), followed by Newman-Keuls *post-hoc test* (Prism 5.0 GraphPad Software, USA).

3. Results

3.1. Hepato-protective effect and anti-fibrotic effect of HD in CCl_4-induced mouse hepatic fibrosis model

First, we determined the functional roles of HD in progressive

Table 1
Primer sequences used in Real-time PCR.

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)
A. Mouse		
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
α-SMA	GTCCCAGACATCAGGGAGTAA	TCGGATACTTCAGCGTCAGGA
Col1α1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
Col3α1	CATGTTCAGCTTTGTGGACCT	GCAGCTGACTTCAGGGATGT
TIMP-1	GCAACTCGGACCTGGTCATAA	CGGCCCGTGATGAGAAACT
MCP-1	GGGCCTGCTGTTCACAGTT	CCAGCCTACTCATTGGGAT
TNF-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
IL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
Gli-1	CCTGGTGGCTTTCATCAACT	ACACAGGGCTGGACTCCATA
Gli-2	TCACATCAGCCAACCAAGAG	GCAGCCTCCATTCTGTTCAT
Shh	CGGCAGATATGAAGGGAAGA	TCATCACAGATGGCCAAGG
Smo	TGCCACCAGAAGAACAAGCCA	CCTCCATTAGGTTAGTGCGG
B. Human		
β-actin	GGCATTCACGAGACCACCTAC	CGACATGACGTTGTTGGCATAC
α-SMA	GTGTTGCCCCTGAAGAGCAT	GCTGGGACATTGAAAGTCTCA
Col1α1	GTGCGATGACGTGATCTGTGA	CGGTGGTTTCTTGGTCGGT
Col3α1	CCTGGTCCTTGCTGTGGTGGTGT	GCAGTTTCTAGCGGGGTTTTTACG
TIMP-1	CTTCTGCAATTCCGACCTCGT	ACGCTGGTATAAGGTGGTCTG
PDGF	CTCGATCCGCTCCTTTGATGA	CGTTGGTGCGGTCTATGAG
Gli-1	GCCCTATGTGAAGCCCTATTT	TCCTACCAGAGTCCCAAGTTTC
Gli-2	AGCATCTCTTGCCACCATTC	ACGGAGGTAGTGCTCCATGT

hepatic fibrosis induced by CCl_4 in mice. Fig. 1A showed the molecular structural formula of HD. Histologically, hematoxylin & eosin (H&E) staining revealed that hepatic fibrosis mice with HD treatment (25, 50 and 100 mg/kg) dose-dependently inhibited hepatocyte necrosis, inflammatory cell infiltration and mouse liver fibrogenesis (Fig. 1B). Previous studies confirmed that Colchicine administration in hepatic fibrosis characterized by significantly anti-inflammatory, anti-fibrotic and immunomodulatory effects, therefore, colchicine (0.1 mg/kg) was used as a positive control in this study [32,33]. Additionally, serum levels of ALT, AST and ALP were reduced after hepatic fibrosis mice treated with HD (Fig. 1C, D and E). Indicating HD exhibited hepatoprotective effect in CCl_4-induced mouse chronic hepatic fibrosis model, in vivo. Next, we investigated whether HD has a role in collagen matrix protein degradation. As shown in Fig. 1F, masson staining showed that collagen deposition was markedly inhibited in the injured liver tissues from HD-treated hepatic fibrosis mice compared with hepatic fibrosis mice. Strikingly, the treatment of hepatic fibrosis mice with HD further decreased serum levels of TGF-β1 and HA (Fig. 1G and H), a corresponding with reduction of Hyp in liver tissues (Fig. 1I), in vivo. Taken together, HD has a significantly inhibitory effect on live fibrogenesis in CCl_4-induced hepatic fibrosis mice.

3.2. HD negatively regulates fibrotic responses in primary HSCs

We further confirmed the functional roles of HD on HSCs, HSCs activate or transdifferentiate into myofibroblast-like cells, which are the main cell type in ECM deposition during hepatic fibrosis [22]. As shown in Fig. 2A, immunohistochemical assay revealed that α-SMA^+ myofbroblasts were increased in CCl_4-induced hepatic fibrosis mice compared with vehicle mice. However, hepatic fibrosis mice with HD treatment significantly decreased α-SMA expression in liver tissues with a dose-dependent manner. Consistently, mRNA levels of fibrogenic genes (α-SMA, Col1α1, Col3α1 and TIMP-1) were reduced in primary HSCs isolated from HD-treated mice compared with hepatic fibrosis mice (Fig. 2B). Besides, it was further confirmed at the protein expression of α-SMA and Col1α1 by western blot (Fig. 2C). These findings suggest HD mediated the activation of HSCs, contributing to the inhibition of liver fibrogenesis initiation in mice.

3.3. HD protects against CCl_4-induced inflammatory responses in mice

As shown in Fig. 3A, immunohistochemistry showed that CCl_4-induced hepatic fibrosis mice with HD treatment significantly reduced $F4/80^+$ macrophages infiltration. It is well accepted that F4/80 and CD11b are markers for pan-macrophages [25]. As shown in Fig. 3B, liver macrophages isolated from mice were defined as $F4/80^+CD11b^+$ cells by flow cytometric analysis. mRNA levels of inflammatory genes (MCP-1, TNF-α, IL-1β and IL-6) were decreased in HD-treated mice compared with CCl_4-induced hepatic fibrosis mice by a dose-dependent manner (Fig. 3C). Consistently, we evaluated the circulating levels of pro-inflammatory cytokines in serum by ELISA. The serum levels of TNF-α and IL-1β were notably reduced in HD-treated mice compared with hepatic fibrosis mice (Fig. 3D). Similarly, immunoblotting verified the suppression of TNF-α and IL-1β protein expression in hepatic fibrosis mice with HD treatment (Fig. 3E). Besides, we detected HD remarkably inhibited the phosphorylation and activation of NF-κB-P65 (Fig. 3E). These results demonstrate that hepatic fibrosis mice treatment with HD regulated the infiltration of liver macrophages, contributing to the suppression of inflammatory responses in CCl_4-induced hepatic fibrosis, in vivo.

3.4. Effects of HD on TGF-β1-induced fibrotic responses in LX-2 cells, in vitro

Next, we assessed the effects of HD on TGF-β1-activated LX-2 cells. As shown in Fig. 4A, result of MTT assay indicated that HD, with a

Table 2
siRNA sequences used in this study.

siRNA	Forward primer (5′-3′)	Reverse primer (5′-3′)
si-Gli-1	GGCUCAGCUUGUGUGUAAUdTd	AUUACACACAAGCUGAGCCdTd
Negative control	UUCUCCGAACGUGUCACGUdTd	ACGUGACACGUUCGGAGAAdTd

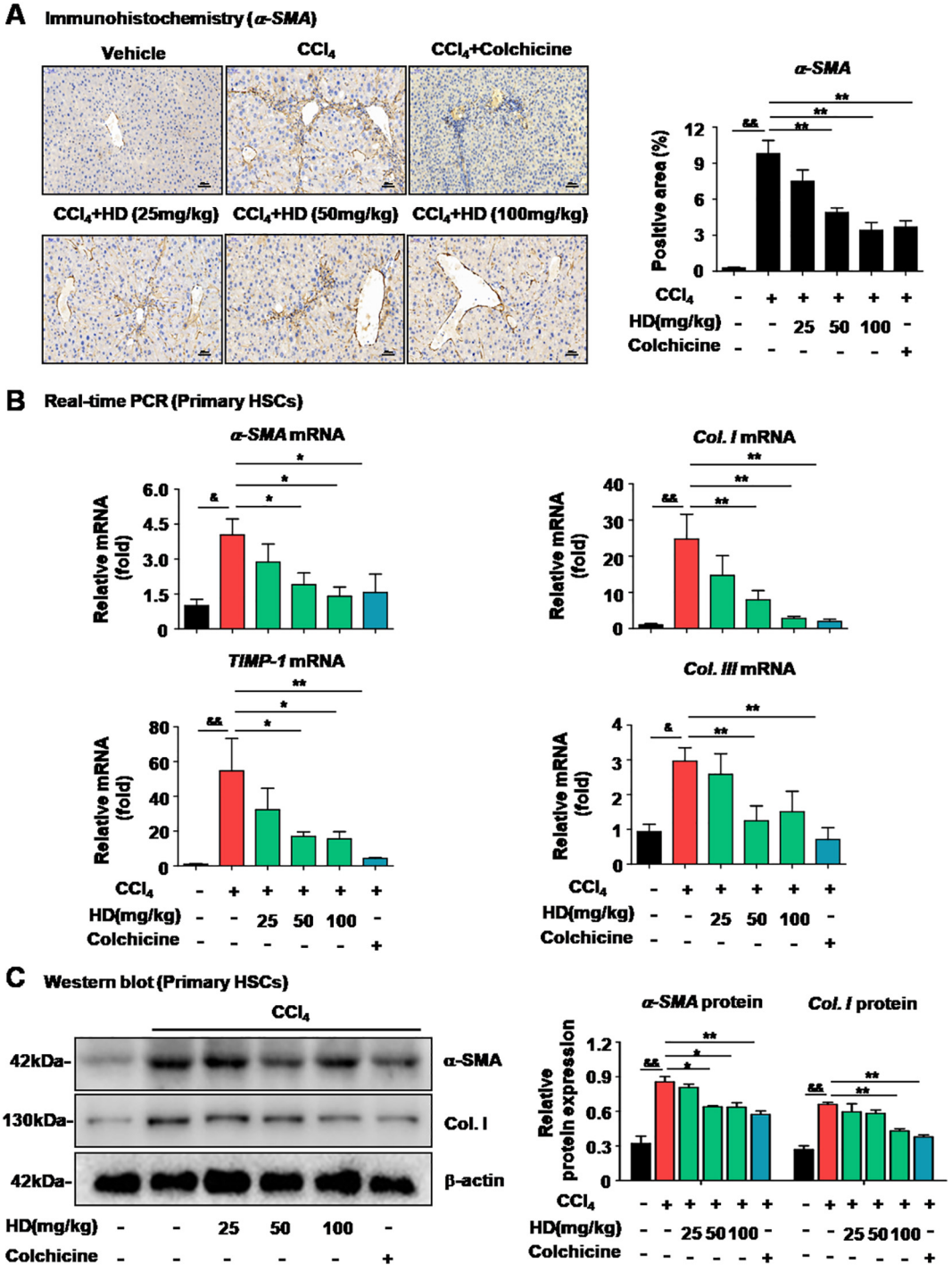

Fig. 2. HD down-regulates fibrotic responses in primary HSCs. (A) Immunohistochemical staining of α-SMA, the positive staining areas were measured by Ipwin32 software. Representative views were presented, scale bar, 50 μM; magnification, 20×. (B) Real-time PCR analyses of α-SMA, Col1α1, Col3α1 and TIMP-1 mRNA levels in primary HSCs. (C) Immunoblottings of α-SMA and Col1α1 protein expression in primary HSCs from mice. Bars represent mean ± SEM for six mice. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ versus vehicle; $^{*}p < 0.05$, $^{**}p < 0.01$ versus CCl_4.

concentration no higher than 16 μM, had a limited suppressive effect on the viability of LX-2 cells. Besides, we found concentration of HD higher than 1 μM showed significantly anti-fibrotic effect in TGF-β1-activated LX-2 cells, we then selected concentrations of 1, 2 and 4 μM used in subsequent experiments, in vitro. In Fig. 4B, results of western blot showed that HD suppressed the protein expression of α-SMA and Col1α1 and inhibited mRNA levels of α-SMA, Col1α1, Col3α1, TIMP-1 and PAI-1 in TGF-β1-activated LX-2 cells with a dose-dependent manner (Fig. 4D). Besides, double-immunofluorescence analysis showed that α-SMA and Col1α1 were consistently decreased in TGF-β1-induced LX-2 cells with 2 μM HD-treatment (Fig. 4C).

3.5. HD attenuates CCl_4-induced mouse liver fibrogenesis and TGF-β1-induced fibrotic responses in LX-2 cell via Gil-1 dependent mechanisms

Moreover, we investigated the mechanism of HD anti-fibrotic effects. Recently report revealed that functions of hesperetin on renal fibrosis is associated with hedgehog signaling [11]. First, we confirmed

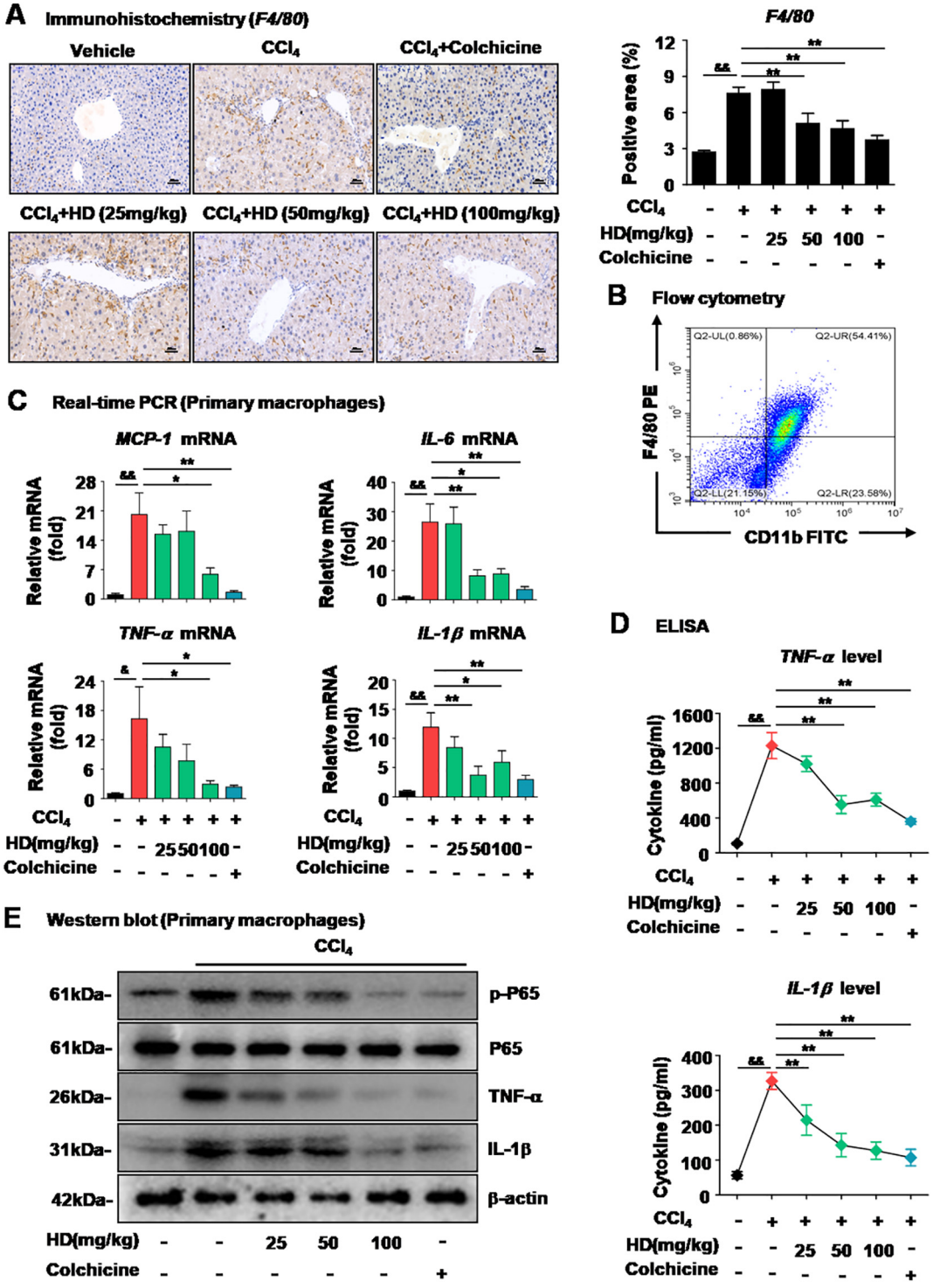

Fig. 3. HD attenuates inflammatory responses in hepatic fibrosis mice. (A) Immunohistochemical staining of F4/80, the positive staining areas were measured by Ipwin32 software. Representative views were presented, scale bar, 50 μM; magnification, 20×. (B) Flow cytometric analysis of $F4/80^{+}CD11b^{+}$ primary macrophages isolated from mice. (C) Real-time PCR analyses of MCP-1, TNF-α, IL-1β and IL-6 mRNA levels in primary macrophages from mice. (D) ELISA analysis of circulation level of pro-inflammatory cytokines TNF-α and IL-1β in serum. (E) Immunoblottings of p-P65, P65, TNF-α and IL-1β protein expression in primary macrophages from mice. Bars represent mean ± SEM for six mice. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ versus vehicle; $^{*}p < 0.05$, $^{**}p < 0.01$ versus CCl_4.

the effects of HD on the expression of hedgehog signaling relate factors Gli-1, Gli-2, Sonic hedgehog (Shh) and Smoothened (Smo) in primary HSCs isolated from mice. As shown in Fig. 5A and B, real-time PCR and immunoblotting assays showed that hepatic fibrosis mice with HD treatment markedly inhibited mRNA levels and protein expression of Gli-1 and Shh, indicating that HD may relate to these two factors. Notably, double-immunofluorescence revealed the co-localization of Gli-1 (green) and myofibroblast marker α-SMA (red) immunoreactivity in liver tissues, and expression of Gli-1 was decreased in HD treated mice compared with hepatic fibrosis mice, in vivo (Fig. 5C).

In addition, LX-2 cells were exposed to GANT61 (an inhibitor of Gli), the effects of GANT61 on Gli-1 mRNA level and protein expression

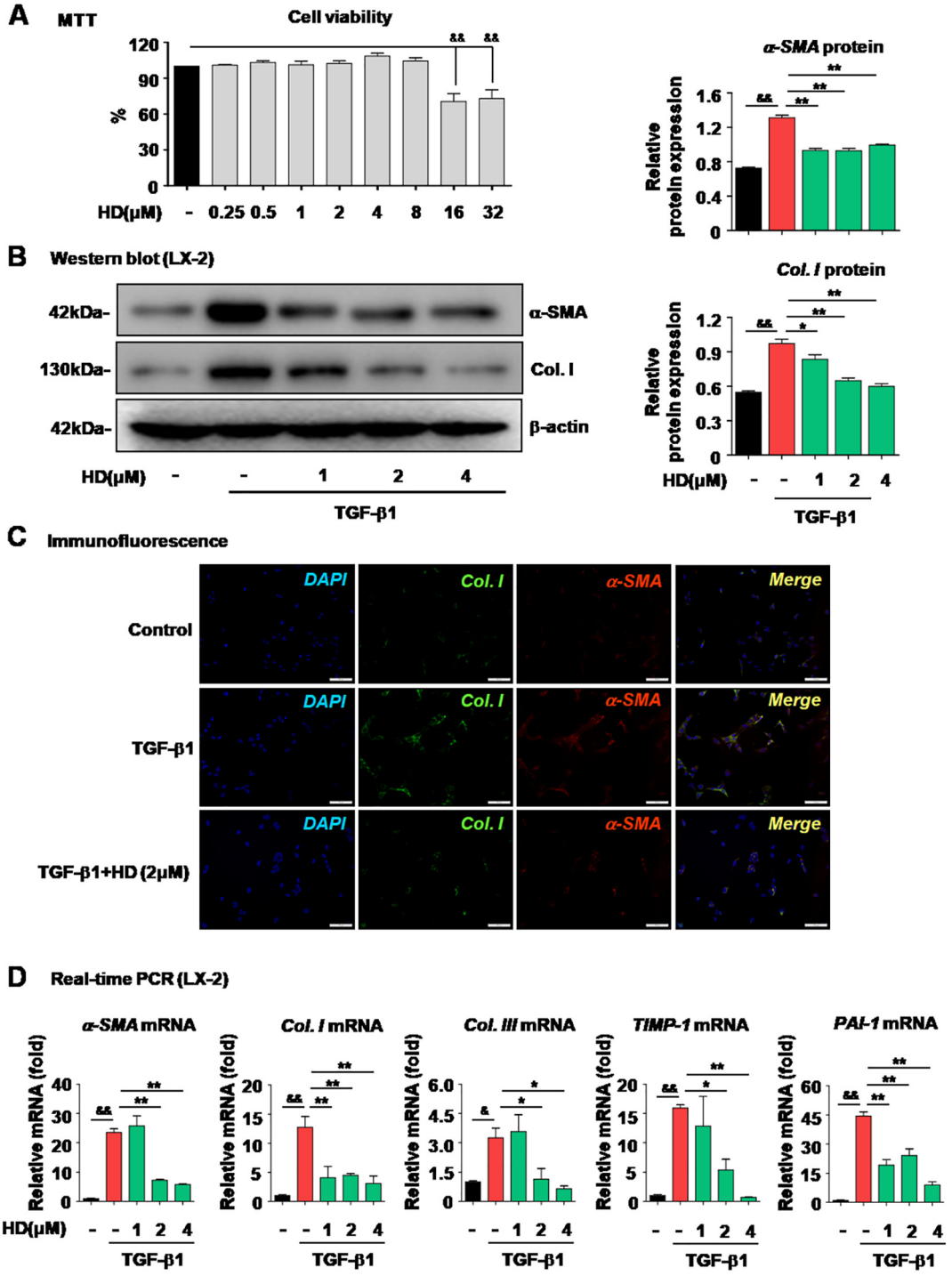

Fig. 4. Effects of HD on TGF-β1-induced fibrotic responses in LX-2 cells. (A) Effect of different concentrations of HD on LX-2 cells viability by MTT assay. (B) Immunoblottings of α-SMA and Col1α1 protein expression in LX-2 cells. (C) Double-immunofluorescence analysis of α-SMA (red) and Col1α1 (green) expression, the positive staining areas were measured by Ipwin32 software. Representative views were presented, scale bar, 100 μM; magnification, 10×. (D) Real-time PCR analyses of α-SMA, Col1α1, Col3α1, TIMP-1 and PAI-1 mRNA levels in LX-2 cells. Bars represent mean ± SEM for three independent experiments in vitro. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ versus Control; $^{*}p < 0.05$, $^{**}p < 0.01$ versus TGF-β1-induced LX-2 cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were determined (Fig. 6B), and the dosage used of GANT61 showed negligible effect on LX-2 cell viability (Fig. 6A). Results showed that expression of α-SMA and Gli-1 was reduced in TGF-β1-activated LX-2 cells following HD treatment (Fig. 6C). Besides, activation of hedgehog-Gli1 signaling induces platelet-derived growth factor (PDGF) up-regulation, which is a critical driving force to hepatic fibrosis [34]. In this study, we found that PDGF was attenuated both in serum from CCl_4-induced hepatic fibrosis mice treated with HD and mRNA level of TGF-β1-activated LX-2 cells treated with HD (Fig. 6D and E). Interestingly, α-SMA and PDGF levels were restored in TGF-β1-activated LX-2 cells exposed to GANT61 (Fig. 6C and E). Suggesting that HD exhibited anti-fibrotic effects is involved in Gil-1 dependent mechanisms.

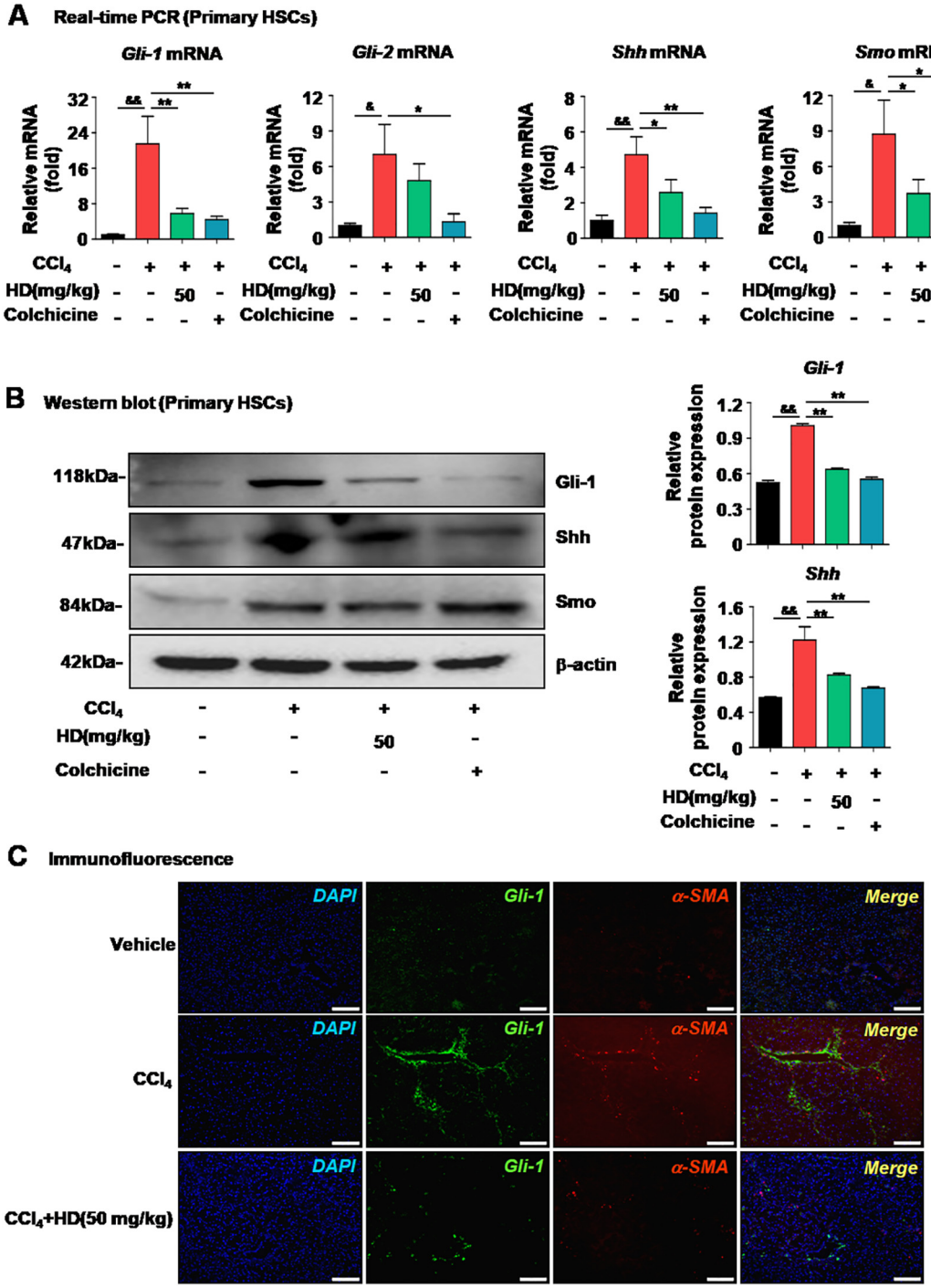

Fig. 5. HD regulates CCl_4-induced live fibrogenesis by Gil-1 signaling. (A) Real-time PCR analyses of Gli-1, Gli-2, Shh and Smo mRNA levels in primary HSCs isolated from mice. (B) Immunoblottings analyses of Gli-1, shh and smo protein expression in primary HSCs isolated from mice. (C) Double-immunofluorescence analysis of α-SMA (red) and Gli-1 (green) expression, and the positive staining areas were measured by Ipwin32 software. Representative views were presented, scale bar, 100 μM; magnification, 10×. Bars represent mean ± SEM for six mice. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ versus vehicle; $^{*}p < 0.05$, $^{**}p < 0.01$ versus CCl_4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. HD promotes apoptosis response in TGF-β1-activated LX-2 cells

Hedgehog signaling relates to the apoptosis of activated HSCs, that is a key event in liver fibrosis resolution [35]. Flow cytometric analysis used PE/AV-FITC staining showed that HD treated TGF-β1-activated LX-2 cells promoted apoptosis response, which was reversed following blockade of Gli-1 (Fig. 7A). This effect was confirmed by immunoblotting analysis of apoptosis-related cleaved-caspase3, Bax and Bcl-2 protein expression (Fig. 7B). Moreover, we constructed siRNA targets Gli-1, the knockdown efficiency of Gli-1 was evaluated after transfection siRNA into LX-2 cells (Fig. 7C). Results showed that HD increased the level of Bax and cleaved-caspase3, while knockdown of Gli-1 substantially reversed the apoptosis-inducing feature of HD on LX-2 cells (Fig. 7D). These results suggest that Gli-1 signaling may be one of the responsible pathway involved in HD-regulated amelioration of hepatic fibrosis pathogenesis.

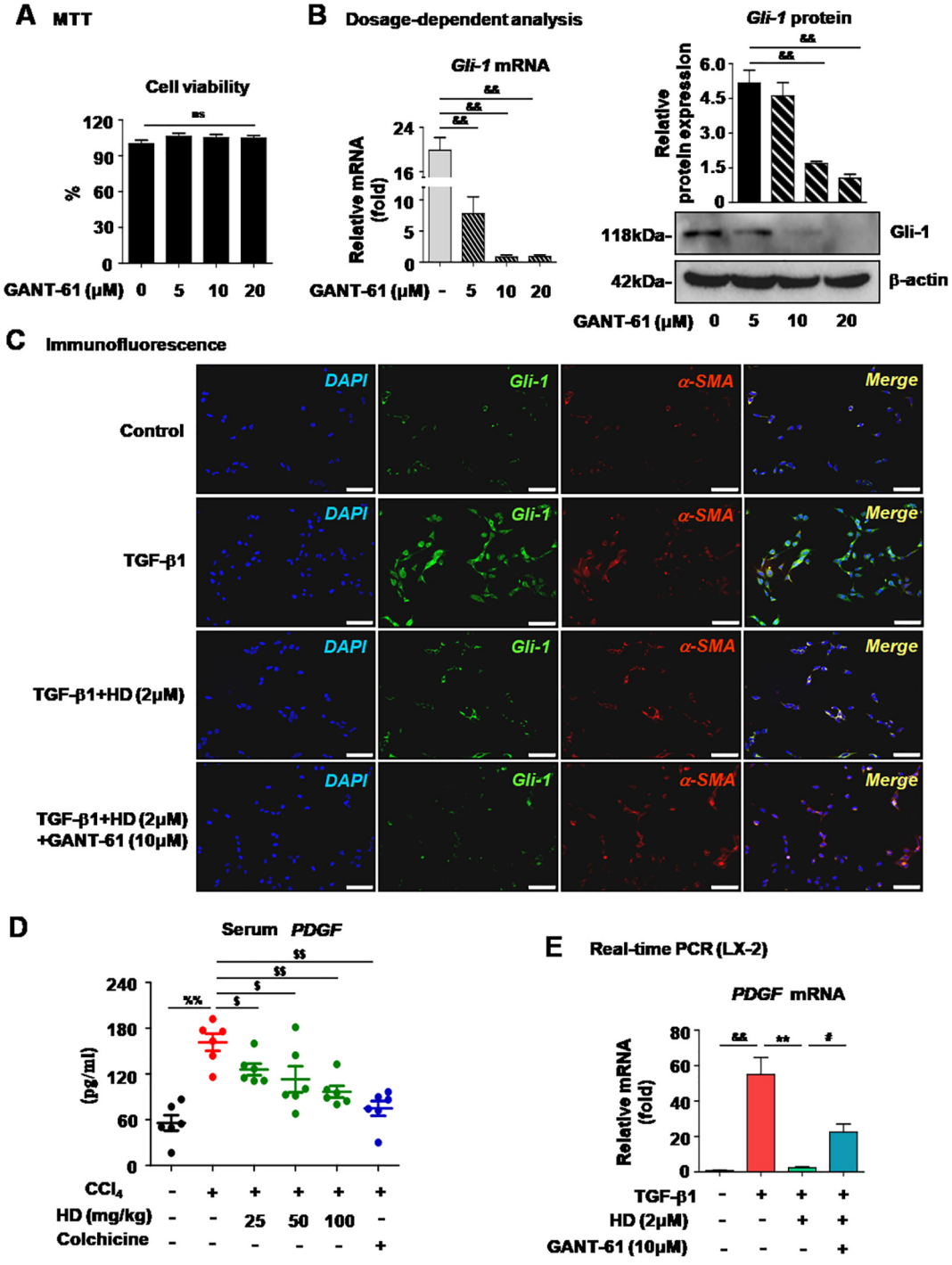

Fig. 6. HD attenuates TGF-β1 induced fibrotic responses in LX-2 cells through Gil-1-dependent mechanism. (A) Effect of different dosages of GANT-61 on cell viability of LX-2 cells by MTT assay. (B) Effect of GANT61 on Gli-1 mRNA level and protein expression was assessed by Real-time PCR and western blot. (C) Double-immunofluorescence analysis of α-SMA (red) and Gli-1 (green) expression in LX-2 cells, and the positive staining areas were measured by Ipwin32 software. Representative views were presented, scale bar, 100 μM; magnification, 10×. (D) Test of serum PDGF level. Bars represent mean ± SEM for six mice. (E) Real-time PCR analysis of PDGF mRNA level. Bars represent mean ± SEM for three independent experiments in vitro. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ versus Control; $^{*}p < 0.05$, $^{**}p < 0.01$ versus TGF-β1-induced LX-2 cells; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus TGF-β1-induced LX-2 cells with HD treatment. $^{\%\%}p < 0.01$ versus vehicle; $^{\$}p < 0.05$ versus CCl_4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Hesperetin, a well-used compound in Traditional Chinese Medicine, has been widely investigated in fibrous diseases. Besides, Hesperetin has been reported to have anti-oxidative, anti-tumorigenic and anti-inflammatory effects [36–38]. Our recent work has revealed that HD exhibited a direct inhibitory effect on inflammation in mice with acute liver injury and LPS-treated RAW264.7 cells [14]. Interestingly, we further explored the pharmacological effects of HD in chronic hepatic fibrosis. In the present study, we found HD attenuated CCl_4-induced progressive liver injury and liver fibrogenesis in hepatic fibrosis mice, indicating the hepato-protective effect of HD on hepatic fibrosis. Furthermore, we investigated HD suppressed collagen-like matrix deposition during liver fibrogenesis with a corresponding inhibition of

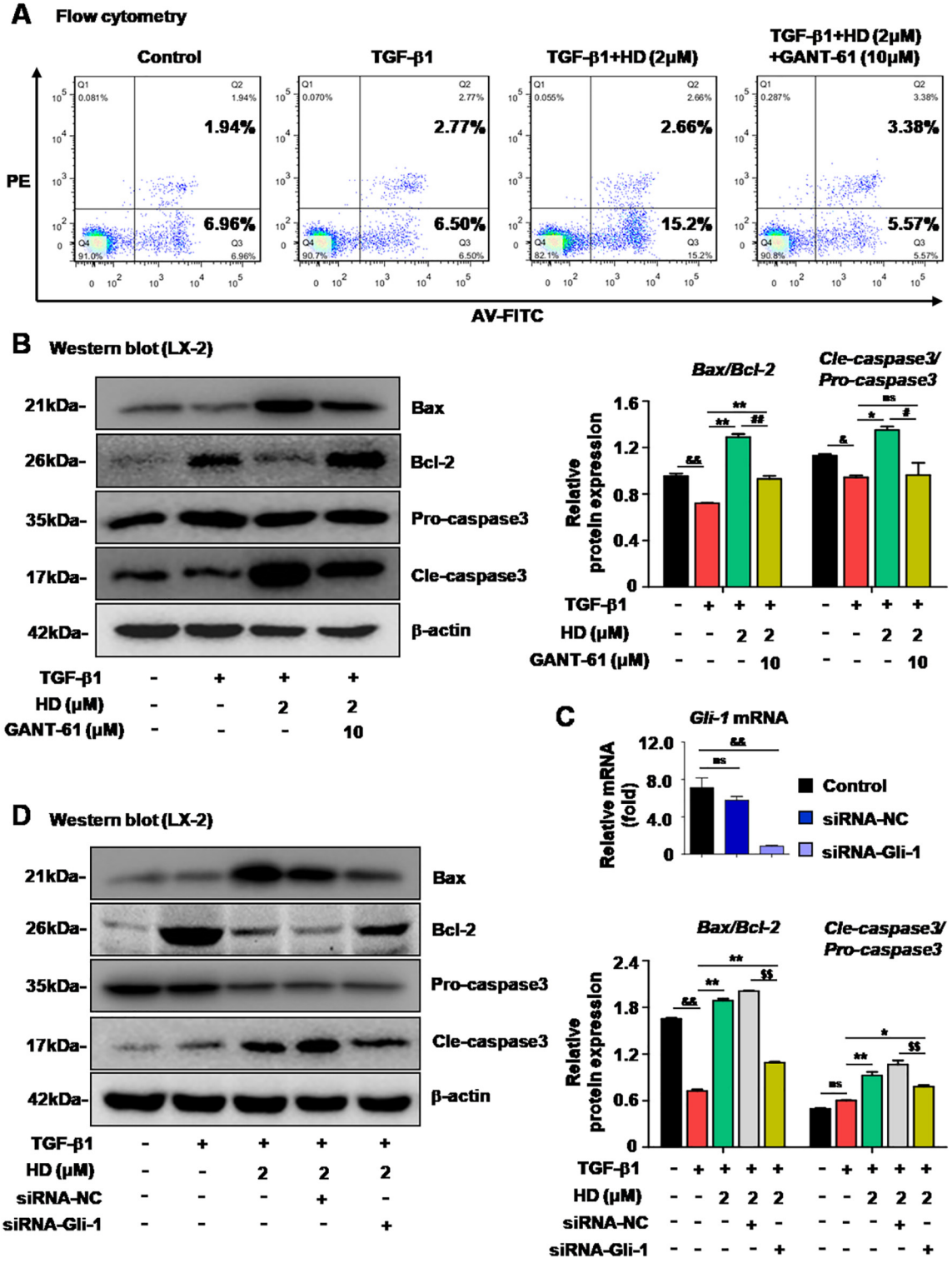

Fig. 7. HD promotes apoptosis response in TGF-β1-induced LX-2 cells. (A) Flow cytometric analysis of PE/AV-FITC in TGF-β1-induced LX-2 cells, the data analysis were performed by FlowJo software. (B) Immunoblottings analysis of Pro-caspase3, Cleaved-caspase3, Bax and Bcl-2 protein expression in LX-2 cells with HD and GANT-61 treatment. (C) Knockdown efficiency of siRNA-Gli-1. (D) Immunoblottings analysis of Pro-caspase3, Cleaved-caspase3, Bax and Bcl-2 protein expression in LX-2 cells with HD and siRNA-Gli-1 transfection. Bars represent mean ± SEM for three independent experiments. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ versus Control; $^{*}p < 0.05$, $^{**}p < 0.01$ versus TGF-β1-induced LX-2 cells; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus TGF-β1-induced LX-2 cells with HD treatment; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ versus TGF-β1-induced LX-2 cells with HD treatment and siRNA-NC transfection.

fibrogenic factors (α-SMA, Col1α1, Col3α1 and TIMP-1) in CCl_4-induced hepatic fibrosis mice. Consistently, HD significantly reduced the expression of α-SMA, Col1α1, Col3α1, TIMP-1 and PAI-1 in TGF-β1-induced LX-2 cells. These observations revealed a regulatory role of HD on the initiation and progression of hepatic fibrosis. Furthermore, we found that HD promoted the apoptosis of LX-2 cells. To date, evidences have confirmed that liver fibrosis resolution is relate to the apoptosis of activated HSCs, which is a key step in onset of fibrosis regression [39].

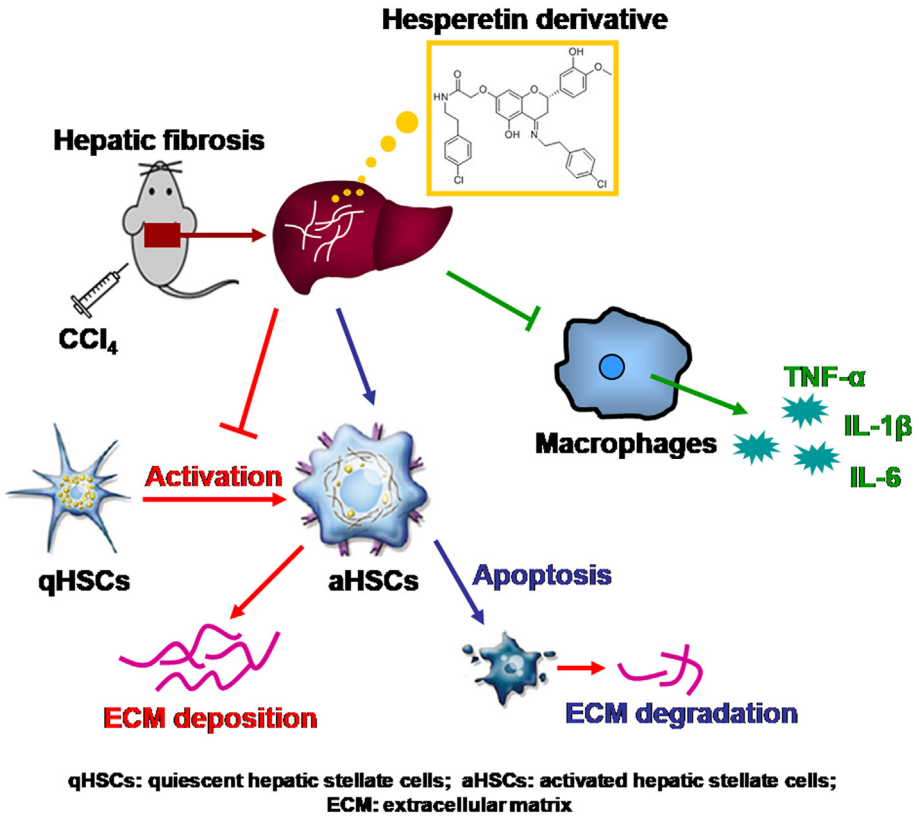

Fig. 8. Hesperetin derivative attenuates CCl_4-induced hepatic fibrosis and inflammation by Gli-1-dependent mechanisms.

Based on these findings, we elucidated the anti-fibrotic effects of HD may partly relate to its anti-inflammatory feature, as hepatic fibrosis is associate with unresolved inflammatory responses [40]. In this study, we found the percentage of $F4/80^+$ macrophages infiltration into liver tissue was remarkably decreased in hepatic fibrosis mice following HD treatment. Additionally, HD reduced the levels of inflammatory factors (MCP-1, TNF-α, IL-1β and IL-6) in mice, contributing to protecting against the inflammatory responses. Further studies are needed to uncover the molecular mechanism involved in the effect of HD on inflammation in hepatic fibrosis.

We also assessed the potential signaling pathway by which HD attenuated hepatic fibrosis. It has been reported that HD showed direct functions on hepatic fibrosis-related hedgehog signaling [41,42]. We further confirmed the role of HD on hedgehog signaling relate factors and found that HD mediated the expression of Gli-1 (a driving force of fibrogenesis [43]) in HSCs. Therefore, we drew a conclusion that effect of HD may depend on hedgehog-Gli-1 signaling, as a deficiency of Gli-1 largely abolished the therapeutic effects of HD on hepatic fibrosis.

Collectively, as shown in Fig. 8, our study firstly identified that HD alleviated liver injury and fibrogenesis in CCl_4-induced hepatic fibrosis mice, and inhibited the expression of fibrogenic genes in activated HSCs. Substantially, HD prevented inflammatory responses and reduced the levels of inflammatory factors in liver macrophages. Besides, anti-fibrotic effects of HD correlated with the Hedgehog signaling, at least in part, by suppressing the excessive activation of Gli-1 during hepatic fibrosis. This study suggests HD may be a potential anti-fibrotic Traditional Chinese Medicine monomer for the treatment of hepatic fibrosis.

Author contributions

JL, XC, XFL, YC designed the manuscript; XC, XFL, YC performed the experiments; SYC, SZ, HDL analyzed the data; FTB, XYP, JNW, CH contributed all samples, reagents and materials; JL contributed to all aspects of this study, data interpretation, and revised the manuscript for publication. All authors have revised and approved the final manuscript.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

References

[1] V. Hernandez-Gea, S.L. Friedman, Pathogenesis of liver fibrosis, Annu. Rev. Pathol. 6 (2011) 425–456.
[2] F. Klingberg, B. Hinz, E.S. White, The myofibroblast matrix: implications for tissue repair and fibrosis, J. Pathol. 229 (2) (2013) 298–309.
[3] C. Qu, D. Zheng, S. Li, Y. Liu, A. Lidofsky, J.A. Holmes, J. Chen, L. He, L. Wei, Y. Liao, H. Yuan, Q. Jin, Z. Lin, Q. Hu, Y. Jiang, M. Tu, X. Chen, W. Li, W. Lin, B.C. Fuchs, R.T. Chung, J. Hong, Tyrosine kinase SYK is a potential therapeutic target for liver fibrosis, Hepatology 68 (2018) 1125–1139.
[4] E. Seki, S. De Minicis, C.H. Osterreicher, J. Kluwe, Y. Osawa, D.A. Brenner, R.F. Schwabe, TLR4 enhances TGF-beta signaling and hepatic fibrosis, Nat. Med. 13 (11) (2007) 1324–1332.
[5] T. Lan, C. Li, G. Yang, Y. Sun, L. Zhuang, Y. Ou, H. Li, G. Wang, T. Kisseleva, D. Brenner, J. Guo, Sphingosine kinase 1 promotes liver fibrosis by preventing miR-19b-3p-mediated inhibition of CCR2, Hepatology 68 (3) (2018) 1070–1086.
[6] A. Wree, M.D. McGeough, M.E. Inzaugarat, A. Eguchi, S. Schuster, C.D. Johnson, C.A. Pena, L.J. Geisler, B.G. Papouchado, H.M. Hoffman, A.E. Feldstein, NLRP3 inflammasome driven liver injury and fibrosis: roles of IL-17 and TNF in mice,

Hepatology 67 (2017) 736–749.
[7] D. Schuppan, M. Ruehl, R. Somasundaram, E.G. Hahn, Matrix as a modulator of hepatic fibrogenesis, Semin. Liver Dis. 21 (3) (2001) 351–372.
[8] K. Bottcher, K. Rombouts, F. Saffioti, D. Roccarina, M. Rosselli, A. Hall, T. Luong, E.A. Tsochatzis, D. Thorburn, M. Pinzani, MAIT cells are chronically activated in patients with autoimmune liver disease and promote profibrogenic hepatic stellate cell activation, Hepatology 68 (1) (2018) 172–186.
[9] I. Mederacke, C.C. Hsu, J.S. Troeger, P. Huebener, X. Mu, D.H. Dapito, J.P. Pradere, R.F. Schwabe, Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology, Nat. Commun. 4 (2013) 2823.
[10] J.P. Pradere, J. Kluwe, S. De Minicis, J.J. Jiao, G.Y. Gwak, D.H. Dapito, M.K. Jang, N.D. Guenther, I. Mederacke, R. Friedman, A.C. Dragomir, C. Aloman, R.F. Schwabe, Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice, Hepatology 58 (4) (2013) 1461–1473.
[11] H.W. Wang, L. Shi, Y.P. Xu, X.Y. Qin, Q.Z. Wang, Hesperetin alleviates renal interstitial fibrosis by inhibiting tubular epithelial-mesenchymal transition in vivo and in vitro, Exp. Ther. Med. 14 (4) (2017) 3713–3719.
[12] R. Kong, N. Wang, H. Luo, J. Lu, Hesperetin mitigates bile duct ligation-induced liver fibrosis by inhibiting extracellular matrix and cell apoptosis via the TGF-beta1/Smad pathway, Curr. Mol. Med. 18 (1) (2018) 15–24.
[13] E. Seyedrezazadeh, S. Kolahian, A.A. Shahbazfar, K. Ansarin, M. Pour Moghaddam, M. Sakhinia, E. Sakhinia, M. Vafa, Effects of the flavanone combination hesperetin-naringenin, and orange and grapefruit juices, on airway inflammation and remodeling in a murine asthma model, Phytother. Res. 29 (4) (2015) 591–598.
[14] X. Chen, H.W. Ding, H.D. Li, H.M. Huang, X.F. Li, Y. Yang, Y.L. Zhang, X.Y. Pan, C. Huang, X.M. Meng, J. Li, Hesperetin derivative-14 alleviates inflammation by activating PPAR-gamma in mice with CCl4-induced acute liver injury and LPS-treated RAW264.7 cells, Toxicol. Lett. 274 (2017) 51–63.
[15] M. Verdelho Machado, A.M. Diehl, The hedgehog pathway in nonalcoholic fatty liver disease, Crit. Rev. Biochem. Mol. Biol. 53 (3) (2018) 264–278.
[16] J.J. Yang, H. Tao, J. Li, Hedgehog signaling pathway as key player in liver fibrosis: new insights and perspectives, Expert Opin. Ther. Targets 18 (9) (2014) 1011–1021.
[17] J. Hyun, S. Wang, J. Kim, K.M. Rao, S.Y. Park, I. Chung, C.S. Ha, S.W. Kim, Y.H. Yun, Y. Jung, MicroRNA-378 limits activation of hepatic stellate cells and liver fibrosis by suppressing Gli3 expression, Nat. Commun. 7 (2016) 10993.
[18] J. Kim, S. Wang, C. Lee, S. Sung, Y. Shin, K.S. Song, H.J. Cha, M. Ock, Y. Jung, Blood-stage Plasmodium Berghei ANKA infection promotes hepatic fibrosis by enhancing hedgehog signaling in mice, Cell. Physiol. Biochem. 50 (4) (2018) 1414–1428.
[19] M. Sabol, D. Trnski, V. Musani, P. Ozretic, S. Levanat, Role of GLI transcription factors in pathogenesis and their potential as new therapeutic targets, Int. J. Mol. Sci. 19 (9) (2018).
[20] K.W. Kinzler, B. Vogelstein, The GLI gene encodes a nuclear protein which binds specific sequences in the human genome, Mol. Cell. Biol. 10 (2) (1990) 634–642.
[21] S. Kar, M. Deb, D. Sengupta, A. Shilpi, S.K. Bhutia, S.K. Patra, Intricacies of hedgehog signaling pathways: a perspective in tumorigenesis, Exp. Cell Res. 318 (16) (2012) 1959–1972.
[22] X. Chen, W.X. Li, Y. Chen, X.F. Li, H.D. Li, H.M. Huang, F.T. Bu, X.Y. Pan, Y. Yang, C. Huang, X.M. Meng, J. Li, Suppression of SUN2 by DNA methylation is associated with HSCs activation and hepatic fibrosis, Cell Death Dis. 9 (10) (2018) 1021.
[23] X. Lin, L.N. Kong, C. Huang, T.T. Ma, X.M. Meng, Y. He, Q.Q. Wang, J. Li, Hesperetin derivative-7 inhibits PDGF-BB-induced hepatic stellate cell activation and proliferation by targeting Wnt/beta-catenin pathway, Int. Immunopharmacol. 25 (2) (2015) 311–320.
[24] Y.H. Paik, K. Iwaisako, E. Seki, S. Inokuchi, B. Schnabl, C.H. Osterreicher, T. Kisseleva, D.A. Brenner, The nicotinamide adenine dinucleotide phosphate oxidase (NOX) homologues NOX1 and NOX2/gp91(phox) mediate hepatic fibrosis in mice, Hepatology (Baltimore, Md) 53 (5) (2011) 1730–1741.
[25] Y. Yang, X.Q. Wu, W.X. Li, H.M. Huang, H.D. Li, X.Y. Pan, X.F. Li, C. Huang, X.M. Meng, L. Zhang, X.W. Lv, H. Wang, J. Li, PSTPIP2 connects DNA methylation to macrophage polarization in CCL4-induced mouse model of hepatic fibrosis, Oncogene 37 (47) (2018) 6119–6135.
[26] R. Dong, Y. Yang, Z. Shen, C. Zheng, Z. Jin, Y. Huang, Z. Zhang, S. Zheng, G. Chen, Forkhead box A3 attenuated the progression of fibrosis in a rat model of biliary atresia, Cell Death Dis. 8 (3) (2017) e2719.
[27] H.Y. Lan, P. Hutchinson, G.H. Tesch, W. Mu, R.C. Atkins, A novel method of microwave treatment for detection of cytoplasmic and nuclear antigens by flow cytometry, J. Immunol. Methods 190 (1) (1996) 1–10.
[28] X.M. Meng, X.R. Huang, A.C. Chung, W. Qin, X. Shao, P. Igarashi, W. Ju, E.P. Bottinger, H.Y. Lan, Smad2 protects against TGF-beta/Smad3-mediated renal fibrosis, J. Am. Soc. Nephrol. 21 (9) (2010) 1477–1487.
[29] J. Fu, M. Rodova, S.K. Roy, J. Sharma, K.P. Singh, R.K. Srivastava, S. Shankar, GANT-61 inhibits pancreatic cancer stem cell growth in vitro and in NOD/SCID/IL2R gamma null mice xenograft, Cancer Lett. 330 (1) (2013) 22–32.
[30] T. Mazumdar, J. Devecchio, A. Agyeman, T. Shi, J.A. Houghton, Blocking hedgehog survival signaling at the level of the GLI genes induces DNA damage and extensive cell death in human colon carcinoma cells, Cancer Res. 71 (17) (2011) 5904–5914.
[32] J.L. Poo, G. Feldmann, A. Moreau, C. Gaudin, D. Lebrec, Early colchicine administration reduces hepatic fibrosis and portal hypertension in rats with bile duct ligation, J. Hepatol. 19 (1) (1993) 90–94.
[33] A. Floreani, S. Lobello, M. Brunetto, V. Aneloni, M. Chiaramonte, Colchicine in chronic hepatitis B: a pilot study, Aliment. Pharmacol. Ther. 12 (7) (1998) 653–656.
[34] V. Kumar, G. Mondal, R. Dutta, R.I. Mahato, Co-delivery of small molecule hedgehog inhibitor and miRNA for treating liver fibrosis, Biomaterials 76 (2016) 144–156.
[35] J. Hu, G. Cao, X. Wu, H. Cai, B. Cai, Tetramethylpyrazine inhibits activation of hepatic stellate cells through hedgehog signaling pathways in vitro, Biomed. Res. Int. 2015 (2015) 603067.
[36] J.Y. Kim, K.J. Jung, J.S. Choi, H.Y. Chung, Modulation of the age-related nuclear factor-kappaB (NF-kappaB) pathway by hesperetin, Aging Cell 5 (5) (2006) 401–411.
[37] L. Ye, F.L. Chan, S. Chen, L.K. Leung, The citrus flavonone hesperetin inhibits growth of aromatase-expressing MCF-7 tumor in ovariectomized athymic mice, J. Nutr. Biochem. 23 (10) (2012) 1230–1237.
[38] B. Morin, L.A. Nichols, K.M. Zalasky, J.W. Davis, J.A. Manthey, L.J. Holland, The citrus flavonoids hesperetin and nobiletin differentially regulate low density lipoprotein receptor gene transcription in HepG2 liver cells, J. Nutr. 138 (7) (2008) 1274–1281.
[39] J.P. Iredale, R.C. Benyon, J. Pickering, M. McCullen, M. Northrop, S. Pawley, C. Hovell, M.J. Arthur, Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors, J. Clin. Invest. 102 (3) (1998) 538–549.
[40] E. Kaffe, R. Fiorotto, F. Pellegrino, V. Mariotti, M. Amenduni, M. Cadamuro, L. Fabris, M. Strazzabosco, C. Spirli, Beta-catenin and interleukin-1beta-dependent chemokine (C-X-C motif) ligand 10 production drives progression of disease in a mouse model of congenital hepatic fibrosis, Hepatology 67 (5) (2018) 1903–1919.
[41] S.I. Chung, H. Moon, H.L. Ju, K.J. Cho, D.Y. Kim, K.H. Han, J.W. Eun, S.W. Nam, S. Ribback, F. Dombrowski, D.F. Calvisi, S.W. Ro, Hepatic expression of sonic hedgehog induces liver fibrosis and promotes hepatocarcinogenesis in a transgenic mouse model, J. Hepatol. 64 (3) (2016) 618–627.
[42] A. Omenetti, S. Choi, G. Michelotti, A.M. Diehl, Hedgehog signaling in the liver, J. Hepatol. 54 (2) (2011) 366–373.
[43] E.F. Moshai, L. Wemeau-Stervinou, N. Cigna, S. Brayer, J.M. Somme, B. Crestani, A.A. Mailleux, Targeting the hedgehog-glioma-associated oncogene homolog pathway inhibits bleomycin-induced lung fibrosis in mice, Am. J. Respir. Cell Mol. Biol. 51 (1) (2014) 11–25.