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ORIGINAL ARTICLE

Loss of heat shock factor 1 promotes hepatic stellate cell activation and drives liver fibrosis

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Abstract

Liver fibrosis is an aberrant wound healing response that results from chronic injury and is mediated by hepatocellular death and activation of hepatic stellate cells (HSCs). While induction of oxidative stress is well established in fibrotic livers, there is limited information on stress-mediated mechanisms of HSC activation. Cellular stress triggers an adaptive defense mechanism via master protein homeostasis regulator, heat shock factor 1 (HSF1), which induces heat shock proteins to respond to proteotoxic stress. Although the importance of HSF1 in restoring cellular homeostasis is well-established, its potential role in liver fibrosis is unknown. Here, we show that HSF1 messenger RNA is induced in human cirrhotic and murine fibrotic livers. Hepatocytes exhibit nuclear HSF1, whereas stellate cells expressing alpha smooth muscle actin do not express nuclear HSF1 in human cirrhosis. Interestingly, despite nuclear HSF1, murine fibrotic livers did not show induction of HSF1 DNA binding activity compared with controls. HSF1-deficient mice exhibit augmented HSC activation and fibrosis despite limited pro-inflammatory cytokine response and display delayed fibrosis resolution. Stellate cell and hepatocyte-specific HSF1 knockout mice exhibit higher induction of profibrogenic response, suggesting an important role for HSF1 in HSC activation and fibrosis. Stable expression of dominant negative HSF1 promotes fibrogenic activation of HSCs. Overactivation of HSF1 decreased phosphorylation of JNK and prevented HSC activation, supporting a protective role for HSF1. Our findings identify an unconventional role for HSF1 in liver fibrosis. *Conclusion*: Our results show that deficiency of HSF1 is associated with exacerbated HSC activation promoting liver fibrosis, whereas activation of HSF1 prevents profibrogenic HSC activation.

INTRODUCTION

Liver fibrosis is characterized by excessive accumulation of extracellular matrix (ECM) leading to scar tissue formation due to hepatocellular injury caused by various etiologies such as alcohol abuse, viral hepatitis B and C, and nonalcoholic steatohepatitis (NASH).[1] Fibrotic livers can often progress to cirrhosis, which is irreversible and leads to hepatocellular carcinoma, making it one of the leading cause of mortality and morbidity in the United States.[2] Activation of hepatic stellate cells (HSCs) is the hallmark to development of fibrogenesis. In response to profibrogenic stimuli, HSCs undergo trans-differentiation and activation to a proliferative myofibroblast-like phenotype that secretes components of ECM.[3] Regardless of the etiology, fibrogenic responses are regulated by cellular stress pathways, including endoplasmic reticulum stress[4] and oxidative stress.[5] Generation of reactive oxygen species (ROS) contributes to activation of HSCs.[5] In addition, overproduction of reactive nitrogen species and hepatic glutathione are also implicated in profibrogenic activation.[6] These studies indicate the involvement of cellular stress pathways, leading us to hypothesize that stress-mediated heat shock factor 1 (HSF1) plays a crucial role in liver fibrosis.

HSF1 is a transcription factor that acts as an integrated sensory mechanism to modulate protein synthesis, folding, and quality control to facilitate cellular response to environmental stress. Exposure to cellular stressors activates HSF1 and induces heat shock proteins (HSPs) to restore homeostasis.[7] Recent studies have assigned a role for HSF1 in regulation of metabolic pathways[8] and inflammation.[9] Notably, *in vitro* studies show that HSF1 regulates HSP47, collagen-specific chaperone in stellate cells.[10,11] We previously reported an anti-inflammatory function of HSF1 in human monocytes,[12] in models of lipopolysaccharide (LPS)–induced liver injury in mice, and during chronic alcohol exposure in liver and murine macrophages.[13,14] However, the precise function of HSF1 in the pathogenesis of liver fibrosis *in vivo* and its potential impact on HSC activation remains undefined.

Here, we report increased HSF1 expression but loss of DNA binding activity in fibrotic livers. Nuclear HSF1 is observed in hepatocytes but not in activated HSCs in human cirrhotic livers. Furthermore, we demonstrate that deficiency of HSF1 *in vivo* exacerbates liver fibrosis and delays resolution. Hepatocyte-specific deletion of HSF1 exacerbates hepatocyte cell death, and similar to HSC-specific HSF1 deficiency, induces a profibrogenic response to CCl_4, suggesting its important role in HSC activation and fibrosis. We established that HSF1-deficient primary HSCs as well as stable expression of dominant negative HSF1 exhibit increased HSC activation and profibrogenic responses *in vitro*. Finally, we show that activation of HSF1 significantly ameliorates profibrogenic gene expression in HSCs via decreased phosphorylation of c-Jun NH2-terminal kinases (JNK), confirming a protective role for HSF1 in preventing HSC activation and liver fibrosis.

METHODS

Human liver samples

Normal, NASH cirrhotic, and alcoholic cirrhotic human livers were provided by the Liver Tissue Cell Distribution System (Division of Pediatric Gastroenterology and Nutrition, University of Minnesota, Minneapolis) from the patients who received transplantation. Normal liver tissue was the noninvolved surrounding tissue, obtained from patients undergoing partial hepatectomy for liver cancer (Table 1).

Study approval

The study was approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Chan Medical School. All animals received proper care

TABLE 1 Physical and biochemical parameters associated with the study's control and patients with NASH and alcoholic cirrhosis

	Control	NASH cirrhosis	Alcoholic cirrhosis
Number (male/female)	17 (12/5)	10 (4/6)	10 (8/2)
Age (years), median (range)	53.5 (38–78)	62 (48–68)	47 (38–66)
Aspartate aminotransferase (IU/L), median (range)	18 (13–51)	58 (48–89)	50.5 (31–133)
Bilirubin (mg/dl), median (range)	–	4.35 (0.7–25.1)	7.35 (1.5–20.7)
Alkaline phosphatase (IU/L), median (range)	–	213.5 (82–289)	131 (74–326)
Creatinine (mg/dl), median (range)	–	1.23 (0.46–4.98)	1.19 (0.62–2.27)
Albumin (g/dl), median (range)	–	2.7 (1.7–3.8)	2.75 (2.2–3.6)

in accordance with the *Guide for the Care and Use of Laboratory Animals* from the National Institutes of Health.

Liver injury models

Hsf1-deficient mice (*Hsf1*$^{-/-}$; male) aged 8–12 weeks old on a mixed B6/CD1 background and corresponding wild-type (WT) littermates were used for the study. Homozygous *Hsf1*-deficient mice were obtained from Dr. Ashok Saluja, University of Miami Health System, on CD1 background, backcrossed 8–10 generations on C57Bl/6, and *Hsf1*-deficient offspring were confirmed by real-time polymerase chain reaction (PCR) and electrophoretic mobility-shift assay (EMSA) (Supporting Methods). To generate stellate-specific HSF1-deficient (*Hsf1*$^{fl/fl}$ *LratCre*$^{+/-}$) mice and hepatocyte-specific HSF1-deficient (*Hsf1*$^{fl/fl}$ *AlbCre*$^{+/-}$) mice, *Hsf1*$^{fl/fl}$ were obtained from Dr. Chengkai Dai (National Cancer Institute) and cross bred with *LratCre* transgenic mice or *AlbCre* transgenic mice (Jackson Laboratory). *Hsf1*$^{fl/fl}$Cre-negative littermates served as WT controls. Cell-specific knockouts were confirmed by isolation of liver cells and messenger RNA (mRNA) expression. Fibrosis was induced by intraperitoneal injection of 25% CCl_4 (Sigma-Aldrich) in corn oil (0.5 μl/g body weight), twice a week, for either for 2 or 6 weeks.[15] Mice injected with corn oil served as controls. Acute toxic injury was induced by giving a single intraperitoneal injection of 1 μl/g body weight of 25% CCl_4, in corn oil,[16] and livers were collected after 48 h. Fibrosis was also induced by diet containing 0.1% 3,5-diethoxycarbonyl-1, 4-dihydrocollidine (DDC; Dyets Inc.) for 4 weeks.[17] Cholestatic liver fibrosis was induced by mice by common bile duct ligation (BDL) for 2 weeks as described.[18]

Supplemental methods

Supplemental methods include biochemical assays, EMSA, HSC isolation, peritoneal exudate cell (PEC) isolation and stimulation, LX-2 cell stimulations, chemical genetic modulation of HSF1 in LX-2, small interfering RNA (siRNA) transfection, quantitative PCR, western blotting, immunohistochemistry, and terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining.

Statistical analysis

Data are expressed as mean ± SEM and considered statistically significant at $p < 0.05$. Statistical analyses were performed using GraphPad Prism 8.1. Differences between two groups were assessed using a Student's *t*-test. One-way or two-way analysis of variance was used to assess differences between multiple groups.

RESULTS

HSF1 expression is increased but not activity in liver fibrosis

The role of cellular oxidative pathways in liver fibrosis is well-established.[6] Oxidative stress can induce and activate proteostasis regulator, HSF1, which acts as a protective mechanism to restore cellular homeostasis.[19] Clinical relevance of HSF1 in the pathogenesis of liver fibrosis is unknown. Here we observed that *HSF1* was increased in both alcoholic cirrhotic and NASH cirrhotic livers when compared with normal liver biopsies and positively correlated with alpha-smooth muscle actin (α*SMA*) (Figure 1A). Nuclear localization of HSF1 in the hepatocytes was observed in the cirrhotic livers (Figure 1B). Elevated *αSMA*, collagen α1(I) (*COL1A1*), and *HSP47* in the human cirrhotic livers validated the clinical features (Figure S1A,B). Transcriptional up-regulation of HSPs is a hallmark of HSF1 activation.[20] Elevated *HSP40*, *HSPA1A*, and *HSP90AA1* confirmed the presence of active HSF1 in both alcoholic and NASH cirrhotic human livers (Figure 1C).

Murine models of liver fibrosis including CCl_4 intoxication for 6 weeks[15] and methionine-choline–deficient diet for 8 weeks[21] exhibited hepatic *Hsf1* induction compared with respective controls (Figure 1D). The murine fibrotic livers also exhibited elevated *Hsp40*, *Hspa1a*, and *Hsp90aa1* (Figure 1E), similar to human cirrhotic livers (Figure 1C). While HSF1 and HSP90AA1 protein levels were elevated in fibrotic livers, HSPA1A was not significantly altered compared with controls (Figure 1F). Similar to the CCl_4 intoxicated animals, the DDC diet–fed fibrotic liver also demonstrated elevated hepatic HSF1 (Figure 1F). Interestingly, we found that DNA binding activity of HSF1 in fibrotic livers demonstrated a trend of decrease compared with the controls (Figure 1G). Acute CCl_4-induced liver injury leads to activation of early oxidative stress responses.[16] After 48 h of acute CCl_4, we observed induction of *Hsf1*, *Hsp40*, *Hspa1a*, and *Hsp90aa1* (Figure 1F), concomitant to up-regulation of *αSma*, *Col1a1*, platelet-derived growth factor receptor beta (*Pdgfrβ*), and *Hsp47* (Figure S1C). At 72 h, *Hsf1* and HSP genes decreased significantly, suggesting a transient induction of proteostasis responses (Figure 1F). Collectively, these results suggest a role for HSF1 in the pathophysiology of liver fibrosis.

Activated HSCs fail to activate HSF1-mediated proteostasis response

Activation of HSCs and differentiation to myofibroblasts is the hallmark of liver fibrosis.[3] Having observed increased HSF1 in human cirrhotic and mouse fibrotic livers, we sought to determine whether HSF1 and HSPs are induced in HSCs. Nuclear localization of

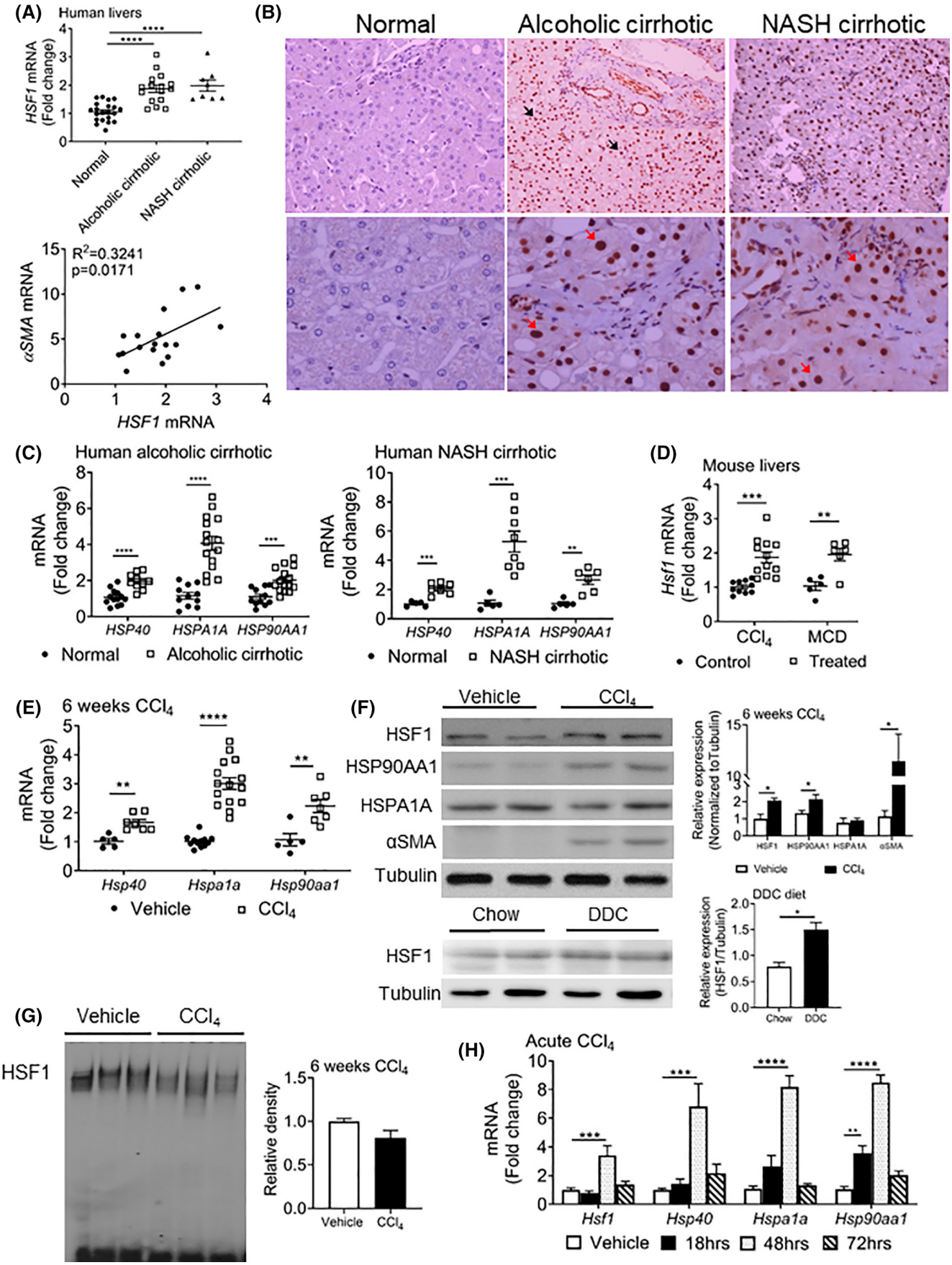

(A)
Human livers
HSF1 mRNA (Fold change)
Normal
Alcoholic cirrhotic
NASH cirrhotic
αSMA mRNA
R²=0.3241
p=0.0171
HSF1 mRNA
(B)
Normal
Alcoholic cirrhotic
NASH cirrhotic
(C)
Human alcoholic cirrhotic
mRNA (Fold change)
HSP40
HSPA1A
HSP90AA1
Normal
Alcoholic cirrhotic
Human NASH cirrhotic
NASH cirrhotic
(D)
Mouse livers
Hsf1 mRNA (Fold change)
CCl4
MCD
Control
Treated
(E)
6 weeks CCl4
Hsp40
Hspa1a
Hsp90aa1
Vehicle
CCl4
(F)
Vehicle
CCl4
HSF1
HSP90AA1
HSPA1A
αSMA
Tubulin
Relative expression (Normalized to Tubulin)
6 weeks CCl4
Chow
DDC
DDC diet
Relative expression (HSF1/Tubulin)
(G)
Vehicle
CCl4
HSF1
6 weeks CCl4
Relative density
(H)
Acute CCl4
Hsf1
Hsp40
Hspa1a
Hsp90aa1
Vehicle
18hrs
48hrs
72hrs

FIGURE 1 Heat shock factor 1 (HSF1) and heat shock proteins (HSPs) are elevated in human cirrhotic and mouse fibrotic livers. Normal livers ($n = 20$), and livers from patients with alcoholic ($n = 16$) and nonalcoholic steatohepatitis (NASH; $n = 8$) cirrhosis were assessed for expression of *HSF1* and its correlative analyses with alpha smooth muscle actin (*αSMA*; $n = 18$) (A), immunohistochemical staining for HSF1 (top panel, original magnification ×20; bottom panel, original magnification ×40) (B), and *HSP40*, *HSPA1A*, and *HSP90AA1* messenger RNA (mRNA) (C). (D) *Hsf1* in mouse fibrotic livers induced by CCl_4 and methionine-choline–deficient (MCD) diet. (E) mRNA expression of *Hsp40*, *Hspa1a*, and *Hsp90aa1*. (F) Protein levels of HSF1, HSP90AA1, HSPA1A, and α-SMA (quantitated graphs on right) and HSF1 protein level in livers of 3,5-diethoxycarbonyl-1, 4-dihydrocollidine (DDC) diet–fed animals. (G) HSF1 DNA binding activity in mice injected with CCl_4 for 6 weeks ($n = 12$–16). (H) *Hsf1*, *Hsp40*, *Hspa1a*, and *Hsp90aa1* after 18, 48, and 72 h in mice intoxicated with acute CCl_4 ($n = 4$). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

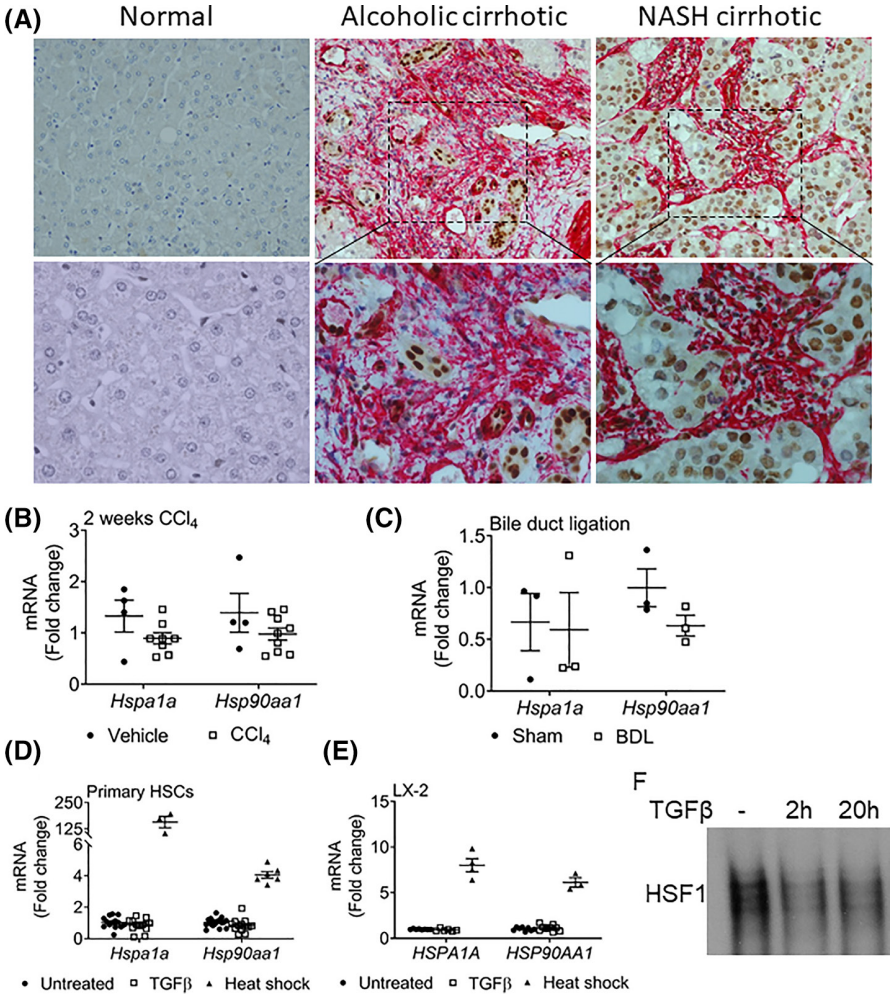


FIGURE 2 HSF1 is not activated in myofibroblast. (A) Immunohistochemical staining of HSF1 (brown nuclei) and αSMA (red) in normal, alcoholic cirrhotic, and NASH cirrhotic human livers ($n = 8$) (top panel, original magnification ×20; bottom panel, original magnification ×40). (B,C) *Hspa1a* and *Hsp90aa1* in hepatic stellate cells (HSCs) isolated from mice injected with corn oil or CCl_4 injected twice a week for 2 weeks (B) and sham or bile duct ligated (BDL) mice (C) ($n = 3$). (D,E) *Hspa1a* and *Hsp90aa1* in transforming growth factor β (TGFβ)–stimulated primary HSCs ($n = 15$) (D) and LX-2 cells ($n = 8$) (E). HSCs and LX-2 cells subjected to heat shock followed by a recovery phase of 4 h served as positive control. (F) Electrophoretic mobility-shift assay (EMSA) depicting DNA binding activity in the TGFβ-treated LX-2 cells for 2 h and 20 h.

HSF1, which confirms its stress-mediated activation,[20] was not observed in αSMA-positive cells (blue nuclei) in fibrotic septae, but was observed in hepatocytes (brown nuclei) of human cirrhotic livers (Figure 2A). Furthermore, *αSma* and *Col1a1* expressing primary HSCs isolated from mice intoxicated with CCl_4 for 2 weeks (Figure S2A), and BDL (Figure S2B) failed to express *Hspa1a* and *Hsp90aa1* (Figure 2B,C). We

also found that transforming growth factor β (TGFβ)–induced activation of HSCs, confirmed by induction of *αSma* and *Col1a1* in primary HSCs (Figure S2C) and LX-2 cells (Figure S2D), failed to induce *Hspa1a* and *Hsp90aa1* (Figure 2D,E). We found decreased DNA binding activity of HSF1 in LX-2 cells exposed to TGFβ (Figure 2F). Previous studies have reported that JNK activation can suppress HSF1 transcriptional activity.[22] We found that TGFβ mediated JNK phosphorylation in activated LX-2 cells (Figure S2E), suggesting that JNK may likely mediate inactivation of HSF1 following exposure to profibrogenic stimulus. Our data show that lack of HSF1 activation in HSCs is linked to fibrosis, implying a protective role for HSF1 in HSC activation.

Deficiency of HSF1 exacerbates liver fibrosis

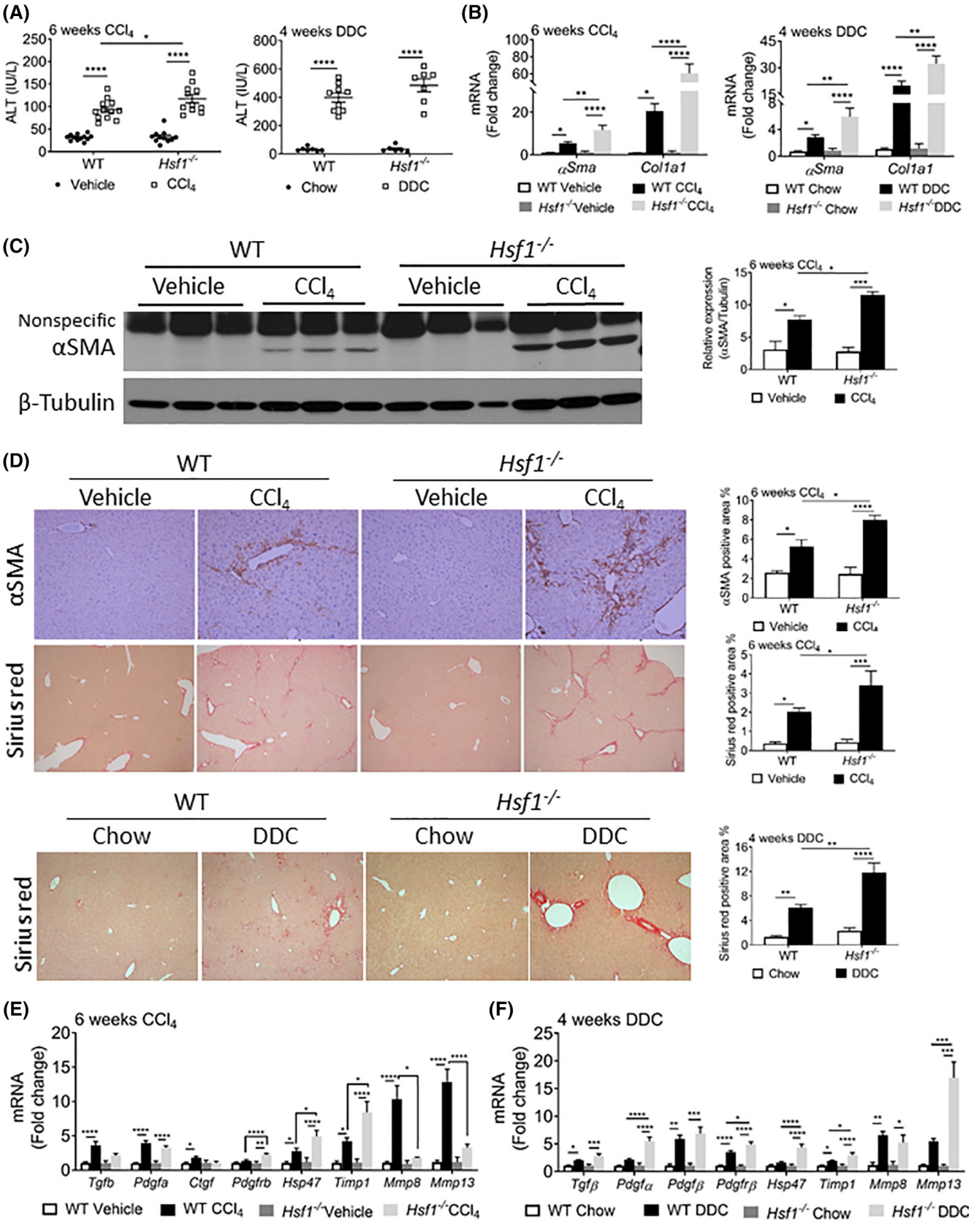
Next, we sought to understand whether deficiency of HSF1 *in vivo* would have an impact on induction of liver fibrosis. We used two models of liver fibrosis: biweekly intoxication with CCl_4 for 6 weeks or chronic feeding with a diet containing 0.1% DDC for 4 weeks in *Hsf1*$^{-/-}$ and their WT counterparts. *Hsf1*$^{-/-}$ mice were confirmed by real-time PCR (Figure S3A). Also, lack of DNA binding activity in livers of *Hsf1*$^{-/-}$ mice confirmed the absence of functional HSF1 (Figure S3B). Furthermore, CCl_4 intoxication of WT mice demonstrated elevated hepatic *Hspa1a* and *Hsp90aa1*, whereas no induction was observed in the *Hsf1*$^{-/-}$ mice (Figure S3C,D), confirming no functional activation of HSF1. Following CCl_4 intoxication, *Hsf1*$^{-/-}$ mice demonstrated higher serum alanine transaminase (ALT) (Figure 3A). Although an increase in the trend of serum ALT was observed in the *Hsf1*$^{-/-}$ DDC-fed mice, the difference between WT and *Hsf1*$^{-/-}$ mice was not statistically significant (Figure 3A). Both CCl_4 intoxication and DDC diet resulted in higher *αSma* and *Col1a1* in *Hsf1*$^{-/-}$ mice (Figure 3B). Western blotting and immunohistochemical staining revealed elevated hepatic αSMA in *Hsf1*$^{-/-}$ intoxicated with CCl_4 for 6 weeks (Figure 3C,D) and 2 weeks (Figure S3E). Furthermore, both CCl_4 intoxication and DDC diet led to higher hepatic collagen accumulation and increased bridging fibrosis in *Hsf1*$^{-/-}$ (Figure 3D).

Growth factors like *Tgfβ*, *Pdgf*, and connective tissue growth factor (*Ctgf*) induce trans-differentiation of HSCs and promote fibrosis.[3] CCl_4 intoxication resulted in increase of *Tgfβ* in *Hsf1*$^{-/-}$ fibrotic livers at 2 weeks (Figure S3F). Six weeks of CCl_4 intoxication induced *Tgfβ* and *Pdgfα* in *Hsf1*$^{-/-}$ fibrotic livers to some extent (Figure 3E). On the other hand, *Pdgfβ* was significantly increased in *Hsf1*$^{-/-}$ after acute CCl_4 intoxication (Figure S3E). Furthermore, *Hsf1*$^{-/-}$ fibrotic livers did not exhibit induction of *Ctgf* (Figure 3E). PDGFRβ expressed by activated HSCs binds to the PDGF ligands to activate mitogenic pathways in the stellate cells.[23] Our data show that *Pdgfrβ* is increased in *Hsf1*$^{-/-}$ fibrotic livers after 6 weeks (Figure 3E), acute, and 2 weeks of CCl_4 administration (Figure S3F). The collagen-specific chaperone, *Hsp47*, was also elevated in *Hsf1*$^{-/-}$ and parallels increased collagen synthesis (Figure 3E). The balance between tissue inhibitors of metalloproteinases (TIMP) and matrix metalloproteinases (MMP) determines the extent of ECM deposition during hepatic fibrosis.[24] *Hsf1*$^{-/-}$ demonstrated higher tissue inhibitor of metalloproteinase 1 (*Timp1*) (Figure 3E) and decreased *Mmp8* and *Mmp13* (Figure 3E). In the DDC diet–induced fibrosis model, *Hsf1*$^{-/-}$ mice demonstrate elevated *Tgfβ* and *Pdgfβ* (Figure 3F). In addition, *Hsf1*$^{-/-}$ fibrotic livers from DDC-fed mice showed increase in *Pdgfα*, *Pdgfβ, Pdgfrβ*, *Hsp47*, and *Timp1* (Figure 3F). However, expression of *Mmp8* and *Mmp13* remained high in these livers (Figure 3F). Collectively, our data indicate that—regardless of the etiological factors—HSF1 deficiency promotes HSC activation, profibrogenic responses, and ECM deposition.

Deficiency of HSF1 impedes resolution of fibrosis

Having established that absence of HSF1 accelerates the progression of liver fibrosis, we sought to understand whether HSF1 deficiency hinders resolution of fibrosis. WT and *Hsf1*$^{-/-}$ mice subjected to CCl_4 intoxication for 6 weeks were allowed to recover for 4 weeks. Significant reduction in αSMA was observed in WT compared with *Hsf1*$^{-/-}$ mice after 4 weeks of recovery (Figure 4A). The *Hsf1*$^{-/-}$ mice following recovery still exhibit significant ECM deposition (Figure 4B) and *Col1a1* (Figure 4C). The WT mice exhibited no induction of *Hsp47* concomitant to reduced *Col1a1*, whereas the *Hsf1*$^{-/-}$ recovery

FIGURE 3 Deficiency of HSF1 exacerbates liver fibrosis. Wild-type (WT) and *Hsf1*$^{-/-}$ mice were either injected with corn oil or CCl_4, twice weekly for 6 weeks, or fed with diet containing 0.1% DDC. (A) Hepatic injury was assessed by serum alanine aminotransferase (ALT). (B) Hepatic fibrosis was quantitated by *αSma* and collagen α1(I) (*Col1a1*) expression. (C) αSMA immunoblotting was quantified by normalizing to tubulin using ImageJ. (D) αSMA immunohistochemistry (original magnification ×10) was quantitated as percentage area demonstrating αSMA by ImageJ; sirius red staining (original magnification ×10) with graphs represent percentage fibrotic area quantitated by ImageJ. (E) Expression of *Tgfβ*, platelet-derived growth factor receptor beta (*Pdgfα*), connective tissue growth factor (*Ctgf*), *Pdgfrβ*, *Hsp47*, tissue inhibitor of metalloproteinase 1 (*Timp1*), matrix metalloproteinase 8 (*Mmp8*), and *Mmp13* in CCl_4 intoxicated mouse liver. (F) Expression of *Tgfβ*, *Pdgfα*, *Pdgfβ, Pdgfrβ*, *Hsp47*, *Timp1*, *Mmp8*, and *Mmp13* in DDC-fed mouse liver (n = 8–10 per treatment group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.



group of mice demonstrated higher *Hsp47*, correlating with higher collagen deposition (Figure 4D). *Timp1* was reduced significantly in the livers of WT, but *Hsf1*$^{-/-}$ mice displayed high *Timp1* even after a 4-week recovery (Figure 4E). Induction of *Timp1* provides pro-survival signals to activated HSCs, [25] and its expression in the

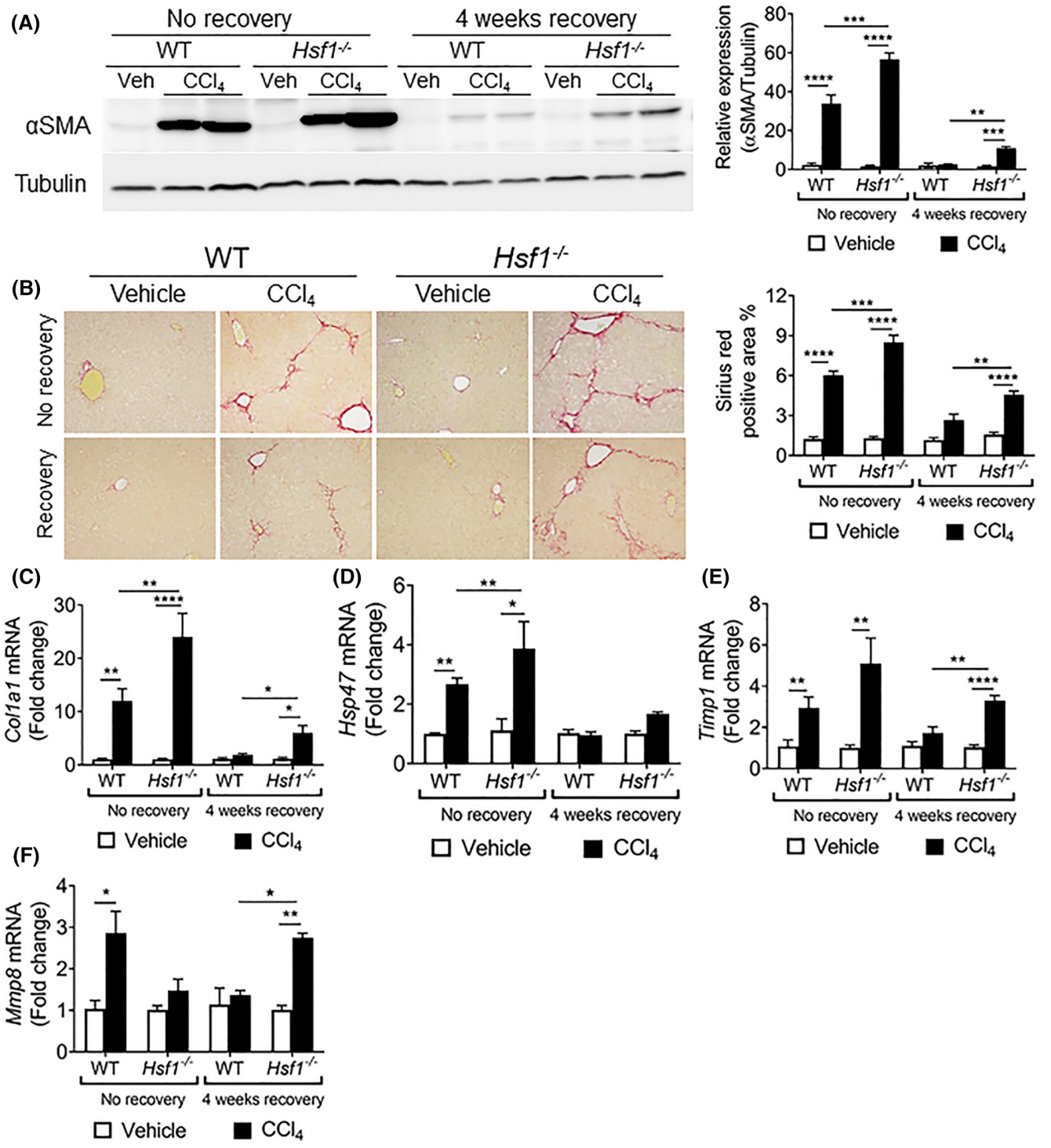


FIGURE 4 Deficiency of HSF1 delays resolution of liver fibrosis. WT and *Hsf1*$^{-/-}$ mice injected with corn oil or CCl_4 for 6 weeks were allowed to recover for 4 weeks. Fibrosis was evaluated by αSMA immunoblotting and quantified by densitometry after normalization to tubulin using ImageJ (A), sirius red staining (original magnification ×10) and quantified by ImageJ (B), and *Col1a1* (C), *Hsp47* (D), *Timp1* (E), and *Mmp8* (F) expression (n = 4–7 per treatment group per genotype). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Hsf1$^{-/-}$ livers after 4 weeks of recovery strengthens the presence of activated HSCs in these livers. Moreover, expression of *Mmp8* in the *Hsf1*$^{-/-}$ recovery group of animals suggests likely ongoing resolution after 4 weeks of recovery (Figure 4F). These results indicate that lack of HSF1 significantly hampers resolution of liver fibrosis.

Deficiency of HSF1 induces selective inflammatory response and facilitates neutrophil recruitment

Exacerbated chronic pro-inflammatory response is prerequisite for liver fibrosis.[26] Because HSF1 and HSPs interact and regulate signaling intermediates involved in innate and adaptive immune responses,[27] we evaluated inflammatory chemokines and cytokines implicated in hepatic fibrogenesis. Monocyte chemoattractant protein 1 (*Mcp1*), important in myeloid cell recruitment and crucial in fibrosis,[28] was elevated in *Hsf1*$^{-/-}$ in both the CCl_4-intoxicated (Figure 5A,C) and DDC diet–fed mice (Figure 5B). In contrast, CC-chemokine receptor 2 (CCR2), a MCP1-specific receptor,[15] was decreased in *Hsf1*$^{-/-}$ fibrotic livers in both models (Figure 5A,B). Chemokine (C-C motif) ligand 5, crucial for stellate cell migration and proliferation during liver fibrosis,[29] was induced to a similar level in both WT and *Hsf1*$^{-/-}$. Macrophage inflammatory protein I alpha (*Mip1α*), also known to promote liver fibrosis,[30] was reduced in the *Hsf1*$^{-/-}$ fibrotic livers, in both models (Figure 5A,B). Although tumor necrosis factor alpha (*Tnfα*) and interleukin (*Il)1β* were decreased in the CCl_4-intoxicated *Hsf1*$^{-/-}$ (Figure 5A), these were significantly elevated in the *Hsf1*$^{-/-}$ fibrotic livers induced by

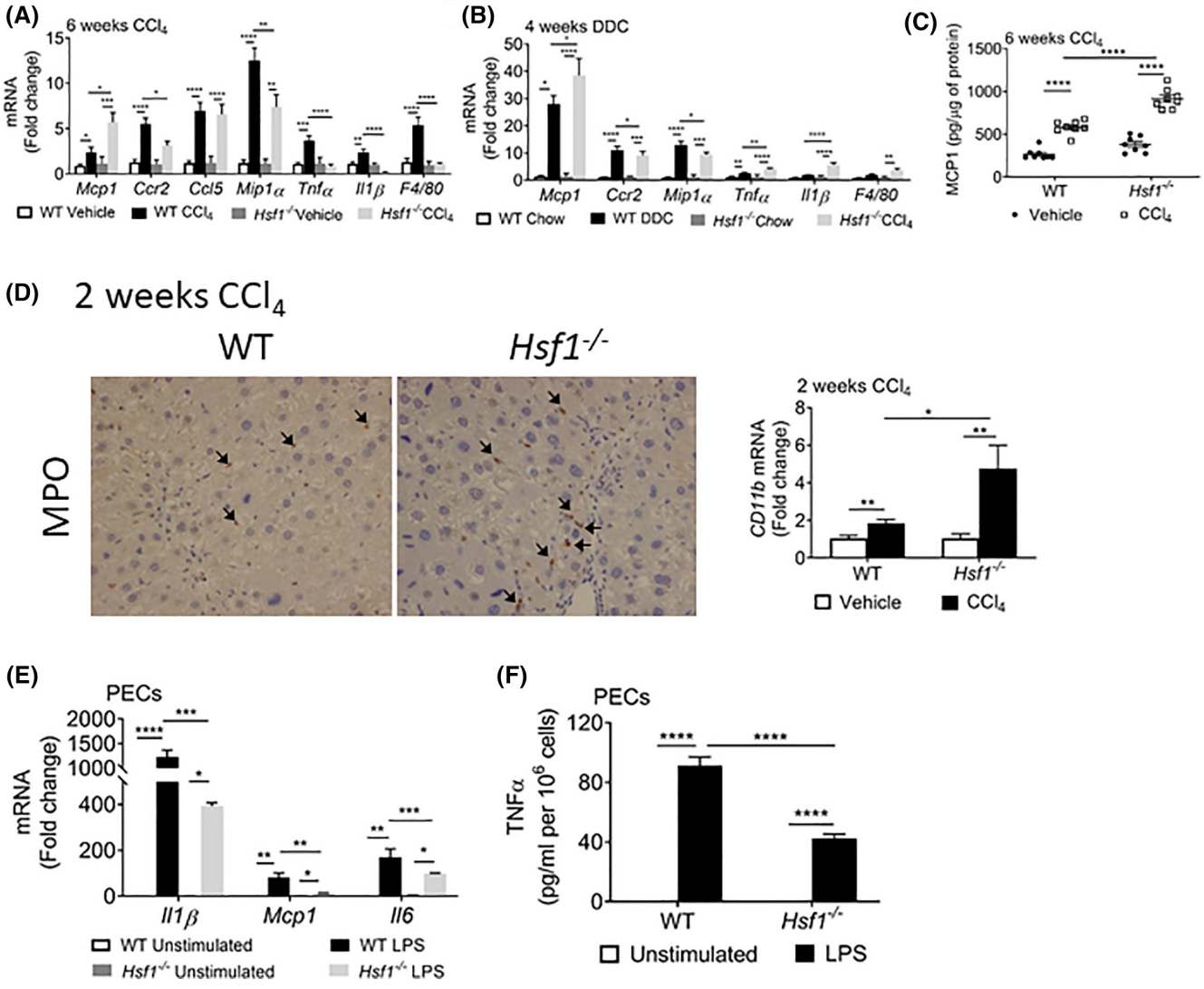


FIGURE 5 *Hsf1*$^{-/-}$ affects intrahepatic proinflammatory cytokines during liver injury. (A,B) WT and *Hsf1*$^{-/-}$ mice were either injected with corn oil or CCl_4 for 6 weeks or fed with diet containing 0.1% DDC. Inflammation was evaluated by expression of monocyte chemoattractant protein 1 (*Mcp1*), CC-chemokine receptor 2 (*Ccr2*), chemokine (C-C motif) ligand 5 (*Ccl5*), macrophage inflammatory protein I alpha (*Mip1α*), tumor necrosis factor α (*Tnfα*), interleukin (*Il)1β*, and *F4/80* in CCl_4-intoxicated mice (A) or DDC diet–fed mice (B). (C) Hepatic MCP1 was assessed by enzyme-linked immunosorbent assay (ELISA) in CCl_4-intoxicated mice (n = 8–10). (D) Livers of WT and *Hsf1*$^{-/-}$ mice intoxicated with CCl_4 for 2 weeks were stained with myeloperoxidase (MPO; original magnification ×40) and assessed for *CD11b* expression (n = 4). (E,F) Peritoneal exudate cell (PEC)–formed WT and *Hsf1*$^{-/-}$ mice were stimulated with lipopolysaccharide (LPS) for 2 h and levels of *Il1β*, *Il6*, and *Mcp1* were evaluated (E), and for 18 h to evaluate TNFα by ELISA in the culture supernatant (F) (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

DDC diet (Figure 5B). After 6 weeks of CCl_4 administration, *F4/80* expression (Figure 5B) was significantly reduced, whereas DDC diet in *Hsf1*$^{-/-}$ fibrotic livers showed a trend toward increased *F4/80* (Figure 5B). As macrophages contribute to the pool of *Tnfα* and *Il1β*, we predict that discrepancy in both models can be due to differences in *F4/80*-expressing macrophages. Similarly, *Hsf1*$^{-/-}$ mice challenged with CCl_4 for 2 weeks displayed a decreasing trend in mRNA expression of *F4/80* when compared with WT (Figure S4A). Recent studies have established that neutrophils promote HSC activation, and activated HSCs promote the survival of neutrophils.[31] Increased myeloperoxidase staining and *CD11b* indicated elevated neutrophil infiltration in *Hsf1*$^{-/-}$ mice at 2 weeks (Figure 5D) and 6 weeks of CCl_4 administration (Figure S4B). Cellular cross-talk between HSF1 and inflammatory pathways is well established.[13,27,32] PECs from *Hsf1*$^{-/-}$ displayed lower *Il1β*, *Mcp1*, *Il6* (Figure 5E) and TNFα (Figure 5F), suggesting dysfunctional macrophage activity. Collectively, our data suggest that neutrophils, and not macrophages, contribute to elevated fibrosis in *Hsf1*$^{-/-}$ mice.

Absence of HSF1 promotes TGFβ-mediated activation of HSCs

Previous studies have reported TGFβ as a major profibrogenic factor. Also, we observed higher induction of *Tgfβ* in the *Hsf1*$^{-/-}$ CCl_4-administered mice (Figure S3E). Thus, to determine the significance of HSF1 in HSC activation, we used different approaches to knock out HSF1 in HSCs and used TGFβ as a profibrogenic mediator *in vitro*. Primary HSCs were isolated from WT and *Hsf1*$^{-/-}$ mice, and a representative image is depicted in Figure S5A. HSF1-deficient primary HSCs stimulated with TGFβ displayed increased *αSma* and *Col1a1* (Figure 6A). Similar results were observed in TGFβ-stimulated LX-2 cells transiently transfected with HSF1 siRNA (si*HSF1*), in which significant up-regulation of *aSMA* and *COL1A1* was observed compared to cells transfected with scrambled siRNA (scram) (Figure 6B). Knockdown efficiency of *siHSF1* was approximately 80% (Figure S5B).

In another approach, we stably transduced LX-2 cells with a dominant negative, constitutively active version of HSF1 (dn-cHSF1) fused to a destabilized variant of *E. coli* dihydrofolate reductase (DHFR.dn-cHSF1.LX-2 cells). Addition of the small molecule Trimethoprim (TMP) to these cells stabilizes the N-terminal DHFR domain of the fusion protein and prevents proteasomal degradation, resulting in an increased pool of DHFR.dn-cHSF1 (Figure 6C), which selectively inhibits endogenous HSF1 activity.[33] Adding TMP to DHFR.dn-cHSF1.LX-2 cells significantly decreased heat shock–mediated induction of *HSPA1A* (Figure 6C), validating the regulatable inhibition of endogenous HSF1 by DHFR.dn-cHSF1. Exposure of DHFR.dn-cHSF1.LX-2 cells to TMP followed by stimulation with TGFβ significantly elevated *αSMA* and *COL1A1* compared with the non-TMP exposed cells (Figure 6D), confirming that HSF1 inhibition promotes HSC activation.

HSC and hepatocyte-specific HSF1 deficiency enhances profibrogenic response

Having observed an exacerbated profibrogenic response in HSF1-deficient HSCs, we sought to determine the effects of cell-specific HSF1 deficiency *in vivo* on CCl_4-mediated liver injury. We generated HSC-specific HSF1-deficient mice by cross-breeding HSF1-floxed (HSF1$^{fl/fl}$) mice with Lrat Cre transgenic mice. To characterize and evaluate specificity of HSF1 deletion, hepatocytes, Kupffer cells (KCs), HSCs, and bone marrow–derived macrophages (BMDMs) were isolated from *Hsf1*$^{fl/fl}$ and *Hsf1*$^{fl/fl}$ *LratCre*$^{+/-}$ mice subjected to heat shock at 42°C, and induction of *Hspa1a* was analyzed. HSCs isolated from *Hsf1*$^{fl/fl}$ *LratCre*$^{+/-}$ mice demonstrated about 85% decrease in HSF1 activity (Figure 7A). Hepatocytes, KCs, and BMDMs demonstrate similar induction of *Hspa1a* (Figure S6A), confirming that HSF1 deletion is restricted only to HSCs. Primary HSCs isolated from *Hsf1*$^{fl/fl}$ *LratCre*$^{+/-}$ mice stimulated with TGFβ showed higher *αSma* and *Col1a1* compared with *Hsf1*$^{fl/fl}$ (Figure S6B), demonstrating HSC activation similar to *Hsf1*$^{-/-}$ (Figure 6A). Using the acute CCl_4 model that establishes a pro-inflammatory and profibrotic milieu in the liver, we observed increased *αSma*, *Pdgfβ, Pdgfrβ*, and *Mcp1* in *Hsf1*$^{fl/fl}$ *LratCre*$^{+/-}$ mice compared with *Hsf1*$^{fl/fl}$ (Figure 7B). Elevated *Tnfα* and *Il1β* were also noted in CCl_4-treated *Hsf1*$^{fl/fl}$ *LratCre*$^{+/-}$ (Figure 7B). Chronic administration of CCl_4 to *Hsf1*$^{fl/fl}$ and *Hsf1*$^{fl/fl}$ *LratCre*$^{+/-}$ mice for 6 weeks resulted in similar increase in serum ALT (Figure S6C). Interestingly, αSMA (Figure S6D) and ECM deposition (Figure 7C) showed comparable induction in both groups of mice. Inflammatory markers such as *Mcp1*, *Ccr2*, *Mip1α*, *Tnfα*, *Il1β* and *F4/80* (Figure S6E), and profibrogenic markers like *Tgfβ, Pdgfα*, *Pdgfrβ* and *Mmp13* (Figure 7D), were increased after CCl_4 similar to *Hsf1*$^{fl/fl}$ mice. These data highlight the importance of HSF1 in early HSC activation and fibrosis.

Apart from HSCs, hepatocytes significantly contribute to the pathogenesis of liver fibrosis through hepatocellular cell death and oxidative stress. To evaluate the importance of hepatocellular HSF1 in liver fibrosis, we generated hepatocyte-specific HSF1-deficient mice by cross-breeding HSF1 floxed (*Hsf1*$^{fl/fl}$) mice with *AlbCre* transgenic mice. Hepatocyte-specific deletion of HSF1 was confirmed in *Hsf1*$^{fl/fl}$ *AlbCre*$^{+/-}$ mice by reduced *Hspa1a* in the heat shocked primary hepatocytes, when compared with the *Hsf1*$^{fl/fl}$ mice (Figure 7E).

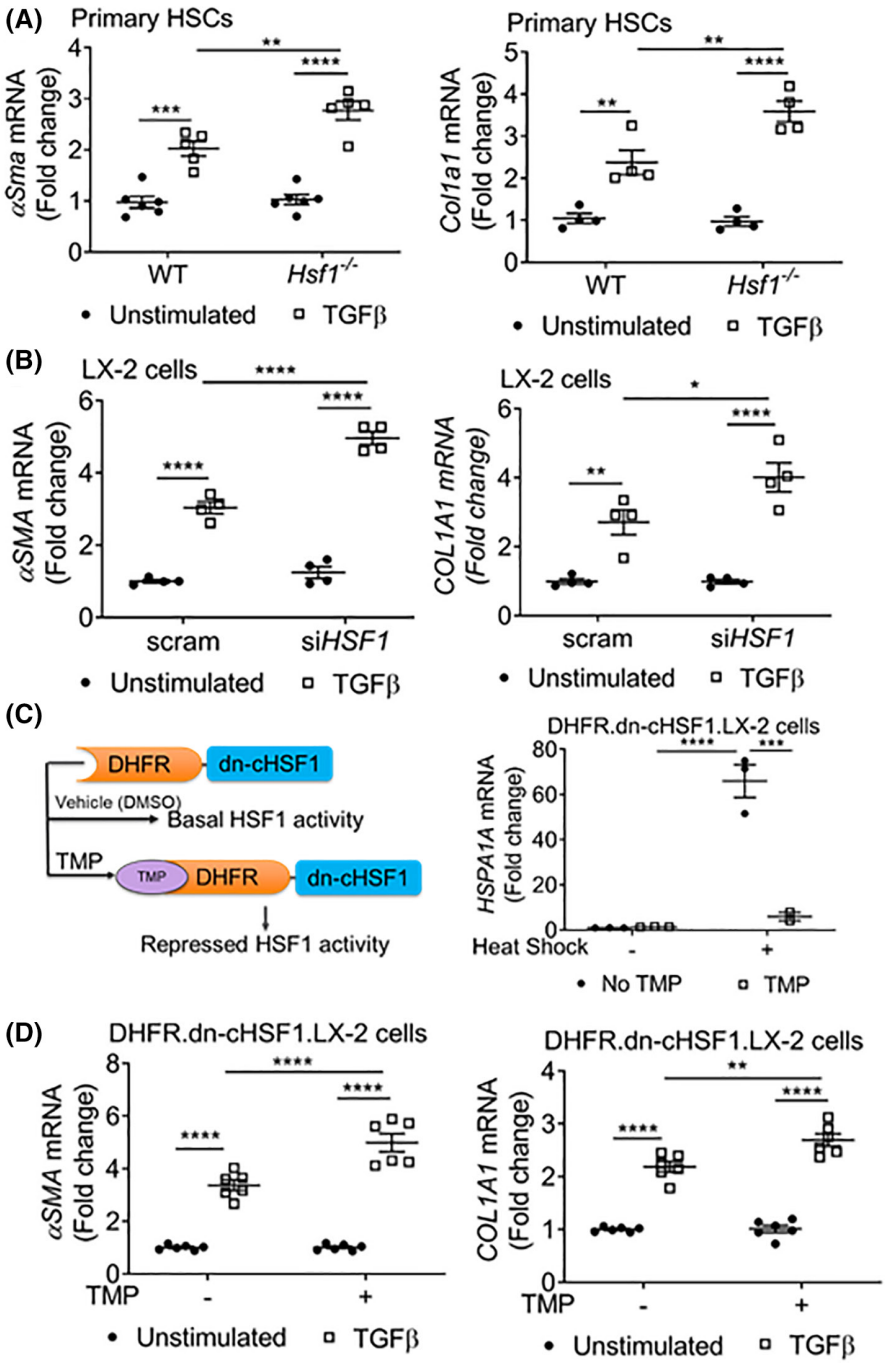
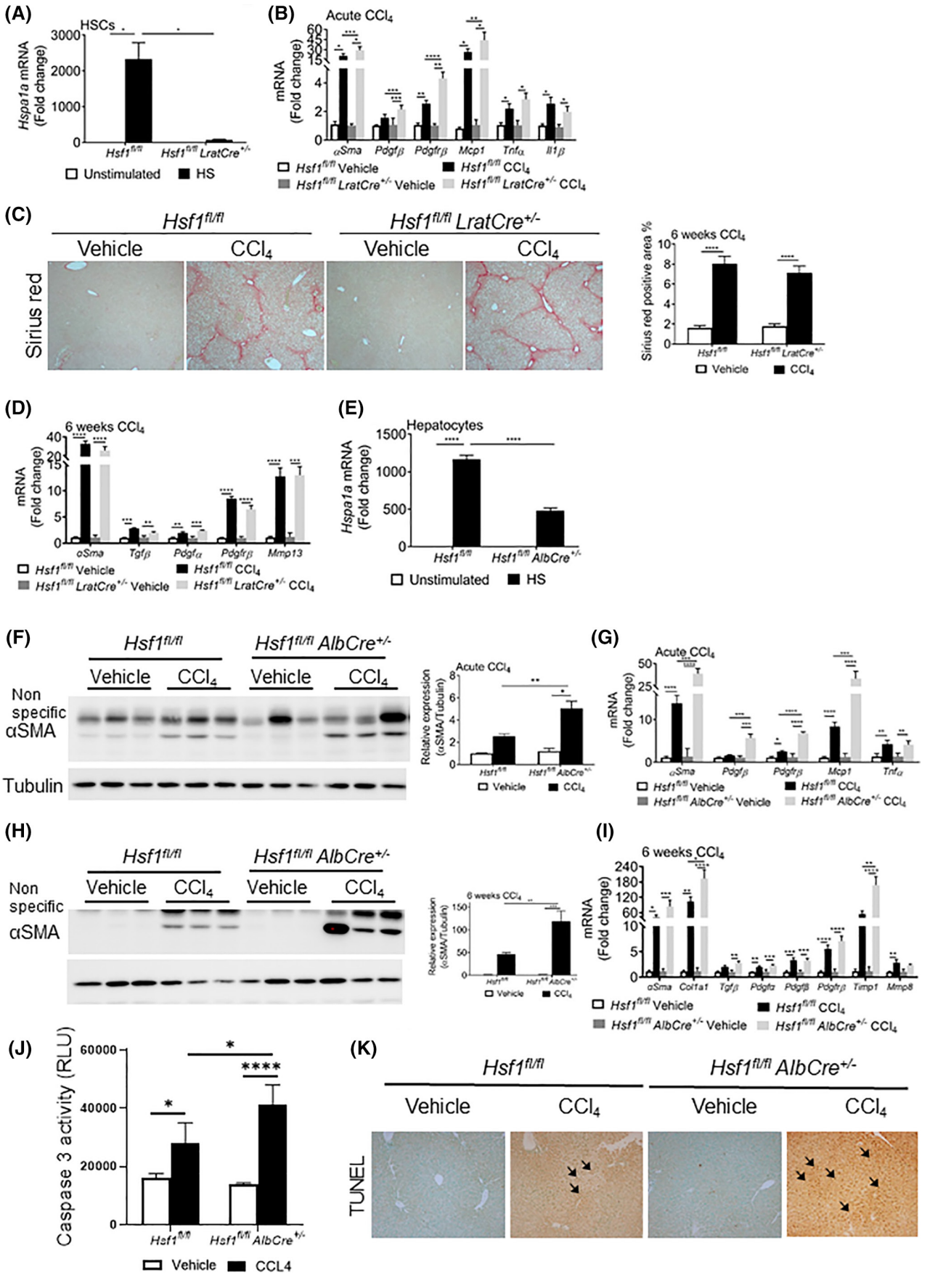


FIGURE 6 Deficiency of HSF1 increases the profibrogenic gene response to TGFβ in HSCs. (A,B) *αSma* and *Col1a1* in TGFβ-stimulated primary HSCs isolated from WT and *Hsf1*$^{-/-}$ mice ($n = 4$) (A) and LX-2 cells transfected with si*HSF1* or scram sequences ($n = 4$) (B). (C) Schematic representation of DHFR.Dn-cHSF1 variant of LX-2 cells and *HSPA1A* levels in the heat-shocked DHFR.Dn-cHSF1. LX2 cells in the presence or absence of TMP ($n = 3$). (D) *αSMA* and *COL1A1* in TGFβ stimulated DHFR.Dn-cHSF1.LX-2 cells in the presence or absence of TMP ($n = 6$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. TMP, Trimethoprim.

Administration of acute CCl_4 to *Hsf1*$^{fl/fl}$ *AlbCre*$^{+/-}$ mice resulted in elevated αSMA protein (Figure 7F) as well as *αSma, Pdgfβ*, *Pdgfrβ*, and *Mcp1* mRNA (Figure 7G). Similar increase in αSMA was observed in *Hsf1*$^{fl/fl}$ *AlbCre*$^{+/-}$ mice after 6 weeks of CCl_4 (Figure 7H). Profibrogenic genes (Figure 7I) and pro-inflammatory cytokines (Figure S6F) was also increased in 6-week CCl_4-treated *Hsf1*$^{fl/fl}$ *AlbCre*$^{+/-}$ mice. Assessment of hepatocyte cell death revealed significant increase in caspase-3 activity (Figure 7J) and TUNEL-positive cells (Figure 7K) in CCl_4-treated *Hsf1*$^{fl/fl}$ *AlbCre*$^{+/-}$ mice. Collectively, our data suggest that deficiency of HSF1 in stellate cell and hepatocytes enhances profibrogenic responses in the liver.


(A)
HSCs
Hspa1a mRNA (Fold change)
Hsf1fl/fl
Hsf1fl/fl LratCre+/-
Unstimulated
HS
(B)
Acute CCl4
mRNA (Fold change)
αSma
Pdgfβ
Pdgfrβ
Mcp1
Tnfα
Il1β
Hsf1fl/fl Vehicle
Hsf1fl/fl CCl4
Hsf1fl/fl LratCre+/- Vehicle
Hsf1fl/fl LratCre+/- CCl4
(C)
Hsf1fl/fl
Hsf1fl/fl LratCre+/-
Vehicle
CCl4
Sirius red
6 weeks CCl4
Sirius red positive area %
(D)
6 weeks CCl4
mRNA (Fold change)
αSma
Tgfβ
Pdgfα
Pdgfrβ
Mmp13
(E)
Hepatocytes
Hspa1a mRNA (Fold change)
Hsf1fl/fl AlbCre+/-
(F)
Hsf1fl/fl
Hsf1fl/fl AlbCre+/-
Non specific αSMA
Tubulin
Acute CCl4
Relative expression (αSMA/Tubulin)
(G)
Acute CCl4
mRNA (Fold change)
αSma
Pdgfβ
Pdgfrβ
Mcp1
Tnfα
Hsf1fl/fl AlbCre+/- Vehicle
Hsf1fl/fl AlbCre+/- CCl4
(H)
Hsf1fl/fl
Hsf1fl/fl AlbCre+/-
Non specific αSMA
6 weeks CCl4
Relative expression (αSMA/Tubulin)
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6 weeks CCl4
mRNA (Fold change)
αSma
Col1a1
Tgfβ
Pdgfα
Pdgfβ
Pdgfrβ
Timp1
Mmp8
(J)
Caspase 3 activity (RLU)
Vehicle
CCL4
(K)
Hsf1fl/fl
Hsf1fl/fl AlbCre+/-
Vehicle
CCl4
TUNEL

FIGURE 7 HSC-specific and hepatocyte-specific deletion of HSF1 increases profibrogenic response to acute CCl_4. (A) *Hspa1a* in heat-shocked HSCs isolated from *Hsf1*$^{fl/fl}$ and *Hsf1*$^{fl/fl}$ *LratCre*$^{+/-}$ mice. (B) Mice were injected with an acute dose of corn oil or CCl_4 and sacrificed after 48 h to assess *αSma*, *Pdgfβ*, *Pdgfrβ*, *Mcp1*, *Tnfα*, and *Il1β*. (C,D) *Hsf1*$^{fl/fl}$ and *Hsf1*$^{fl/fl}$ *LratCre*$^{+/-}$ mice were injected with corn oil or CCl_4, for 6 weeks. (C) Fibrosis was assessed by *αSma* and sirius red staining (original magnification ×10) and are presented as percentage fibrotic area. (D) Levels of *αSma, Tgfβ*, *Pdgfα*, *Pdgfrβ*, and *Mmp13*. (E) *Hspa1a* in heat-shocked HSCs isolated from *Hsf1*$^{fl/fl}$ and *Hsf1*$^{fl/fl}$ *AlbCre*$^{+/-}$ mice. (F,G) *Hsf1*$^{fl/fl}$ and *Hsf1*$^{fl/fl}$ *AlbCre*$^{+/-}$ mice were injected with an acute dose of corn oil or CCl_4 (F) and hepatic *αSma*, *Pdgfβ*, *Pdgfrβ*, *Mcp1*, and *Tnfα* (G). (H) *Hsf1*$^{fl/fl}$ and *Hsf1*$^{fl/fl}$ *AlbCre*$^{+/-}$ mice were injected with corn oil or CCl_4 for 6 weeks, and fibrosis was assessed by αSMA immunoblotting quantified by normalizing to tubulin using ImageJ. (I) Hepatic *αSma*, *Col1a1, Tgfβ, Pdgfα, Pdgfβ*, *Pdgfrβ*, *Timp1*, and *Mmp8*. (J) liver caspase-3 activity. (K) Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining of formalin-fixed liver sections (n = 6–8 mice per treatment group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Activation of HSF1 ameliorates TGFβ-mediated induction of profibrogenic genes

Because both *Hsf1*$^{-/-}$ and *in vitro* inhibition of HSF1 escalated HSC activation, we sought to understand the effects of HSF1 activation on induction of profibrogenic genes in response to TGFβ. In the first approach, LX-2 cells were heat shocked (HS) to activate HSF1, allowed to recover for 1 h, and exposed to TGFβ. Heat shock–mediated activation of HSF1 decreased *αSMA* and *COL1A1* in response to TGFβ (Figure 8A). Elevated *HSPA1A* at 2, 4, and 8 h after HS confirmed the activation of HSF1 (Figure S7A). In another approach, we used celastrol, a pharmacological inducer of HSF1 activation with kinetics similar to HS.[34] Treatment of TGFβ-stimulated LX-2 cells with celastrol at a concentration of 1.5 μM minimally affected cell viability (Figure S7B) and decreased *αSMA* and *COL1A1* (Figure 8B). Activation of HSF1 by celastrol was confirmed by the increased *HSPA1A* in the celastrol-treated LX-2 cells (Figure S7C).

To better understand whether HSF1 activation can reduce HSC activation, we used a chemical method to induce HSF1 activity independent of stress stimuli. We engineered LX-2 cells to stably express a previously reported constitutively active HSF1 variant (cHSF1) fused to a ligand-regulated, destabilized version of FKBP (FKBP.cHSF1.LX-2 cells).[35] In the absence of the small molecule ligand Shield-1, the FKBP.cHSF1 fusion protein was rapidly degraded by the proteasome. Addition of Shield-1 prevented proteasomal degradation of FKBP.cHSF1 and resulted in transcriptional upregulation of HSF1 target genes,[33] *HSP40, HSPA1A*, and *HSP90AA1* in the absence of HS, confirming stress-independent, small molecule–regulated activation of HSF1 (Figure 8C). FKBP.cHSF.LX-2 cells treated with vehicle and then exposed to TGFβ displayed induction of *αSMA* and *COL1A1* similar to that seen in the parental LX-2 cells. Addition of Shield-1 significantly attenuated the TGFβ-mediated expression of *αSMA* and *COL1A1* (Figure 8D). Previous studies reported that TGFβ induces JNK phosphorylation, which is required for HSC activation.[36] The FKBP.cHSF.LX-2 cells stimulated with TGFβ increased phosphorylated JNK (p-JNK) (Figure 8D), similar to LX-2 cells (Figure S2D). Notably, addition of Shield-1 activated HSF1 and diminished p-JNK (Figure 8E), which correlated with decreased profibrogenic response (Figure 8D). Thus, we can conclude that HSF1 activity is directly responsible for the attenuation of these profibrogenic genes via inhibition of JNK activity. Overall, our studies identify that HSF1 can regulate and curb HSC activation promoting anti-fibrotic responses in the liver.

DISCUSSION

Traditionally characterized as a sensor of cellular stress caused by protein misfolding, HSF1 functions as a transcriptional regulator of HSPs to restore proteostasis. Here we established that loss of HSF1 activity facilitates HSC activation and profibrogenic gene expression. We identified that HSF1 and HSPs are elevated in human cirrhotic and mouse fibrotic livers. However, fibrotic livers and myofibroblasts associated with ECM did not show activated HSF1. Also, whole-body, hepatocyte, and stellate cell–specific HSF1 deficiency exacerbates liver fibrosis by increased HSC activation. *Hsf1*-deficient stellate cells *in vitro* and stellate cell–specific HSF1 knockout mice exhibited enhanced HSC activation. On the other hand, activation of HSF1 caused a significant reduction in HSC activation likely via decreased JNK activation. Overall, our studies demonstrate that TGFβ-mediated JNK may induce loss of HSF1 activity and facilitate hepatic fibrosis, whereas activation of HSF1 reduces HSC activation likely via inhibition of JNK and induces antifibrotic responses (Figure 8F).

Overactivation of HSF1 in alcoholic murine macrophages has been previously demonstrated by our group.[12,14] Recent studies point to a role for HSF1 in metabolic processes[8] and demonstrate that HSF1 is required for maintaining oxidized nicotinamide adenine dinucleotide NAD+ and adenosine triphosphate in hepatic cells.[37] Transition of quiescent HSCs to myofibroblast is triggered by the activation of NOX and generation of ROS.[5] The oxidant byproducts such as ROS and reactive nitrogen species results in decline of cell homeostasis and detrimental alterations leading to pathological conditions.[38] HSF1 and HSPs are regulators of the cytoplasmic proteostasis network

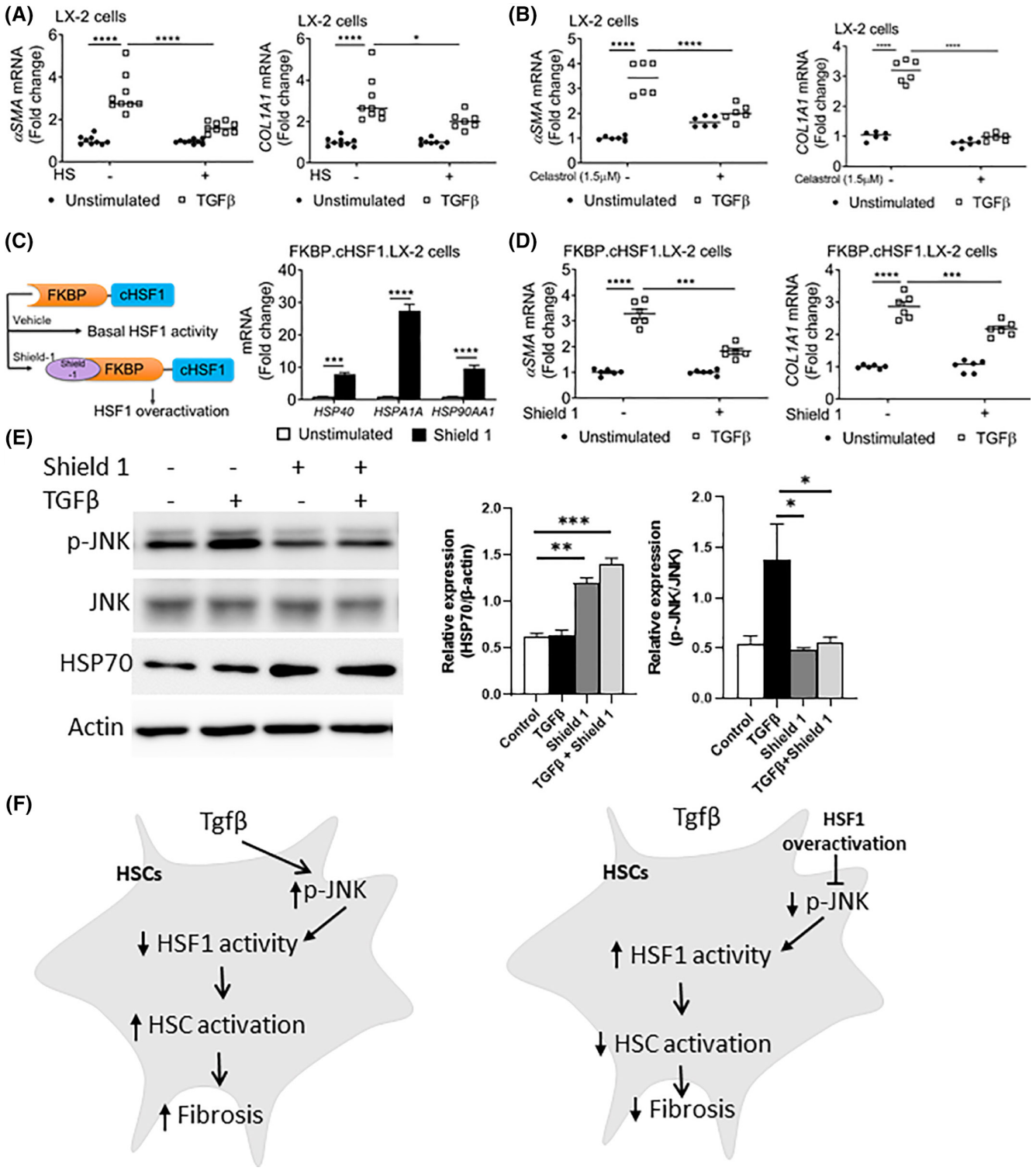

FIGURE 8 Activation of HSF1 ameliorates TGFβ-mediated LX-2 cell activation. (A,B) Expression of *αSMA* and *COL1A1* in TGFβ-stimulated heat-shocked LX-2 cells (A), and LX-2 cells treated with celastrol (B). (C) Schematic representation of FKBP.cHSF1 variant of LX-2 cells and assessment of *HSP40, HSPA1A*, and *HSP90AA1* in the FKBP.cHSF1.LX-2 cells in the presence or absence of Shield-1 ($n = 6$). (D) *αSMA* and *COL1A1* in TGFβ-stimulated FKBP.cHSF1.LX2 cells in the presence or absence of Shield-1. (E) Lysates from FKBP.cHSF1.LX2 stimulated with TGFβ were assessed for p-JNK, JNK, HSP70, and β-actin by immunoblotting and quantified by ImageJ. (F) Schematic representation demonstrating the effects of HSF1 deficiency on HSC activation ($n = 6–9$ per treatment group). *$p < 0.05$, ****$p < 0.0001$.

that senses cellular stress to restore homeostasis.[20] Increased HSF1 and HSPs in human cirrhotic and murine fibrotic livers is likely a defense mechanism that cells adapt to restore the cellular homeostasis.

Although nuclear HSF1 is observed in hepatocytes, activated stellate cells did not exhibit nuclear *HSF1* and downstream *HSPs*. Similar cell-specific differences in proteostasis mediators are described previously in the brain.[39] Activation of JNK is known to suppress HSF1 activity.[22] In the CCl_4-induced fibrotic livers, JNK activation in activated myofibroblast and not hepatic parenchyma is reported.[36] Our data suggest that TGFβ-induced p-JNK during HSC activation[36] may inhibit HSF1 activity. Activation of proteostasis sensors is required for cell survival during stressful conditions and maintenance of cellular homeostasis. Hence, the inability to activate HSF1 in HSCs is likely detrimental, leading to fibrosis.

Using *Hsf1*$^{-/-}$ mice, we revealed that absence of HSF1 exacerbates liver fibrosis as evidenced by increased HSC activation, ECM deposition, and profibrogenic gene activation. Previous studies demonstrate a positive relation between HSF1 activation and expression of HSP47, a collagen-specific chaperone in LX-2 cells.[10] Despite the deficiency of HSF1, we observed high expression of *Hsp47*, concomitant to increased *Col1a1* in *Hsf1*$^{-/-}$ fibrotic livers. These data suggest that HSP47 may be regulated by transcription factors other than HSF1, such as SP1 and ZF9, which are previously implicated in HSC activation.[40,41] Expression of *Timp1* is increased in *Hsf1*$^{-/-}$ fibrotic livers, whereas *Mmp8* and *13* are decreased, suggesting that elevated TIMP1 may result in increased ECM via down-regulation of MMPs. Liver macrophages are the predominant source of MMP8 and MMP13.[42] *Hsf1*$^{-/-}$ mice inherently exhibit reduced migration and lack of terminal differentiation of macrophages.[43] It is likely that reduced macrophages contribute to decreased MMPs and increased ECM deposition in *Hsf1*$^{-/-}$ mice.

In addition to their contribution to fibrogenic processes, macrophages are also crucial mediators of resolution of liver fibrosis.[42] Decreased macrophages in *Hsf1*$^{-/-}$ fibrotic livers after 6 weeks of CCl_4 intoxication may further account for the delay in the resolution of liver fibrosis in *Hsf1*$^{-/-}$ mice. Studies demonstrate that regression of liver fibrosis in *Hspa1a/b*$^{-/-}$ mice was strongly accelerated compared with WT mice, likely due to apoptosis of inactivated HSCs.[44] Interestingly, *Hsf1*$^{-/-}$ mice exhibit a lesser degree of resolution, suggesting that HSF1, which is a transcriptional regulator of HSPA1A, may provide a protective regulatory function in reversal of activated HSCs in addition to its antiapoptotic properties. Thus, HSF1 may play a central role in driving antifibrotic responses by modulating HSC activation.

Hepatic inflammation mediated by macrophages is tightly linked to HSC activation and development of fibrosis.[26] *Hsf1*$^{-/-}$ mice show elevated *Mcp1* and decreased *Ccr2* and *Mip1α*. Following liver injury, MCP1 is secreted by hepatocytes, KCs, and activated stellate cells.[14,21] It is likely that increased *Mcp1* in *Hsf1*$^{-/-}$ mice is predominantly contributed by hepatocytes and activated HSCs. TNFα and IL-1β are two potent inflammatory cytokines implicated in liver fibrosis.[3] Although the DDC model of liver fibrosis demonstrated elevated *Tnfα* and *Il1β* expression, we did not observe induction of *Tnfα* and *Il1β* in *Hsf1*$^{-/-}$ fibrotic livers induced by CCl_4 intoxication, indicating no significant contribution of macrophages in inflammation in *Hsf1*$^{-/-}$ mice. This can be attributed either to differences in the stage of disease progression in both fibrosis models or the defect in monocyte differentiation in *Hsf1*$^{-/-}$ mice. Previous studies have shown that absence of HSF1 decreases the transcription of the *Spl1*/*Pu.1* gene required for monocyte to macrophage differentiation, resulting in about 50% decrease in macrophage activation.[43] In line with this, our data revealed that *Hsf1*$^{-/-}$ PECs stimulated with LPS displayed decreased induction of pro-inflammatory cytokines. Notably, we observed increased hepatic infiltration of neutrophils in CCl_4-administered *Hsf1*$^{-/-}$ mice. Our finding is supported by Chen et al., who demonstrated an increase in hepatic neutrophils in LPS-administered *Hsf1*$^{-/-}$ mice due to greater surface expression of P-selectin glycoprotein ligand-1 that promotes leukocyte recruitment.[45] Neutrophils promote HSC activation, and, in turn, activated HSCs increase the survival of neutrophils in the model of high-fat diet and binge ethanol.[31] It is likely that decreased HSF1 activity in the liver promotes higher neutrophil activation, and this will be evaluated in the future. Our data establish that *Hsf1*$^{-/-}$ fibrotic livers demonstrate selective increase in inflammatory response, likely facilitated by neutrophils.

Because HSF1 activity is dramatically reduced in activated HSCs, we investigated the role of HSF1 in HSC activation. We observed that HSF1-deficient HSCs show exacerbated profibrogenic gene expression *in vitro*. Likewise, our data show that stellate cell–specific HSF1 deficiency confers elevated fibrosis genes in an acute CCl_4 model of liver injury. In fact, primary HSF1-deficient stellate cells isolated from *Hsf1*$^{fl/fl}$ *LratCre*$^{+/-}$ mice also show increased *aSma* and *Col1a1* when stimulated with TGFβ *in vitro*. We did not use PDGFB as a profibrogenic stimuli, as previous studies have reported that HSF1 deficient mouse embryonic fibroblasts are resistant to PDGFB mediated proliferation.[46] Furthermore, our data show similar extent of fibrosis after 6 weeks of CCl_4 administration between *Hsf1*$^{fl/fl}$ and *Hsf1*$^{fl/fl}$ *LratCre*$^{+/-}$ mice, suggesting that HSF1 in hepatocytes may also contribute to augmented fibrosis in whole-body HSF1 knockouts. We further investigated this possibility by generating *Hsf1*$^{fl/fl}$ *AlbCre*$^{+/-}$ mice, which demonstrated elevated liver injury in response to acute CCl_4. Hepatocellular injury leads to higher ROS production via nicotinamide adenine dinucleotide phosphate (reduced form) oxidases,

leading to its interaction with surrounding inflammatory cells and HSCs facilitating the progression of liver fibrosis.[47] Because HSF1 is required for the expression of anti-apoptotic gene HSPA1A,[48] and to combat oxidative stress,[19] we confirmed increased hepatocellular cell death in *Hsf1*$^{fl/fl}$ *AlbCre*$^{+/-}$ mice, which likely induces profibrogenic gene response. These data suggest that active HSF1 plays a beneficial role in ameliorating liver fibrosis via regulation of cell death pathways and myofibroblast differentiation in a cell-specific manner.

The beneficial effects of HSF1 activation on obesity, insulin resistance, and liver steatosis in mice exposed to high-fat diet have been previously reported.[8] Augmentation of HSF1 activity via celastrol decreases inflammation and prevents liver dysfunction in response to thioacetamide exposure.[49] In our study, activation of HSF1 down-regulated TGFβ-mediated profibrogenic genes in HSCs. TGFβ promotes JNK phosphorylation in HSCs, and pharmacological inhibition of JNK results in decreased HSC activation and fibrosis.[36] HSF1 is known to suppresses activity of JNK.[50] Our studies demonstrate that constitutive activation of HSF1 leads to decreased phosphorylation of JNK in response to TGFβ stimulation, which in turn leads to decreased TGFβ-mediated profibrogenic response (Figure 8F).

In summary, we establish that absence of HSF1 promotes HSC activation and liver fibrosis. HSF1 deficiency hampers resolution of liver fibrosis, and its activation prevents TGFβ-mediated expression of profibrogenic genes. Thus, up-regulation of HSF1 activity as a potential therapeutic for liver fibrosis is attractive. Our study introduces a paradigm in understanding the role of proteostasis mediators in pathogenesis of liver fibrosis. Future studies dissecting the stellate cell–specific antifibrogenic transcriptional program regulated by HSF1 will uncover underlying mechanisms in liver fibrosis and identify potential therapeutic targets.

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CONFLICT OF INTEREST

Nothing to report.

REFERENCES

1. Weiskirchen R, Weiskirchen S, Tacke F. Recent advances in understanding liver fibrosis: bridging basic science and individualized treatment concepts. F1000Res. 2018;7:921.
2. Yoon YH, Chen CM. Liver cirrhosis mortality in the United States: national, state, and regional trends, 2000–2015 National Institute on Alcohol Abuse and Alcoholism Division of Epidemiology and Prevention Research Alcohol Epidemiologic Data System 2018. Surveillance Report. 2018;111:1–73.
3. Seki E, Schwabe RF. Hepatic inflammation and fibrosis: functional links and key pathways. Hepatology. 2015;61:1066–79.
4. Maiers JL, Malhi H. Endoplasmic reticulum stress in metabolic liver diseases and hepatic fibrosis. Semin Liver Dis. 2019;39:235–48.
5. Liang S, Kisseleva T, Brenner DA. The role of NADPH oxidases (NOXs) in liver fibrosis and the activation of myofibroblasts. Front Physiol. 2016;7:17.
6. Diesen DL, Kuo PC. Nitric oxide and redox regulation in the liver. Part II: Redox biology in pathologic hepatocytes and implications for intervention. J Surg Res. 2011;167:96–112.
7. Calderwood SK. HSF1, a versatile factor in tumorogenesis. Curr Mol Med. 2012;12:1102–7.
8. Ma X, Xu L, Alberobello AT, Gavrilova O, Bagattin A, Skarulis M, et al. Celastrol protects against obesity and metabolic dysfunction through activation of a HSF1-PGC1alpha transcriptional Axis. Cell Metab. 2015;22:695–708.
9. Choudhury AMP. Chaperones in sterile inflammation and injury. In: Asea A, Kaur P, editors. Chaperokine activity of heat shock proteins. Heat Shock Proteins. Volume 16. Cham, Switzerland: Springer; 2019.
10. Park SJ, Sohn HY, Park SI. TRAIL regulates collagen production through HSF1-dependent Hsp47 expression in activated hepatic stellate cells. Cell Signal. 2013;25:1635–43.
11. Sasaki H, Sato T, Yamauchi N, Okamoto T, Kobayashi D, Iyama S, et al. Induction of heat shock protein 47 synthesis by TGF-beta and IL-1 beta via enhancement of the heat shock element binding activity of heat shock transcription factor 1. J Immunol. 2002;168:5178–83.
12. Muralidharan S, Ambade A, Fulham MA, Deshpande J, Catalano D, Mandrekar P. Moderate alcohol induces stress proteins HSF1 and hsp70 and inhibits proinflammatory cytokines resulting in endotoxin tolerance. J Immunol. 2014;193:1975–87.
13. Ambade A, Catalano D, Lim A, Mandrekar P. Inhibition of heat shock protein (molecular weight 90 kDa) attenuates proinflammatory cytokines and prevents lipopolysaccharide-induced liver injury in mice. Hepatology. 2012;55:1585–95.
14. Mandrekar P, Catalano D, Jeliazkova V, Kodys K. Alcohol exposure regulates heat shock transcription factor binding and heat shock proteins 70 and 90 in monocytes and macrophages: implication for TNF-alpha regulation. J Leukoc Biol. 2008;84:1335–45.
15. Seki E, de Minicis S, Inokuchi S, Taura K, Miyai K, van Rooijen N, et al. CCR2 promotes hepatic fibrosis in mice. Hepatology. 2009;50:185–97.
16. Iracheta-Vellve A, Petrasek J, Satishchandran A, Gyongyosi B, Saha B, Kodys K, et al. Inhibition of sterile danger signals, uric acid and ATP, prevents inflammasome activation and protects from alcoholic steatohepatitis in mice. J Hepatol. 2015;63:1147–55.
17. Pradhan-Sundd T, Vats R, Russell JO, Singh S, Michael AA, Molina L, et al. Dysregulated bile transporters and impaired tight junctions during chronic liver injury in mice. Gastroenterology. 2018;155:1218–32. e1224.
18. Mu X, Pradere JP, Affò S, Dapito DH, Friedman R, Lefkovitch JH, et al. Epithelial transforming growth factor-β signaling does not contribute to liver fibrosis but protects mice from cholangiocarcinoma. Gastroenterology. 2016;150:720–33.
19. Ahn SG, Thiele DJ. Redox regulation of mammalian heat shock factor 1 is essential for Hsp gene activation and protection from stress. Genes Dev. 2003;17:516–28.
20. Dai C, Sampson SB. HSF1: guardian of proteostasis in cancer. Trends Cell Biol. 2016;26:17–28.

21. Baeck C, Wehr A, Karlmark KR, Heymann F, Vucur M, Gassler N, et al. Pharmacological inhibition of the chemokine CCL2 (MCP-1) diminishes liver macrophage infiltration and steatohepatitis in chronic hepatic injury. Gut. 2012;61:416–26.
22. Dai R, Frejtag W, He B, Zhang Y, Mivechi NF. C-Jun NH2-terminal kinase targeting and phosphorylation of heat shock factor-1 suppress its transcriptional activity. J Biol Chem. 2000;275:18210–8.
23. Kocabayoglu P, Lade A, Lee YA, Dragomir AC, Sun X, Fiel MI, et al. Beta-PDGF receptor expressed by hepatic stellate cells regulates fibrosis in murine liver injury, but not carcinogenesis. J Hepatol. 2015;63:141–7.
24. Duarte S, Baber J, Fujii T, Coito AJ. Matrix metalloproteinases in liver injury, repair and fibrosis. Matrix Biol. 2015;44–46:147–56.
25. Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Nakatani T, et al. Tissue inhibitor of metalloproteinases-1 attenuates spontaneous liver fibrosis resolution in the transgenic mouse. Hepatology. 2002;36:850–60.
26. Czaja AJ. Hepatic inflammation and progressive liver fibrosis in chronic liver disease. World J Gastroenterol. 2014;20:2515–32.
27. Muralidharan S, Mandrekar P. Cellular stress response and innate immune signaling: integrating pathways in host defense and inflammation. J Leukoc Biol. 2013;94:1167–84.
28. Marra F, DeFranco R, Grappone C, Milani S, Pastacaldi S, Pinzani M, et al. Increased expression of monocyte chemotactic protein-1 during active hepatic fibrogenesis: correlation with monocyte infiltration. Am J Pathol. 1998;152:423–30.
29. Berres ML, Koenen RR, Rueland A, Zaldivar MM, Heinrichs D, Sahin H, et al. Antagonism of the chemokine Ccl5 ameliorates experimental liver fibrosis in mice. J Clin Invest. 2010;120:4129–40.
30. Heinrichs D, Berres ML, Nellen A, Fischer P, Scholten D, Trautwein C, et al. The chemokine CCL3 promotes experimental liver fibrosis in mice. PLoS One. 2013;8:e66106.
31. Zhou Z, Xu MJ, Cai Y, Wang W, Jiang JX, Varga ZV, et al. Neutrophil-hepatic stellate cell interactions promote fibrosis in experimental steatohepatitis. Cell Mol Gastroenterol Hepatol. 2018;5:399–413.
32. Choudhury A, Bullock D, Lim A, Argemi J, Orning P, Lien E, et al. Inhibition of HSP90 and activation of HSF1 diminish macrophage NLRP3 inflammasome activity in alcohol-associated liver injury. Alcohol Clin Exp Res. 2020;44:1300–11.
33. Moore CL, Dewal MB, Nekongo EE, Santiago S, Lu NB, Levine SS, et al. Transportable, chemical genetic methodology for the small molecule-mediated inhibition of heat shock factor 1. ACS Chem Biol. 2016;11:200–10.
34. Westerheide SD, Bosman JD, Mbadugha BN, Kawahara TL, Matsumoto G, Kim S, et al. Celastrols as inducers of the heat shock response and cytoprotection. J Biol Chem. 2004;279:56053–60.
35. Shoulders MD, Ryno LM, Cooley CB, Kelly JW, Wiseman RL. Broadly applicable methodology for the rapid and dosable small molecule-mediated regulation of transcription factors in human cells. J Am Chem Soc. 2013;135:8129–32.
36. Kluwe J, Pradere JP, Gwak GY, Mencin A, De Minicis S, Osterreicher CH, et al. Modulation of hepatic fibrosis by c-Jun-N-terminal kinase inhibition. Gastroenterology. 2010;138:347–59.
37. Qiao A, Jin X, Pang J, Moskophidis D, Mivechi NF. The transcriptional regulator of the chaperone response HSF1 controls hepatic bioenergetics and protein homeostasis. J Cell Biol. 2017;216:723–41.
38. Pajares M, Jimenez-Moreno N, Dias IHK, Debelec B, Vucetic M, Fladmark KE, et al. Redox control of protein degradation. Redox Biol. 2015;6:409–20.
39. Takeuchi T, Suzuki M, Fujikake N, Popiel HA, Kikuchi H, Futaki S, et al. Intercellular chaperone transmission via exosomes contributes to maintenance of protein homeostasis at the organismal level. Proc Natl Acad Sci USA. 2015;112:E2497–506.
40. Rippe RA, Almounajed G, Brenner DA. Sp1 binding activity increases in activated Ito cells. Hepatology. 1995;22:241–51.
41. Ratziu V, Lalazar A, Wong L, Dang Q, Collins C, Shaulian E, et al. Zf9, a Kruppel-like transcription factor up-regulated in vivo during early hepatic fibrosis. Proc Natl Acad Sci USA. 1998;95:9500–5.
42. Fallowfield JA, Mizuno M, Kendall TJ, Constandinou CM, Benyon RC, Duffield JS, et al. Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis. J Immunol. 2007;178:5288–95.
43. Jego G, Lanneau D, De Thonel A, Berthenet K, Hazoume A, Droin N, et al. Dual regulation of SPI1/PU.1 transcription factor by heat shock factor 1 (HSF1) during macrophage differentiation of monocytes. Leukemia. 2014;28:1676–86.
44. Kisseleva T, Cong M, Paik Y, Scholten D, Jiang C, Benner C, et al. Myofibroblasts revert to an inactive phenotype during regression of liver fibrosis. Proc Natl Acad Sci USA. 2012;109:9448–53.
45. Chen S, Zuo X, Yang M, Lu H, Wang N, Wang K, et al. Severe multiple organ injury in HSF1 knockout mice induced by lipopolysaccharide is associated with an increase in neutrophil infiltration and surface expression of adhesion molecules. J Leukoc Biol. 2012;92:851–7.
46. Dai C, Whitesell L, Rogers AB, Lindquist S. Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. Cell. 2007;130:1005–18.
47. De Minicis S, Seki E, Paik YH, Osterreicher CH, Kodama Y, Kluwe J, et al. Role and cellular source of nicotinamide adenine dinucleotide phosphate oxidase in hepatic fibrosis. Hepatology. 2010;52:1420–30.
48. Jaattela M, Wissing D, Kokholm K, Kallunki T, Egeblad M. Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. EMBO J. 1998;17:6124–34.
49. Sharma S, Mishra R, Walker BL, Deshmukh S, Zampino M, Patel J, et al. Celastrol, an oral heat shock activator, ameliorates multiple animal disease models of cell death. Cell Stress Chaperones. 2015;20:185–201.
50. Su KH, Cao J, Tang Z, Dai S, He Y, Sampson SB, et al. HSF1 critically attunes proteotoxic stress sensing by mTORC1 to combat stress and promote growth. Nat Cell Biol. 2016;18:527–39.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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