Hepatocyte-specific Smad4 deficiency alleviates liver fibrosis via the p38 / p65 pathway

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Abstract

Liver fibrosis is a wound healing response caused by the abnormal accumulation of extracellular matrix, which is produced by activated hepatic stellate cells (HSCs). Most studies have focused on the activated HSCs themselves in liver fibrosis, whether hepatocytes can modulate the process of fibrosis is still unclear. Sma mothers against decapentaplegic homologue 4 (Smad4) is a key intracellular transcription mediator of transforming growth factor-β (TGF-β) during the development and progression of liver fibrosis. However, the role of hepatocyte Smad4 in the development of fibrosis is poorly elucidated. Here, to explore the functional role of hepatocyte Smad4 and the molecular mechanism in liver fibrosis, CCl_4-induced liver fibrosis model was established in mice with hepatocyte-specific Smad4 deletion ($Smad4^{\Delta hep}$). We found that hepatocyte-specific Smad4 deficiency reduced liver inflammation and fibrosis, alleviated epithelial-mesenchymal transition, and inhibited hepatocyte proliferation and migration. Molecularly, Smad4 deletion in hepatocytes suppressed the expression of inhibitor of differentiation 1 (ID1) and the secretion of connective tissue growth factor (CTGF) of hepatocytes, which subsequently activated the p38 and p65 signaling pathways of HSCs in an epidermal growth factor receptor-dependent manner. Taken together, our results clearly demonstrate that the Smad4 expression in hepatocytes plays important role in promoting liver fibrosis and could therefore be a promising target for future anti-fibrotic therapy.

Key Messages

- The expression of Smad4 in hepatocytes was significantly up-regulated in CCl_4-induced liver fibrosis mice.
- Hepatocyte-specific Smad4 deficiency reduced liver inflammation and fibrosis.
- Hepatocyte-specific Smad4 deficiency alleviated epithelial-mesenchymal transition and inhibited hepatocyte proliferation and migration.
- Smad4 promoted expression of ID1 and the secretion of CTGF of hepatocytes, which subsequently activated the p38 and p65 signaling pathways of HSCs in an epidermal growth factor receptor-dependent manner.

Introduction

Liver fibrosis is a wound healing response caused by the abnormal accumulation of extracellular matrix (ECM) in various chronic liver diseases, including viral hepatitis, alcoholic liver disease (ALD), nonalcoholic steatohepatitis (NASH), autoimmune liver disease (AILD), metabolic liver disease and schistosomiasis infection [1–4]. If the fibrotic process is highly progressive, fibrosis can develop into cirrhosis, which accounts for approximately one million deaths per year worldwide [5]; or even hepatocellular carcinoma (HCC) [6], which is the fourth leading cause of cancer related death globally [7]. Therefore, it is important to understand the molecular mechanisms underlying liver fibrosis to improve the prevention and treatment of liver fibrosis and HCC.

In the process of liver fibrosis, ECM mainly comes from activated myofibroblasts [2]. Activated HSCs are the main source of myofibroblasts. Therefore, HSCs activation has been identified as a central driver of liver fibrosis by promoting ECM accumulation [8]. In normal liver, quiescent HSCs (qHSCs) reside in the space of Disse, where they store vitamins. However, persistent liver injury and subsequent inflammatory responses upregulate multiple factors, including cytokines, chemokines, reactive oxygen species (ROS) and iron, which can stimulate HSCs activation and proliferation. Unlike qHSCs, activated HSCs (aHSCs) express alpha smooth muscle actin (α-SMA) and secret ECM components [8, 9]. Thus, inhibiting HSCs activation may be an effective strategy for anti-fibrotic therapy.

In addition to activated HSCs, myofibroblasts in liver fibrosis may originate from epithelial cells including hepatocytes and bile duct cells [10]. Zeisberg et al. demonstrated that hepatocytes can participate in the process of liver fibrosis through epithelial mesenchymal transformation (EMT) in CCl_4 treated transgenic mouse model [11]. Furthermore, studies have shown that EMT of hepatocytes to myofibroblasts is considered to be a key process in liver fibrosis [10, 12].

The liver possesses a rich cellular environment that is mainly composed of parenchymal cells and nonparenchymal cells, which jointly regulate fibrosis formation and regression [13]. Hepatocytes, which are the dominant parenchymal cell type in the liver, actively coordinate the profibrogenic response [14, 15] and play a key role in the process of liver fibrosis [2, 16]. Damaged hepatocytes are "promoters" that participate in the initiation and persistence of HSCs activation by releasing various compounds, such as ROS, cytokines, chemokines, and growth factors [17]. In addition, pro-apoptotic signals induce hepatocyte apoptosis that closely correlates with liver inflammation and HSCs activation. HSCs and Kupffer cells phagocytose hepatocyte-derived apoptotic bodies, thereby enhancing the expression of profibrogenic genes and death ligands, such as FasL [8, 18, 19].

The transforming growth factor (TGF-β) superfamily plays an important role in the development of liver fibrosis and intracellular TGF-β signal transduction is mediated by Smad proteins [20]. The eight members of the mammalian Smad family are divided into distinct classes: receptor-regulated Smad (Smad1, 2, 3, 5, and 8), common Smad (Smad4) and inhibitory Smad (Smad6 and Smad7) [20, 21]. Smad4 is a core mediator of the TGF-β signaling pathway, which interactions with MAPK, PI3K/AKT, NF-κB and WNT/β-catenin signaling pathway. In addition, Smad4 plays a pivotal role in the switch of TGF-β function in liver fibrosis and inflammation [22]. In chronic hepatitis C, liver tissues display higher Smad4 immunopositivity. The expression level of Smad4 in hepatocytes of advanced liver fibrosis stage was higher than that in hepatocytes of early liver fibrosis stage [23]. Similarly, Qin et al. found that hepatocyte-specific Smad4 deletion inhibited lipogenesis, alleviated inflammation and apoptosis in NASH [24]. Yang et al. also confirmed that Smad4 deficiency in hepatocytes weakened spontaneous liver injury, inflammation, fibrosis, and HCC in mice with hepatocyte-specific TAK1 deletion [25]. Although Smad4 deletion in LX-2 cells led to the decreased expression of fibrotic genes, including collagen type I (*Col1a1*), *α-SMA, TGF-β* and tissue inhibitor of metalloproteinases 1 (*TIMP1*) [26], Wang et al. found no significant defects in Smad4 mutant mice before 8 months of age, and only some fibrosis and neutrophil accumulation in the livers over 8 months of age [27]. The malignant progression of liver fibrosis can lead

to the occurrence of HCC. Some evidence demonstrated that knockdown of Smad4 inhibited cell migration and invasion in HCC [28, 29]. Although many studies have investigated the role of Smad4 in liver diseases, the function and mechanism of hepatocyte Smad4 during the early liver fibrosis remains unclear.

In this study, we established a mouse model of hepatocyte-specific Smad4 deletion to explore the functional role and molecular mechanism of Smad4 in liver fibrosis. Our results showed that Smad4 deletion in hepatocytes decreased CCl_4-induced liver fibrosis by regulating the expression of inhibitor of differentiation 1 (ID1) and the secretion of connective tissue growth factor (CTGF) in hepatocytes. Furthermore, hepatocyte-specific Smad4 deletion promoted HSCs activation via the p38/p65 pathway in an epidermal growth factor receptor (EGFR)-dependent manner. Together, our findings demonstrated that Smad4 expression in hepatocytes can promote fibrosis during the pathogenesis of early hepatic fibrosis.

Materials And Methods

Animals

Albumin-Cre (Alb-Cre) and $Smad4^{flox/flox}$ ($Smad4^{fl/fl}$) mice on a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME, USA) [30]. Mice with a conditional Smad4 knockout in Alb-expressing hepatocytes ($Smad4^{\Delta hep}$) were generated by crossing $Smad4^{fl/fl}$ and Alb-Cre mice. All mice genotypes were verified by PCR for three times before subsequent experiments. 8 to 10 weeks old male $Smad4^{\Delta hep}$ mice and control littermate mice were used for the experiments. All animal experiments were performed after being approved by the Institutional Laboratory Animal Care and Use Committee of College of Science, Beijing Jiaotong University. All mice were housed under specific pathogen-free conditions with a 12 h light/dark cycles in humidity 40%-70% and at an ambient temperature 18-26 ℃. Mice were fed regularly with diet pellets and had free access to water.

Carbon tetrachloride (CCl_4)-induced acute liver fibrosis model

To induce acute liver fibrosis, mice were injected intraperitoneally with CCl_4 mixed with corn oil (1:9, Sigma-Aldrich, St. Louis, MO, USA) at a dose of 0.5 μL CCl_4/g body weight twice weekly for 4 weeks, and control mice were injected intraperitoneally with the same dose of corn oil[6]. Twenty-four hours after the final CCl_4 injection, mice were sacrificed and their tissues were harvested.

Immunohistochemistry and immunofluorescence analysis

Paraffin-embedded and frozen sections of liver tissues were prepared as described previously [6]. For immunohistochemical analysis, paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and Sirius Red respectively. For immunofluorescence detection, paraffin sections were incubated with anti-Albumin (Affinity Biosciences, OH, USA), anti-Smad4 (Santa Cruz Biotechnology, Shanghai, China), anti-α-SMA (Abcam, Cambridge, UK), anti-collagen I (Affinity Biosciences, OH, USA) and anti-PCNA (Santa Cruz Biotechnology, Shanghai, China) primary antibodies respectively; frozen sections were

incubated with anti-F4/80, anti-CD11b and anti-Gr-1 primary antibodies (BD Pharmingen, San Diego, CA), respectively, and followed by incubation with Alexa Fluor 488- or 594-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA). Cell nuclei were stained with DAPI. The results were evaluated under the microscope (DP71, OLYMPUS). Image J software was used to quantify the collagen deposition in Sirius Red staining and the positive areas in immunohistochemistry and immunofluorescence, which were presented in the form of percentage.

Western blotting analysis

Western blotting was performed as described previously [31]. Briefly, cells and liver tissue samples were collected and lysed in RIPA buffer supplemented with a protease inhibitor cocktail (Solarbio, Beijing, China). Protein concentration was measured using a BCA protein assay kit (LABLEAD, Beijing, China). Proteins were separated by 10% SDS-PAGE gel at 115 V for 1.2 h, then were transferred to a PVDF membrane at 200 mA for 1 h. The membranes were blocked with 5% milk in TBST for 1 h and incubated overnight at 4°C with primary antibodies. Primary antibodies included anti-Smad4, anti-α-SMA, anti-GAPDH, anti-E-cadherin, anti-ID1, anti-CTGF, anti-p65, anti-p-p65, anti-p38 and anti-p-p38 (Affinity Biosciences, OH, USA). Followed by HRP-conjugated goat anti-mouse and goat anti-rabbit IgG (Solarbio, Beijing, China) were used as secondary antibodies. Protein bands were scanned using a Clinx Science Instrument and quantified with Image J software.

Isolation of mouse primary hepatocytes

Primary mouse hepatocytes were isolated using a two-step collagenase digestion and gradient centrifugation method, as described previously [32]. Filtered cells were centrifuged at 50×g for 3 min to collect hepatocytes, which were then resuspended in 10 mL DMEM and placed on top of 40% percoll and centrifuged at 800×g for 10 min. The hepatocyte fraction at the bottom of the layers was collected and cell viability was examined by Trypan blue exclusion. Both the cell purity and viability were greater than 90%.

Cell culture

Mouse primary hepatocytes were cultured in William's E medium (Gibco, Grand Island US) supplemented with 10% fetal bovine serum (FBS, BI, Israel) and 1% penicillin/streptomycin. AML-12 hepatocyte cells (ATCC, Manassas, VA, USA) were cultured in DMEM/F12 medium (BI, Israel) supplemented with 10% FBS, 1% penicillin/streptomycin, 1% insulin-transferrin-selenium (ITS, Procell, Wuhan, China) and 40 ng/mL dexamethasone (Solarbio, Beijing, China). The human HSC LX-2 cell line was purchased from Xiangya Medical Collage (Changsha, China). LX-2 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C with 5% CO_2. AML-12 cells were treated with 5 ng/mL TGF-β1 (Sino Biological, Beijing, China) for 24 h. LX-2 cells were treated with 200 ng/mL CTGF recombinant protein (rCTGF, Cloud-Clone Corp, Wuhan, China) or 10 μM Erlotinib (MCE, NJ, USA) for 72 h.

Small Interfering RNA (siRNA) interference

Smad4-targeting siRNA (si-Smad4) and control siRNA (si-NC) were purchased from GenePharma (Suzhou, China). AML-12 cells were transfected with 53.3 nM siRNA using si-mate transfection reagent (GenePharma, Suzhou, China) according to the manufacturer's protocols.

Quantitative Real-Time polymerase chain reaction (qPCR)

Total RNA was isolated from liver tissues and cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. cDNA was synthesized using a Primescript RT Master Mix Kit (MCE, Princeton, NJ, USA). qPCR was performed in duplicate with a SYBR Premix Ex TaqTM Kit (MCE, Princeton, NJ, USA). Data were analysed using the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH expression.

Cell viability analysis

AML-12 cells were cultured in a 96-well plate at a density of 5×10^3 cells per well and then transfected with si-Smad4 and stimulated with 5 ng/mL TGF-β1 after 48 h. The viability was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method according to the manufacturer's protocols. The OD value of cells was analysed at 12 h and 24 h, respectively.

Wound-healing assay

AML-12 cells were cultured in 6-well plate and transfected with si-Smad4, and allowed to grow until confluent. The cell layer was scratched with a 200 μL pipette tip. After scratching, cells were washed with serum-free medium, and incubated in complete DMEM/F12 media with 5 ng/mL TGF-β1. The scratch areas were photographed at 0 h and 24 h, respectively. Quantification of wound-healing was performed using Image J software.

Flow cytometry analysis

Flow cytometry was performed as described previously [33]. Single-cell suspensions were collected from liver tissues and spleen tissues, and incubated with the following directly labeled mouse-specific monoclonal antibodys FITC-labeled anti Gr1, APC-labeled anti F4/80, Percp-labeled anti CD11b and APC-labeled anti Gr1. Cells were collected on a FACSCalibur (BD Biosciences, San Diego, CA) and analysed by FlowJo software (TreeStar, Ashland, OR).

RNA sequencing analysis

To explore potential genes involved in liver fibrosis, RNA sequencing analysis was performed as described previously [34]. Total RNA of liver fibrosis tissues from $Smad4^{fl/fl}$ and $Smad4^{\Delta hep}$ mice (n = 2 per group) was extracted with RNeasy Mini Kit (QIAGEN, Dusseldorf, Germany). RNA-sequencing analysis was performed using the BGISEQ-500 sequencer platform by BGI (Shenzhen, China). Differentially expressed genes (DEGs) were identified with a p value of <0.01, and an absolute log2 Ratio of ≥ 1. The Kyoto encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed by using phyper in R. All analyses were conducted on the Dr Tom network platform of BGI (http://report.bgi.com).

Public database analysis

The expression of *Id1* and *Ctgf* in clinical samples from hepatitis, cirrhosis patients and healthy individuals was analysed using raw gene expression data (GSE89377) [35], downloaded from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo).

Statistical analysis

All data were showed as the mean ± SD and were analysed using GraphPad Prism software. Significant differences between mean values were obtained using three independent experiments. Differences between the two groups were compared using two-tailed unpaired Student's t-test analysis. One-way ANOVA tests with a Bonferroni correction were used for multiple comparisons. Statistically significant was set at p 0.05.

Results

Smad4 expression is upregulated in hepatocytes during liver fibrosis.

To investigate the functional role of Smad4 in liver fibrosis, C57BL/6 mice were administered CCl_4 to establish liver fibrosis model, and liver tissues were harvested at 24 h after the last CCl_4 injection (Fig. 1a). Western blotting analysis of Smad4 expression in liver tissues revealed that Smad4 expression was significantly upregulated in mice with liver fibrosis compared with control mice (Fig. 1b, c). Consistently, double immunofluorescence staining further indicated that Smad4 was highly expressed in hepatocytes in liver fibrosis tissues (Fig. 1d). Collectively, these results demonstrated that Smad4 expression is significantly enhanced in hepatocytes during the progression of liver fibrosis.

Hepatocyte-specific Smad4 deficiency attenuates liver fibrosis.

To identify the role of hepatocyte Smad4 in liver fibrosis, transgenic mice expressing Cre recombinase from the Albumin promoter were crossed with $Smad4^{fl/fl}$ mice to achieve hepatocyte-specific Smad4 ablation ($Smad4^{\Delta hep}$). $Smad4^{\Delta hep}$ mice were born at the expected Mendelian ratio. The $Smad4^{fl/fl}$ littermates were used as control mice. The knockout of Smad4 in hepatocytes from $Smad4^{\Delta hep}$ mice was confirmed by double immunofluorescence staining (Fig. 2a, Fig. S1a). H&E and Sirius Red staining demonstrated that inflammatory cell infiltration and collagen deposition decreased in the liver tissues of CCl_4-treated $Smad4^{\Delta hep}$ mice compared with those in $Smad4^{fl/fl}$ mice (Fig. 2b, c; Fig. S1b, c). Consistently, lower collagen I expression was observed in CCl_4-treated $Smad4^{\Delta hep}$ mice liver (Fig. 2d). Moreover, the infiltration of $F4/80^+$ macrophages, $CD11b^+$ macrophages and $Gr1^+$ neutrophils were markedly lower in liver tissues from $Smad4^{\Delta hep}$ mice compared with those from $Smad4^{fl/fl}$ mice following CCl_4 treatment (Fig. S2).

Immunofluorescence staining and qRT-PCR results indicated that hepatocyte-specific Smad4 deletion downregulated the expression of α-SMA (Fig. 2e, f), suggesting that Smad4 deficiency in hepatocytes

might alleviate the activation of HSCs. In addition, Smad4 deficiency in hepatocytes affected the expression of fibrosis-related genes, dramatically reducing the expression of Col1a1 and TIMP1 while increasing the expression of matrix metalloproteinase 9 (MMP9) at the mRNA level (Fig. 2f). Consistently, western blotting results indicated that α-SMA expression was lower in the liver tissues of CCl_4-treated Smad4$^{\Delta hep}$ mice than that in control mice (Fig. 2g). Taken together, these findings suggested that Smad4 knockout in hepatocytes attenuate CCl_4-induced liver fibrosis.

Hepatocyte-specific Smad4 deficiency reduces cell proliferation and EMT

To confirm whether Smad4 affect the proliferation of hepatocytes, PCNA and Albumin in the liver tissues of Smad4$^{\Delta hep}$ and Smad4$^{fl/fl}$ mice were determined by immunofluorescence double staining. The results showed that the proliferation of hepatocytes in CCl_4-treated Smad4$^{\Delta hep}$ mice was significantly decreased compared with that in Smad4$^{fl/fl}$ mice (Fig. 3a). To further elucidate the role of Smad4 in hepatocytes, we used siRNA to knock down Smad4 in AML-12 cells, and then treated the cells with TGF-β1, an effective inducer of liver fibrosis environment in vitro [36], for 12 h and 24 h. To detect the proliferation and migration ability of AML-12 cells by MTT and wound healing assays, we found that Smad4 deletion remarkably inhibited the proliferation and migration of AML-12 cells after 24 h (Fig. 3b-d). It has been reported that EMT of hepatocytes can not only partly become the source of myofibroblasts and promote liver fibrosis [11, 37], but also promote the motility of hepatocytes [38]. Therefore, we speculated that Smad4 might play a role in the EMT of hepatocytes. As expected, we observed much higher expression of E-cadherin in the liver tissues of CCl_4-treated Smad4$^{\Delta hep}$ mice than that in Smad4$^{fl/fl}$ mice (Fig. 3e). Consistently, western blotting results also confirmed that siRNA-mediated Smad4 knockdown blocked TGF-β1-induced E-cadherin downregulation in AML-12 cells (Fig. 3f). Thus, these findings collectively demonstrated that the knockout of Smad4 in hepatocytes suppressed their proliferation and EMT during liver fibrosis.

Hepatocyte-specific Smad4 deficiency reduced ID1 and CTGF expression

To elucidate the detailed changes of gene expression between Smad4$^{\Delta hep}$ and Smad4$^{fl/fl}$ mice, we performed protein-coding mRNA-sequencing analysis of liver tissues derived from CCl_4-induced liver fibrosis mice. A total of 149 DEGs were identified, including 99 upregulated and 50 downregulated genes (Fig. 4a). The top 30 DEGs involved in the occurrence of liver fibrosis were selected and displayed as the heat map, which revealed that ID1 expression was markedly decreased in CCl_4-treated Smad4$^{\Delta hep}$ mice compared with that in Smad4$^{fl/fl}$ mice (Fig. 4b, c). It was reported that the upregulation of ID1 in hepatocytes was accompanied by the upregulation of CTGF expression [39]. To further verify this result, we analysed a public GEO dataset (GSE89377) and found that compared to healthy individuals, the expression of ID1 and CTGF was dramatically increased in the liver tissues of patients with hepatitis and cirrhosis, (Fig. 4e, f). Therefore, we speculated that ID1 and CTGF may play important roles in liver fibrosis of Smad4$^{\Delta hep}$ mice and Smad4$^{fl/fl}$ mice.

To further explore the interaction between Smad4 and ID1, we detected the expression of Albumin and ID1 in the liver tissues of Smad4$^{\Delta hep}$ mice using double immunostaining. As shown in Fig. 5a, a lower expression of ID1 was observed in Albumin^{+} hepatocytes from CCl_4-treated Smad4$^{\Delta hep}$ mice. Consistently, western blotting results confirmed that the expression of ID1 and CTGF was dramatically down-regulated in the liver tissues of Smad4$^{\Delta hep}$ mice compared with those in Smad4$^{fl/fl}$ mice (Fig. 5b). To further verify the above results, we used si-Smad4 to knock down Smad4 in AML-12 cells, followed by TGF-β1 stimulation. qRT-PCR and western blotting analysis revealed that the expression of ID1and CTGF in AML-12 cells was markedly reduced after Smad4 interference (Fig. 5c-e, 5f), consistent with in vivo results. Collectively, these results indicate that Smad4 deficiency in hepatocytes decrease their expression of ID1 and CTGF, which may be involved in the process of liver fibrosis.

CTGF promotes HSCs activation via p38/p65 signaling

Since HSCs activation is a major event in the pathogenesis of liver fibrosis[2], we further explored the underlying mechanism by which Smad4 expression in hepatocytes affected the activation of HSCs. First, AML-12 cells were treated with si-Smad4 and si-NC respectively, followed by stimulation with TGF-β1. Their culture supernatants were collected as conditioned medium (CM). HSCs were incubated with the above CM for 24 h. In the TGF-β1-free CM treatment groups, the expression of *α-SMA* and *Col1a1* in HSCs was not significantly affected by the absence of Smad4 (Fig. 6a, b). Although the CM of TGF-β1-induced AML-12 cells activated the expression of *α-SMA* and *Col1a1* in HSCs, the CM of TGF-β1-induced AML-12 cells with Smad4 knockdown significantly attenuated the expression of *α-SMA* in HSCs, which was consistent with the results of western blotting analysis (Fig. 6c). During liver fibrosis, hepatocytes in damaged liver and hepatocytes cultured in vitro express a large amount of CTGF, which increases the pro-fibrotic effect of TGF-β [40, 41]. To investigate whether Smad4 in hepatocytes can promote the activation of HSCs through regulating CTGF, LX-2 cells were treated with exogenous recombinant protein CTGF (rCTGF) at different concentrations for 24 h. Interestingly, the results indicated that the expression level of α-SMA significantly increased in a concentration-dependent manner in LX-2 cells after rCTGF treatment (Fig. 6d). Recent studies have reported that CTGF plays an important role in renal fibrosis by binding to EGFR on the cell surface [42, 43], we speculated that CTGF may activate HSCs in an EGFR-dependent manner. Therefore, we cultured LX-2 cells with rCTGF and the EGFR-specific inhibitor (Erlotinib) and found that Erlotinib obviously inhibited CTGF-mediated HSCs activation (Fig. 6e).

To determine the molecular mechanism via which hepatocyte-derived CTGF acts on HSCs to promote fibrosis, we further analysed the RNA sequencing results and found that the proteins correlated with MAPK signaling pathway were markedly downregulated in Smad4$^{\Delta hep}$ mice (Fig. 4d). The p38-MAPK and p65-NF-κB pathways have been reported to play key roles in the process of liver fibrosis [44-46]. Consistently, we found that the expression of phosphorylated p38 (p-p38) and p65 (p-p65) in the livers of CCl_4-treated Smad4$^{\Delta hep}$ mice was lower than that in Smad4$^{fl/fl}$ mice (Fig. 6f). In addition, after LX-2 cells were treated with rCTGF, the phosphorylation of p38 and p65 were distinctly enhanced, whereas erlotinib

suppressed this effect (Fig. 6g). Taken together, these results suggest that CTGF promotes HSCs activation through EGFR receptor-mediated p38 and p65 pathways during liver fibrosis.

Discussion

Smad4 is a core mediator of the TGF-β signaling pathway that can interact with Smad2/3 to transmit upstream Smad signals and promote the occurrence and development of liver fibrosis [20]; however, the specific contribution of hepatocyte Smad4 expression during liver fibrosis remains unclear. Here, we used a mouse model of hepatocyte-specific Smad4 deletion to explore its role and molecular mechanism in liver fibrosis. Notably, we found that hepatocyte-specific Smad4 deletion alleviated CCl_4-induced liver fibrosis, and suppressed hepatocyte proliferation and EMT. Furthermore, Smad4 was able to regulate ID1 expression and CTGF secretion in hepatocytes to activate the p38 and p65 signaling pathways in HSCs and thereby promote HSCs activation (Fig. 7).

Accumulating evidence has shown that the dysregulation of the TGF-β1/Smad pathway is a major contributor in the pathogenesis of liver inflammation, fibrosis and HCC. Thus, the imbalance of Smad signal plays an important role in the development of liver fibrosis [47–49]. Additionally, studies have reported that Smad4-mediated signal transduction in different cell types plays different roles in liver fibrosis. For instance, some studies have reported that Smad4 deficiency in hepatocytes does not affect liver development, but gradually results in iron overload and the infiltration of inflammatory cells in the liver and other organs of mice [27, 50]. However, others demonstrated that Smad4 deletion in HSCs attenuated their activation and reduced the expression of pro-fibrotic genes [26]. Qin et al found that the expression of inflammatory markers, fibrotic markers, and lipogenic genes was significantly lower in the liver tissue of hepatocyte-specific Smad4-deficient NASH mice than that in wild-type mice [24]. Yang et al. confirmed that Smad4 deletion in hepatocytes after knocking out TAK1 inhibited the apoptosis of hepatocytes and decreased serum ALT levels, while simultaneously alleviated liver inflammation, fibrosis, and HCC [25]. Xu et al. reported that the expression of fibrotic genes such as *TIMP1* and *TGF-β* in Smad4-knockout mice was dramatically lower than that in WT mice, suggesting that the TGF-β1/Smad signal transduction system was downregulated [51]. Together, our findings support this conclusion and demonstrate that hepatocyte-specific Smad4 deletion reduces CCl_4-induced liver fibrosis.

Hepatocytes, as the most abundant parenchymal cells in the liver, are the initial cells that affect the process of liver fibrosis. During serious liver injury, hepatocytes lose the ability of regeneration and undergo necrosis, apoptosis, or senescence, while activated myofibroblasts in the liver to secrete ECM proteins [52]. Hepatocytes can be transformed into myofibroblasts through EMT, which is an important source of myofibroblasts in the process of liver fibrosis [11]. Some studies have shown that inhibiting the EMT of hepatocytes can reduce liver fibrosis [53, 54]. Importantly, Kaimori et al. reported that TGF-β1 could induce EMT in AML-12 cells in vitro, whereas Smad4 knockdown in AML-12 cells inhibited EMT [37]. Consistently, we found that Smad4 deletion in hepatocytes alleviated EMT and preserved the expression of the epithelial marker, E-cadherin, which suggested that the absence of Smad4 in hepatocytes attenuated the development of liver fibrosis. However, Taura et al. also demonstrated that

hepatocytes did not undergo EMT during liver fibrosis [55], therefore, the function and mechanism of EMT in hepatocytes during liver fibrosis still needs to be further explored.

Damaged hepatocytes secrete inflammatory factors (e.g., IL-33 and NLRP3) and fibrotic factors (e.g., TGF-β1 and CTGF) that are involved in HSCs activation and promote liver inflammation and fibrosis [8, 56, 57]. In this study, we found that the expression of ID1 and CTGF in hepatocytes was markedly downregulated in hepatocyte-specific Smad4 deletion mice with liver fibrosis. ID1 is mainly correlated with tumorigenesis, cell senescence, cell proliferation and survival, and is overexpressed in various cancer cells and can promote tumor development through different signaling pathways[58]. Moreover, Young et al. reported that *Id1* mRNA level was significantly upregulated in liver biopsy specimens from chronic hepatitis C patients, and that phosphorylated Smad1/5 and ID1 expression were dramatically enhanced in HCV-infected hepatoma cells [59]. Meanwhile, Yin et al. also found that *Id1* deletion inhibited cell proliferation and sensitized oxaliplatin-resistant HCC cells to death [60]. Interestingly, liver-specific Smad4 knockout also markedly weakened ID1 expression[61], which is consistent with our findings.

CTGF is a strongly fibrogenic molecule that is overexpressed in fibrotic organs, including liver, lung, kidney, and heart [57]. Kodama et al. previously demonstrated that p53 overexpression in hepatocytes could promote the expression of CTGF to increase hepatocyte apoptosis and spontaneous liver fibrosis [62]. Similarly, Makino et al. found that upregulated CTGF expression was positively correlated with the clinical malignancy of HCC, and that CTGF-specific knockout in HepG2 reduced the size and number of liver tumors. Thus, CTGF derived from HCC appears to be a key factor in activating nearby HSCs and relaying pro-growth signals to HCC [57]. Additionally, CTGF is also reported to be a downstream mediator of TGF-β and its expression is enhanced when stimulating hepatocytes with TGF-β [63]. Here, we verified the correlation between ID1, CTGF and hepatitis, cirrhosis in clinical cases by analyzing the expression of *Id1* and *Ctgf* in 20 healthy individuals, 14 hepatitis patients and 13 cirrhosis patients using the GEO dataset GSE89377. As expected, we found that the expression of *Id1* and *Ctgf* was distinctly increased in patients with hepatitis and cirrhosis.

HSCs activation is a key step in the development of liver fibrosis. As the main effector cells of the fibrosis response, HSCs are particularly important autocrine or paracrine targets, especially in the activated state [8, 63]. Liao et al. demonstrated that ID1 and MAPK signaling pathways were downstream of CTGF signaling, and ID1 partially upregulated CTGF through positive feedback [39]. Here, our results indicated that Smad4 expression in hepatocytes could activate HSCs through improving CTGF secretion and thereby promoted liver fibrosis. Huang and Brigstock also confirmed that CTGF could promote liver fibrosis by promoting proliferation, survival, migration, adhesion and ECM production of activated HSCs [64]. This result is consistent with our findings. It was recently reported that CTGF could regulate renal inflammation, cell growth, and fibrosis by binding to EGFR [42, 43]. Therefore, we speculated that CTGF derived from hepatocytes might stimulate the activation of HSCs via EGFR. Interestingly, our results showed that the presence of Erlotinib (EGFR inhibitor) attenuated HSCs activation stimulated by CTGF, and that the p38/MAPK signaling pathway was downregulated in CCl_4-induced liver fibrotic tissues of $Smad4^{\Delta hep}$ mice. Consistently, Fuchs et al. also found that the erlotinib could inhibit the activation of

HSCs by reducing EGFR phosphorylation in HSCs [65]. p38 is known to play an important role in the process of liver fibrosis [39] and the activation of p38α/MAPK promotes hepatocyte proliferation and chronic liver inflammation [44]. Yan et al. confirmed that phosphorylated p38 was upregulated in activated HSCs [45]. Some studies suggested that p38/MAPK was associated with the inflammatory signaling pathway p65/NK-κB in chronic hepatitis and HCC [46]. Consistently, our study demonstrated that hepatocyte-derived CTGF increased the phosphorylation of p38 and p65 and promoted HSCs activation through EGFR, thereby contributing to liver fibrosis.

In conclusion, our research indicated that Smad4 expression in hepatocytes was closely involved in the development of liver fibrosis. Notably, Smad4 deletion in hepatocytes alleviated CCl_4-induced liver fibrosis and decreased inflammatory cell infiltration in liver tissues. Molecularly, Smad4 expression in hepatocytes upregulated the expression of ID1, and further enhanced the paracrine activity of CTGF, subsequently, mediated by EGFR, CTGF promoted HSCs activation by regulating the p38 and p65 signaling pathway, which in turn led to liver fibrosis. However, we used CCl_4 to induce short-term fibrosis, and the functional role of hepatocyte Smad4 in long-term liver fibrosis needs to be further studied. Collectively, Smad4 may represent a potential candidate target for the prevention and targeted therapy in liver fibrosis.

Declarations

Supplementary Materials: Figure S1: Detection of Smad4-specific deletion in hepatocytes of $Smad4^{\Delta hep}$ and $Smad4^{fl/fl}$ mice. Figure S2: Smad4 deficiency in hepatocytes attenuates liver inflammatory response.

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Competing interests

None.

Data availability statement

All relevant data are presented in this paper.

Ethical Approval

All animal experiments were approved by the Institutional Laboratory Animal Care and Use Committee of College of Science, Beijing Jiaotong University. The institutional Ethics approval code/number is as follows: BJTU2019-01.

Author contributions

Jinhua Zhang, Lingling Hou and Miaomiao Wei participated in the study design; Miaomiao Wei, Xin Xin and Haiqiang Chen performed the experiments. Miaomiao Wei, Xinlong Yan, Xin Xin and Lingling Hou analysed and interpreted the data. Miao miao Wei, Lingling Hou, Jinhua Zhang and Xinlong Yan wrote the manuscript. All authors read and approved the final paper.

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Figures

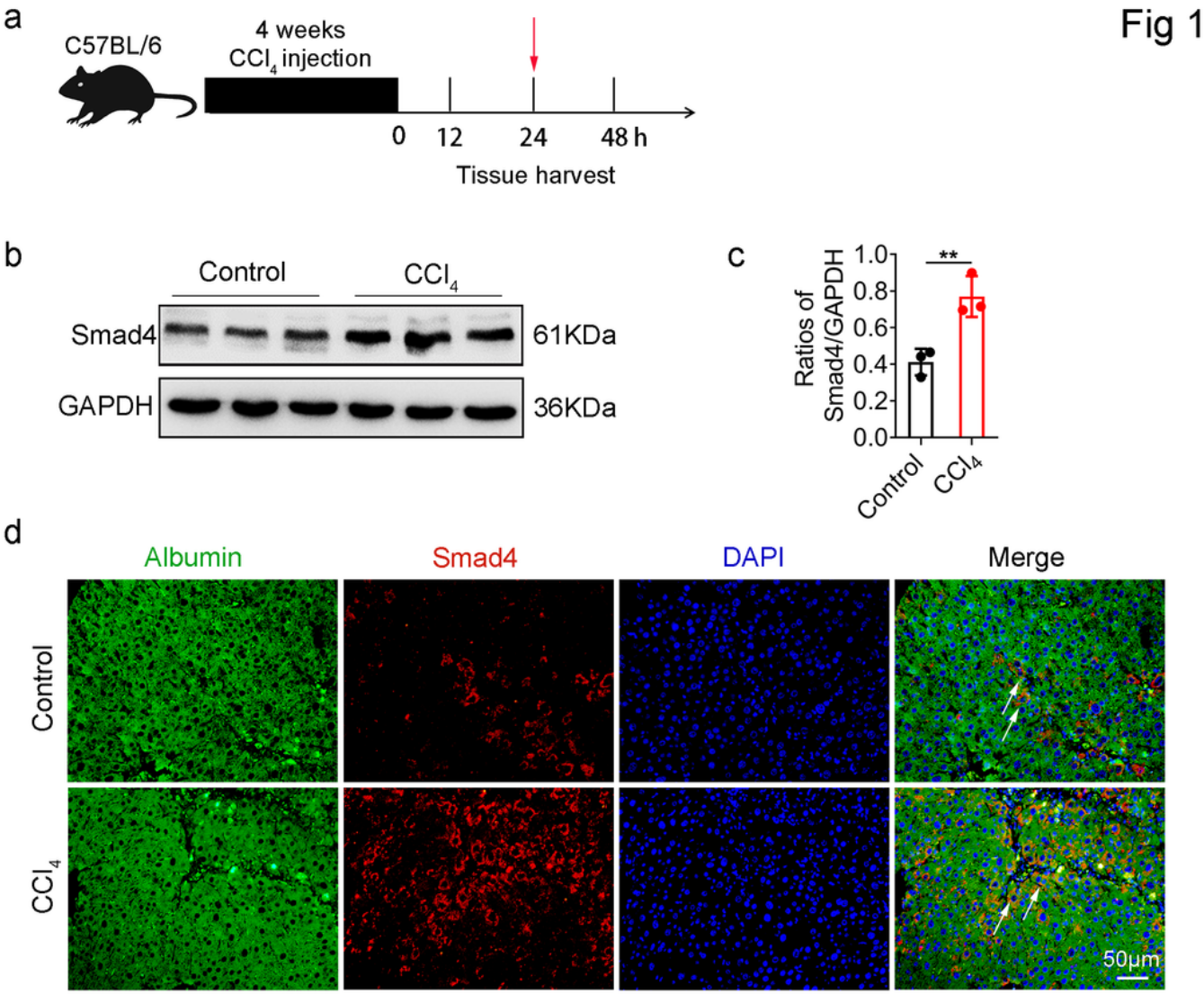

Figure 1

Smad4 expression is upregulated in hepatocytes during liver fibrosis.

Groups of C57BL/6 mice were treated with CCl_4 for 4 weeks to establish a liver fibrosis model (n = 6 per group). Data represent at least three independent experiments. (**a**) Schematic representation of CCl_4-induced liver fibrosis. (**b-c**) Western blotting analysis of Smad4 protein levels in liver fibrosis tissues.

Smad4 expression was normalized to the control GAPDH. (**d**) Double staining of albumin (green) and Smad4 (red) in liver fibrosis tissues (scale bars: 50 μm). Arrowheads indicated the double-positive cells. *p <0.05, **p <0.01.

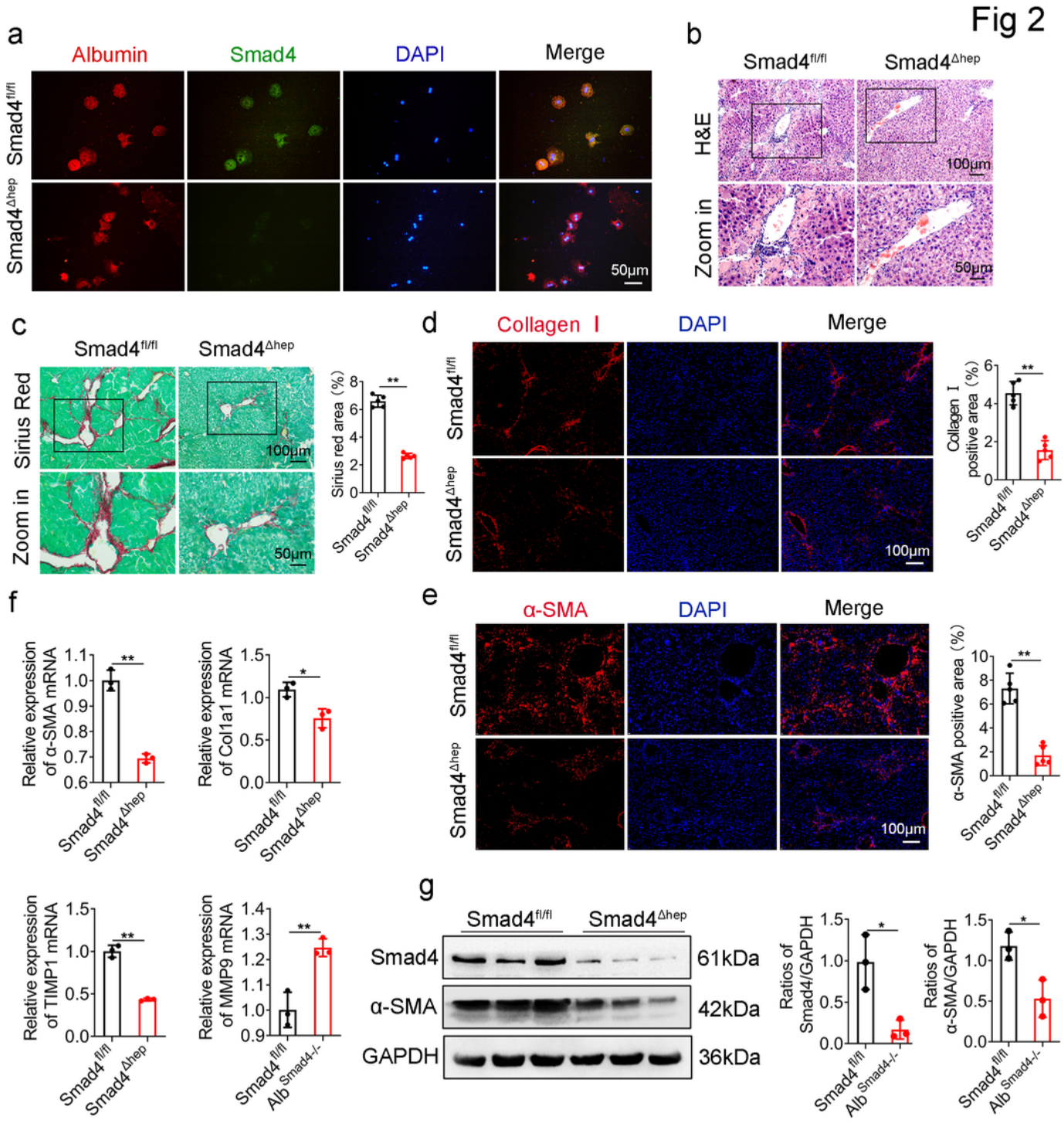

Figure 2

Hepatocyte-specific Smad4 deficiency attenuates liver fibrosis.

Smad4$^{fl/fl}$ and Smad4$^{\Delta hep}$ mice were treated with CCl_4 for 4 weeks to establish a liver fibrosis model (n = 6 per group). (**a**) Double staining of Albumin (red) and Smad4 (green) in primary hepatocytes (scale bars: 50 μm). (**b**) H&E staining of fibrotic liver tissues (scale bars: 100 μm, zoom in: 50 μm). (**c**) Sirius Red staining of fibrotic liver tissues (scale bars: 100 μm, zoom in: 50 μm), quantification of stained areas and statistical analysis. (**d-e**) Immunofluorescence detection of Collagen Ⅰ and α-SMA in fibrotic liver tissues (scale bars: 100 μm), quantification of stained areas and statistical analysis. (**f**) The mRNA levels of *α-SMA, Col1a1, TIMP1* and *MMP9* in fibrotic liver tissues were measured using qRT-PCR analysis. (**g**) Western blotting analysis of Smad4 and α-SMA protein levels in fibrotic liver tissues. Protein density was quantified using densitometry. α-SMA and Smad4 levels were normalized to GAPDH. *p <0.05, **p <0.01.

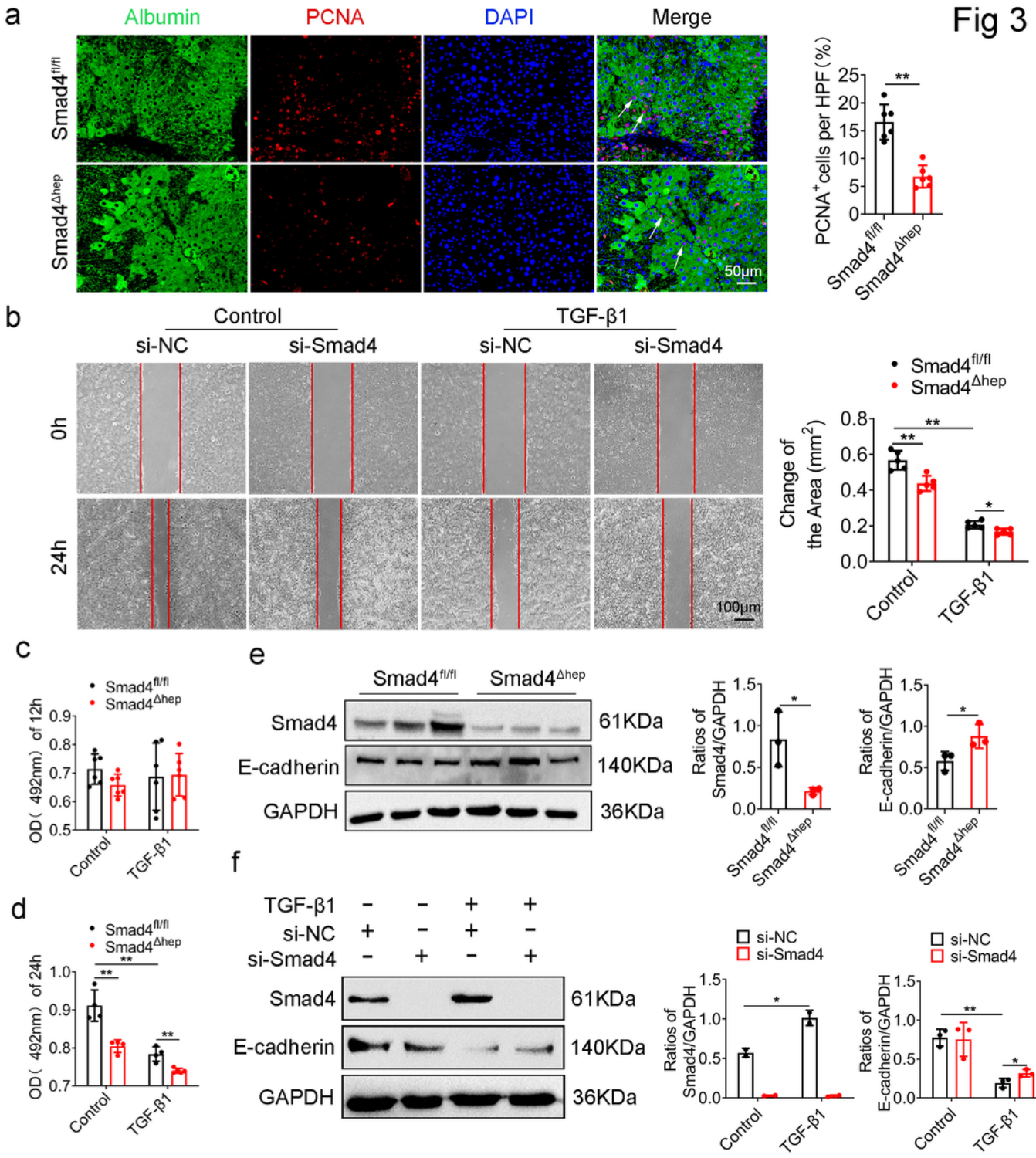

Figure 3

Hepatocyte-specific Smad4 deficiency reduces cell proliferation and EMT.

$Smad4^{fl/fl}$ and $Smad4^{\Delta hep}$ mice were treated with CCl_4 for 4 weeks to establish a liver fibrosis model. AML-12 cells were transfected with control siRNA or Smad4 siRNA. (**a**) Double staining of Albumin (green) and PCNA (red) in fibrotic liver tissues (scale bars: 50 μm) and statistical analysis. Arrowheads

indicated the double-positive cells. (**b**) Representative photographs of wound-healing assay and statistical analysis. AML-12 cells were scratched using pipet tips and treated with TGF-β1 (5 ng/mL) for 24 h. The migration ability of AML-12 cells was evaluated. (**c-d**) AML-12 cells after Smad4 deletion were treated with TGF-β1 (5 ng/mL) for 12 h or 24 h. The MTT assays showed that the proliferation ability of AML-12 cells. (**e**) Smad4 and E-cadherin expression in fibrotic liver tissues of CCl_4-treated Smad4$^{\Delta hep}$ mice were analysed by western blotting. Smad4 and E-cadherin were normalized to GAPDH. (**f**) Smad4 in AML-12 cells were knocked down by siRNA and then treated with TGF-β1(5 ng/mL) for 24 h, Smad4 and E-cadherin expression were analysed by western blotting and normalized to GAPDH. *p <0.05, **p <0.01.

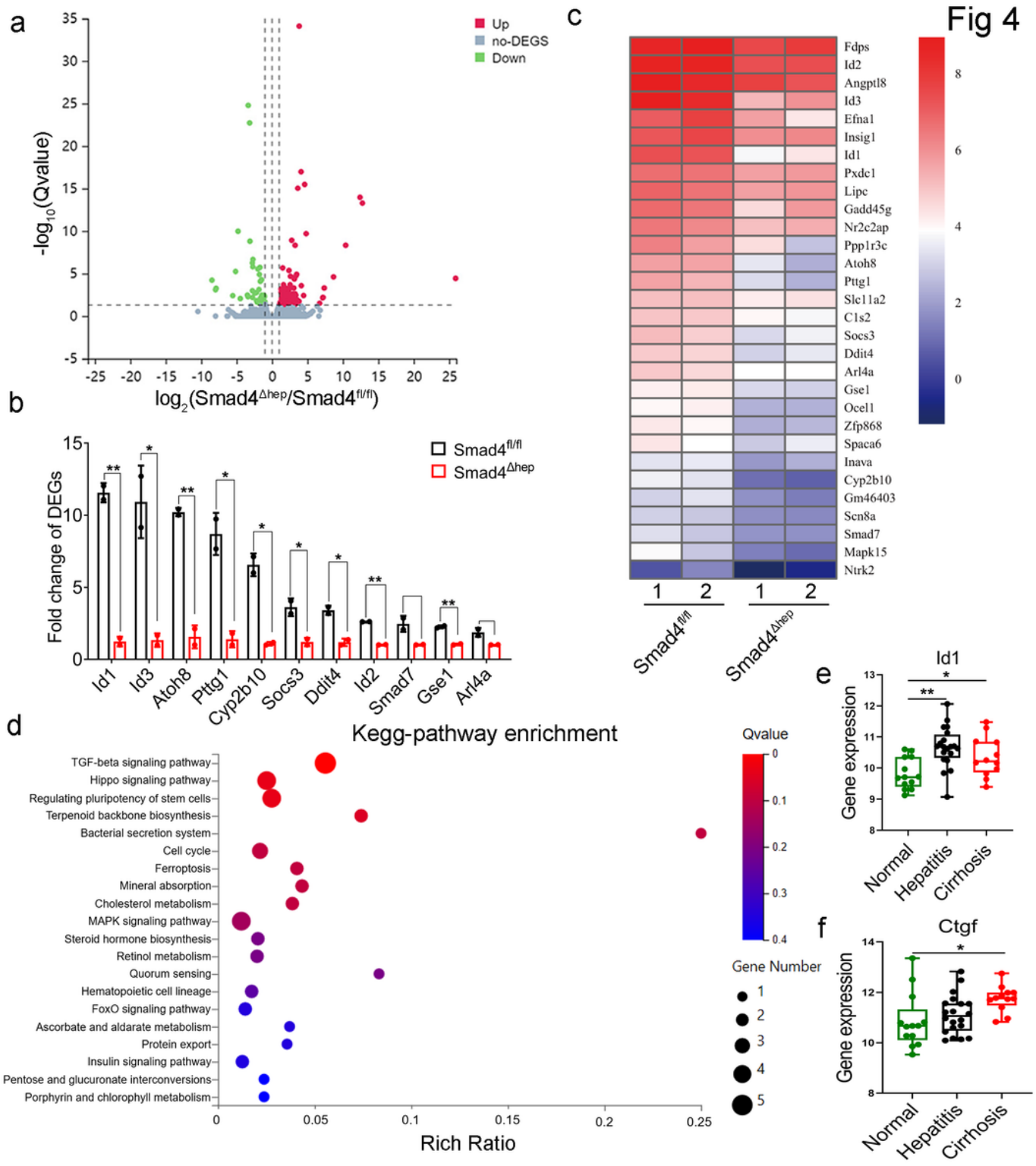

Figure 4

RNA sequencing analysis of DEGs in CCl_4-induced liver fibrosis tissues.

$Smad4^{fl/fl}$ and $Smad4^{\Delta hep}$ mice were treated with CCl_4 for 4 weeks to establish the liver fibrosis model. The fibrotic liver tissues of $Smad4^{fl/fl}$ and $Smad4^{\Delta hep}$ mice were analysed by RNA sequencing (n = 2 per group). (**a**) Volcano diagram of DEGs with $p < 0.05$. (**b**) Analysis of fold change in DEGs. (**c**) Heatmap of

the expression of the most significantly downregulated genes. (**d**) The analysis of the related signal pathways of downregulated genes by KEGG. (**e-f**) The analysis of ID1 and CTGF expression in liver tissues from patients with hepatitis and cirrhosis in the GSE89377 dataset. Normal, n=13; Hepatitis, n=20; Cirrhosis, n=14. *p <0.05, **p <0.01.

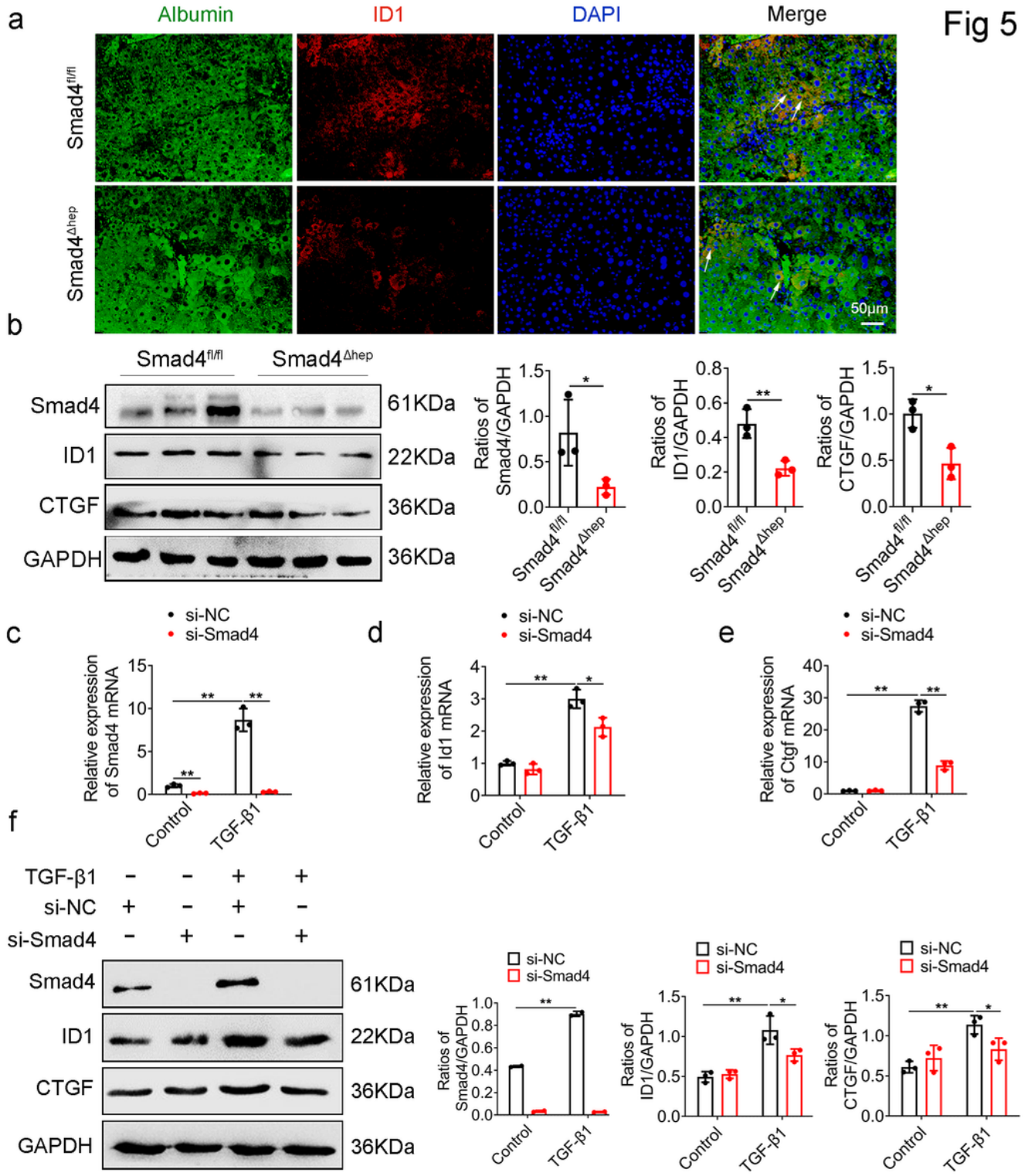

Figure 5

Hepatocyte-specific Smad4 deficiency inhibited the expression of ID1 and CTGF.

$Smad4^{fl/fl}$ and $Smad4^{\Delta hep}$ mice were treated with CCl_4 for 4 weeks to establish the liver fibrosis model (n = 6 per group). AML-12 cells were transfected with control siRNA or Smad4 siRNA respectively and then treated with TGF-β1 (5 ng/mL) for 24 h. (**a**) Double staining of Albumin (green) and ID1 (red) in fibrotic liver tissues (scale bars: 50 μm) and statistical analysis. Arrowhead indicated the double-positive cells. (**b**) Western blotting analysis of protein levels of Smad4, ID1, and CTGF in fibrotic liver tissues. Smad4, ID1, and CTGF were normalized to GAPDH. (**c-e**) The mRNA levels of Smad4, ID1, and CTGF in AML-12 cells were measured using real-time PCR analysis. (**f**) Western blot analysis of protein levels of Smad4, ID1 and CTGF in AML-12 cells. Smad4, ID1, and CTGF were normalized to GAPDH. *p <0.05, **p <0.01.

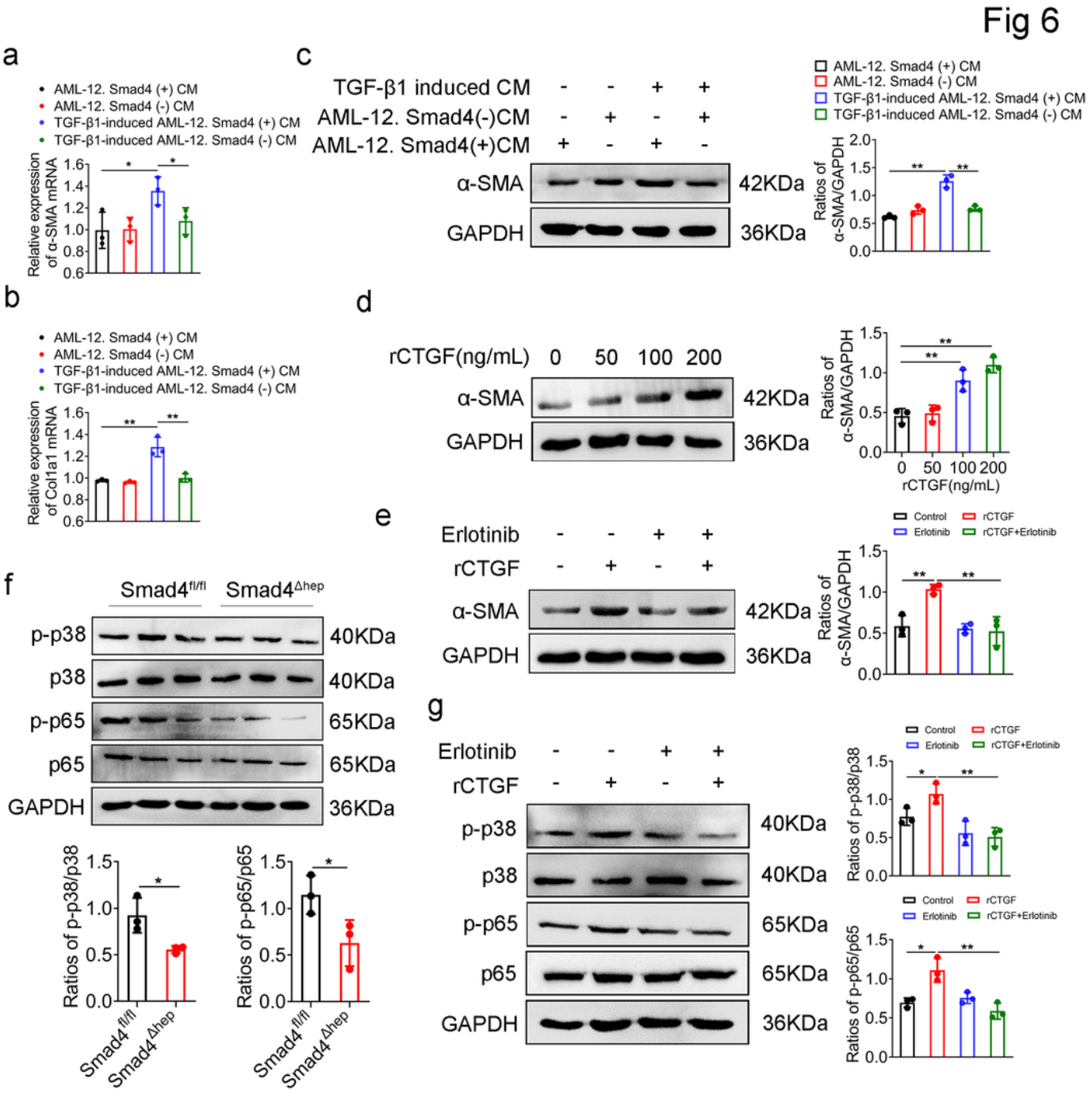

Figure 6

CTGF promotes HSCs activation via the EGFR receptor mediated p38/p65 signaling.

AML-12 cells were transfected with control siRNA or Smad4 siRNA respectively and treated with TGF-β1(5 ng/mL) for 24 h. Subsequently, the conditioned medium was collected to treat LX-2 cells. (**a-b**) The mRNA levels of α-SMA and Col1a1 in LX-2 cells were measured using real-time PCR method. (**c**) Western blotting

analysis of α-SMA protein levels in LX-2 cells. α-SMA was normalized to GAPDH. (**d**) LX-2 cells were treated with recombinant CTGF protein (0 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL) for 48 h. Western blotting analysis of protein levels of α-SMA in LX-2 cells. α-SMA was normalized to GAPDH. (**e**) LX-2 cells were treated with 200 ng/mL recombinant CTGF and 10 nM Erlotinib for 48 h. Western blotting analysis of protein levels of α-SMA in LX-2 cells. α-SMA was normalized to GAPDH. (**f**) Western blotting analysis of protein levels of p-p38, p38, p-p65, and p65 in fibrotic liver tissues. p-p38 and p-p65 were normalized to p38 and p65 respectively. (**g**) LX-2 cells were treated with 200 ng/mL recombinant CTGF and 10 nM Erlotinib for 48 h. Western blotting analysis of protein levels of p-p38, p38, p-p65, and p65 in LX-2 cells. p-p38 and p-p65 were normalized top38 and p65 respectively. *p <0.05, **p <0.01.

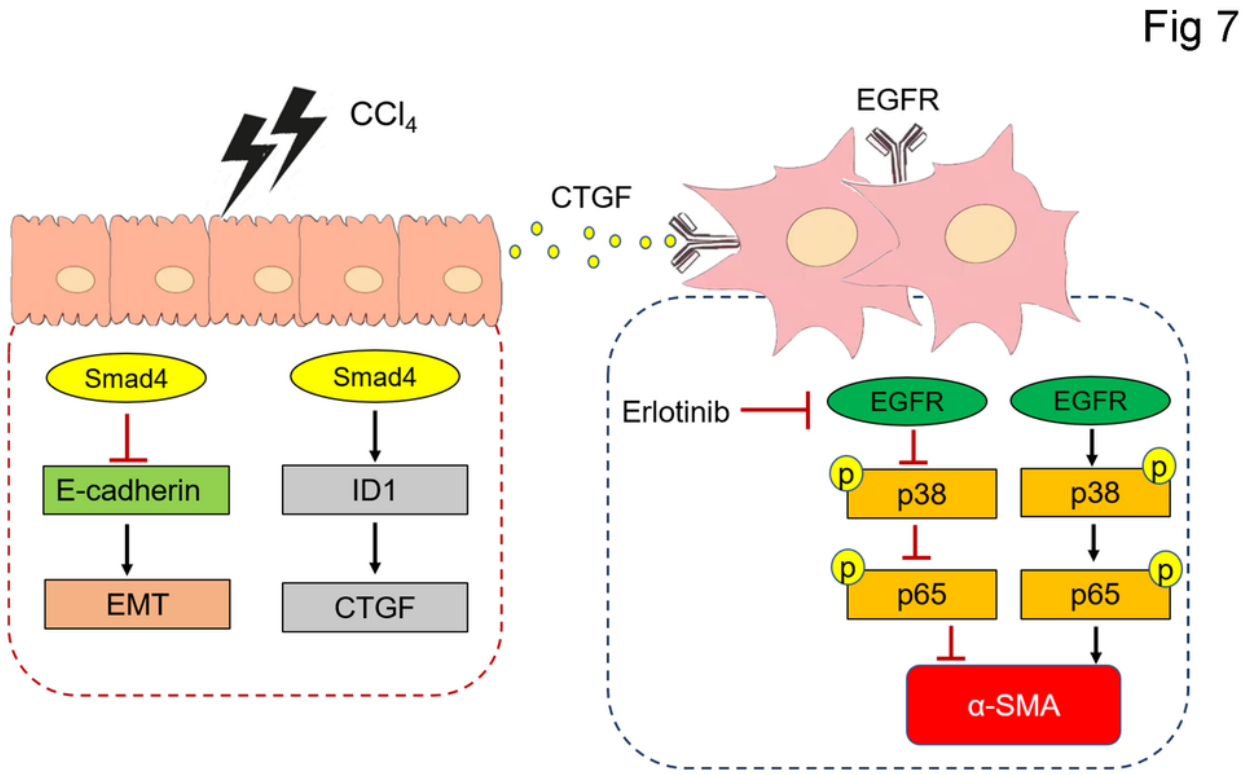

Figure 7

Schematic diagram of the mechanism via which hepatocyte-specific Smad4 deficiency alleviates liver fibrosis.

In liver fibrosis, the expression of Smad4 in hepatocytes was upregulated, leading to increased ID1 and CTGF expression in hepatocyte, thereafter secreted CTGF upregulated the phosphorylation of p38 and

p65 via the EGFR receptor to promote HSC activation and liver fibrosis.

Supplementary Files

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