Journal of Molecular Medicine (2019) 97:1521–1533
https://doi.org/10.1007/s00109-019-01831-8

JMolMed

ORIGINAL ARTICLE

Ablation of serum response factor in hepatic stellate cells attenuates liver fibrosis

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Received: 1 February 2019 / Revised: 19 July 2019 / Accepted: 13 August 2019 / Published online: 21 August 2019

Abstract

Trans-differentiation, or activation, of hepatic stellate cells (HSCs) is a hallmark event in liver fibrosis although the underlying mechanism is not fully appreciated. Serum response factor (SRF) is a pleiotropic sequence-specific transcription factor with a ubiquitous expression pattern. In the present study, we investigated the effect of HSC-specific ablation of SRF on liver fibrosis in vivo and the underlying mechanism. We report that SRF bound to the promoter regions of pro-fibrogenic genes, including collagen type I (*Col1a1/Col1a2*) and alpha smooth muscle actin (*Acta2*), with greater affinity in activated HSCs compared to quiescent HSCs. Ablation of SRF in HSCs in vitro downregulated the expression of fibrogenic genes by dampening the accumulation of active histone marks. SRF also interacted with MRTF-A, a well-documented co-factor involved in liver fibrosis, on the pro-fibrogenic gene promoters during HSC activation. In addition, SRF directly regulated MRTF-A transcription in activated HSCs. More importantly, HSC conditional SRF knockout (CKO) mice developed a less robust pro-fibrogenic response in the liver in response to CCl_4 injection and BDL compared to wild-type littermates. In conclusion, our data demonstrate that SRF may play an essential role in HSC activation and liver fibrosis.

Key messages

- SRF deficiency decelerates activation of hepatic stellate cells (HSCs) in vitro.
- SRF epigenetically activates pro-fibrogenic transcription to promote HSC maturation.
- SRF interacts with MRTF-A and contributes to MRTF-A transcription.
- Conditional SRF deletion in HSCs attenuates BDL-induced liver fibrosis in mice.
- Conditional SRF ablation in HSCs attenuates CCl_4-induced liver fibrosis in mice.

Keywords Liver fibrosis · Hepatic stellate cell · Epigenetics · Transcriptional regulation

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00109-019-01831-8) contains supplementary material, which is available to authorized users.

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Abbreviations

HSC	Hepatic stellate cell
SRF	Serum response factor
BDL	Bile duct ligation
MRTF-A	Myocardin-related transcription factor A
CKO	Conditional knockout
ChIP	Chromatin immunoprecipitation
α-SMA	Alpha smooth muscle actin

Introduction

Fibrogenesis is an integral part of the wound healing process and plays key roles in host defense [1]. A myriad of cells and humoral factors participate in this process, which may result in either restoration of organ function or irreversible disruption of organ structure and organ failure. During fibrogenesis, the extracellular matrix (ECM) undergoes dynamic remodeling. Regardless of the etiologies, myofibroblasts are the primary source of ECM production and secretion in fibrotic organs [2]. The origins of myofibroblast have stirred much debate and remain controversial despite the development and application of sophisticated lineage-tracing techniques [3].

Liver fibrosis is a common pathophysiological event following exposure of the liver to a range of stimuli, including pathogens, toxins, nutrients, and medications [4]. The severity of liver fibrosis correlates with the prognosis of end-stage liver diseases, for which effective therapeutic solutions are still lacking. In the liver, activated hepatic stellate cells (HSCs) are considered the major reservoir of myofibroblasts [5]. Quiescent HSCs function as a storage site for vitamins and lipids; once stimulated by a pro-fibrogenic cue, HSCs transdifferentiate into myofibroblasts acquiring dramatically enhanced ability to synthesize ECM proteins, to proliferate and migrate, and to contract [6]. HSC activation is paralleled by a shift in the transcriptome, which is programmed by an array of transcriptional factors.

Serum response factor (SRF) is a pleiotropic transcription factor involved in the regulation of a wide range of pathophysiological processes [7]. SRF regulates transcription by recognizing and binding to the conserved CArG box located on its target promoters [8]. The ability of SRF to regulate transcription relies on its interaction with a group of co-factors that include myocardin, myocardin-related transcription factor A (MRTF-A), and MRTF-B [9]. Mounting evidence points to a role for SRF in tissue fibrogenesis [10]. Formulations of SRF inhibitors or SRF siRNAs have been shown to prevent conjunctival fibrosis [11–13] and lung fibrosis [14]. SRF protein levels correlate with HSC activation in vitro [15]. Of interest, Zheng et al. have recently shown that the long non-coding RNA HOTTIP can act as a competing endogenous RNA (ceRNA) to sequester miR-150, thereby enhancing the expression of SRF, which in turn promotes HSC activation [16]. You et al. have shown that SRF knockdown significantly antagonizes the miR-125b-induced α-SMA expression [17]. In addition, several independent investigations have implicated SRF in liver fibrosis in vivo [17, 18]. However, there is no direct evidence that links HSC-specific SRF deficiency to (attenuated) liver fibrosis. Here we report that SRF programs HSC activation by recruiting MRTF-A and by directly activating MRTF-A transcription. Importantly, HSC-specific SRF ablation attenuates liver fibrosis in vivo. Therefore, our data suggest that SRF may play an essential role in HSC activation and liver fibrosis.

Materials and methods

Animal studies

All the animal protocols were reviewed and approved by the intramural Ethics Committee on Humane Treatment of Experimental Animals. The $Srf^{flox/flox}$ strain [19] was crossbred with the *GFAP*-Cre strain [20, 21] to generate HSC-specific SRF knockout (CKO) mice. To induce liver fibrosis, 6–8-week-old male mice were subjected to bile duct ligation (BDL) or the sham procedure and sacrificed 2 weeks after surgery as previously described [22]. Alternatively, the mice were injected peritoneally with CCl_4 (1.0 mL/kg body weight as 50%, vol/vol) or corn oil weekly for 4 weeks.

Cell isolation, viral infection, and transient transfection

Primary hepatic stellate cells were isolated and maintained as previously described [23]. The cells were infected with adenovirus carrying GFP or Cre (Biowit, China) and harvested 2 days after infection. Small interfering RNA targeting SRF (GAUGGAGUUCAUCGACAACAA) was transfected with Lipofectamine RNAiMax (Thermo) per vendor's recommendation. Immortalized rat hepatic stellate cells (HSC-T6) were maintained in DMEM supplemented with 10% FBS. SRF expression constructs [24] and human MRTF-A promoter-luciferase construct [25] have been previously described. Transient transfection was performed with Lipofectamine 2000. Cells were harvested 48 h after transfection and reporter activity was measured using a luciferase reporter assay system (Promega) as previously described [26]. Briefly, cells were plated in 12-well culture dishes (~ 60,000 cells/well). The next day, 0.1 μg of reporter construct and 0.1–0.3 μg of effector construct (SRF WT or SRF DN) were transfected into each well. DNA content was normalized by the addition of an empty vector (pcDNA3). For monitoring transfection efficiency and for normalizing luciferase activity, 0.02 μg of GFP construct was transfected into each well.

Protein extraction, immunoprecipitation, and Western blot

Whole cell lysates were obtained by re-suspending cell pellets in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100) with freshly added EDTA-free protease inhibitor tablet (Roche) as previously described [27]. Western blot analyses were performed with anti-SRF (sc-13029, Santa Cruz), anti-collagen type I (600-403-103, Rockland), anti-β-actin (A1978, Sigma), anti-α-SMA (ab5694, Abcam), and anti-MRTF-A (sc-32,909, Santa Cruz) antibodies. All experiments were repeated at least three times.

RNA isolation and real-time PCR

RNA was extracted with the RNeasy RNA isolation kit (Qiagen). Reverse transcriptase reactions were performed as previously described using a SuperScript First-strand Synthesis System (Invitrogen) [28]. Data were normalized with 18S rRNA as an internal control according to manufacturer's protocol and expressed as fold change over the control group. All experiments were repeated at least three times.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed essentially as described before [29–31]. Briefly, chromatin in control and treated cells were cross-linked with 1% formaldehyde. Cells were incubated in lysis buffer (150 mM NaCl, 25 mM Tris pH 7.5, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate) supplemented with protease inhibitor tablet and PMSF. DNA was fragmented into ~ 500 bp pieces using a Branson 250 sonicator. Aliquots of lysates containing 200 μg of protein were used for each immunoprecipitation reaction with anti-acetyl histone H3 (06-599, Millipore), anti-acetyl histone H4 (06-866, Millipore), anti-trimethyl H3K4 (07-473, Millipore), anti-p300 (sc-585, Santa Cruz), anti-ASH2 (A300-489A, Bethyl Laboratories), anti-WDR5 (A302-429A, Bethyl Laboratories), anti-SRF (sc-13,029, Santa Cruz), and anti-MRTF-A (sc-32,909, Santa Cruz) antibodies. Precipitated genomic DNA was amplified by real-time PCR with the following primers: for *Col1a1*, 5′-ATTTGAAGTCCCAGAAAG-3′ and 5′-AGAAACTCCCGTCTGCTC-3′; for *Col1a2*, 5′-CTTCGTGCATGACTTCAGCTTT-3′ and 5′-CGTCCTTTAGCATGGCAAGAC-3′; for *Acta2*, 5′-CCTGTTTCGGGAGCAGAA-3′ and 5′-GGTTATATAGCCCCCTGG-3′; for *Col3a1*, 5′-GACTCTGGCAAAACTCAAAGTATCA-3′ and 5′-TAGGAATGTGCTTTGTGATAGCCT-3′; for *Lox*, 5′-ACGTTTCCAATCACATTACG-3′ and 5′-ACGGTCCTCCTCTCCCCTTT-3′; for *Gapdh*, 5′-ATCACTGCCACCCAGAAGACTGTGGA-3′ and 5′-CTCATACCAGGAAATGAGCTTGACAAA-3′. All experiments were repeated at least three times.

Histology

Histological analyses were performed essentially as described before [32–34]. Briefly, paraffin sections were stained with picrosirius red (Sigma) or Masson's trichrome (Sigma) according to standard procedures. Pictures were taken using an Olympus IX-70 microscope.

Statistical analysis

One-way ANOVA with post hoc Scheffe analyses were performed using an SPSS package. Unless otherwise specified, *p* values smaller than .05 were considered statistically significant (*).

Results

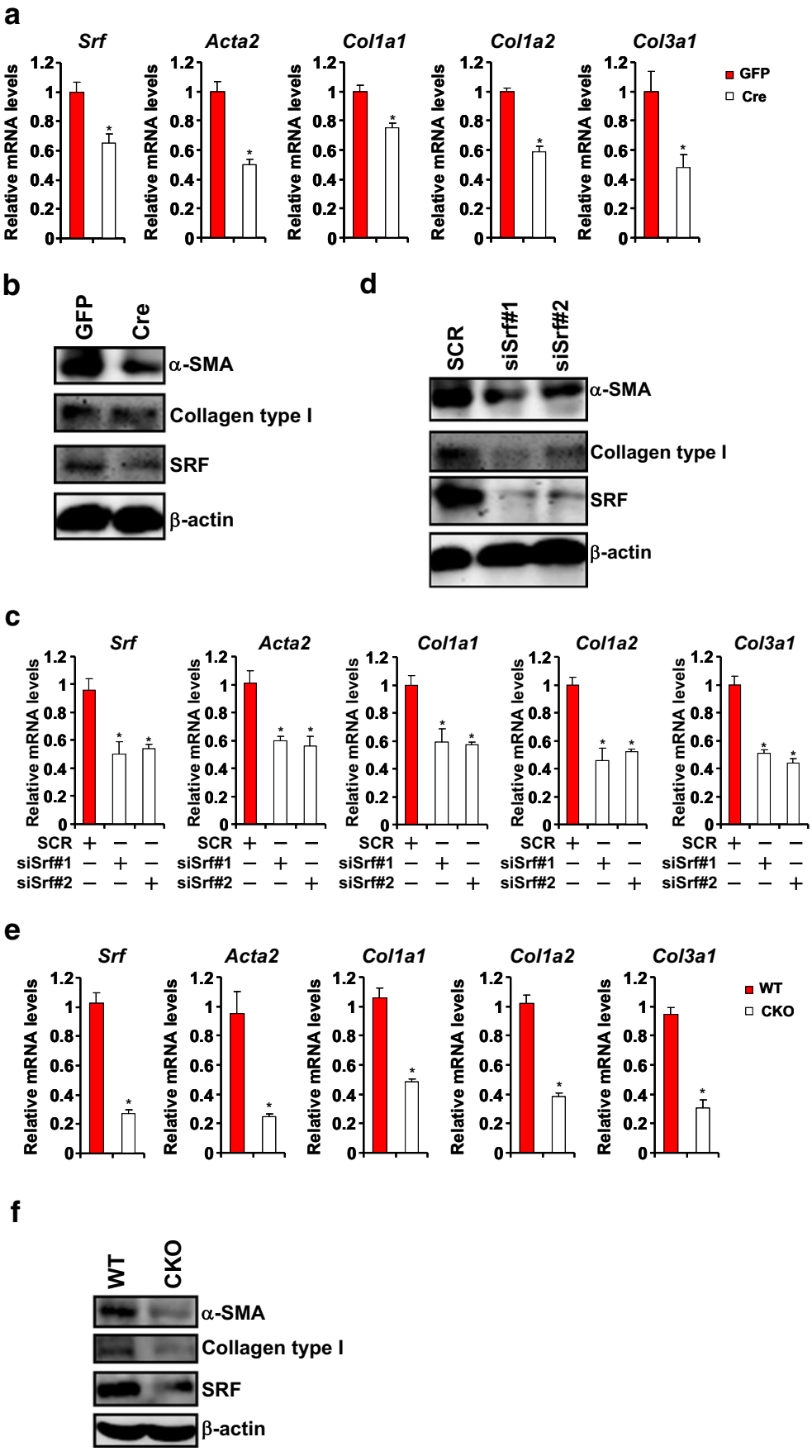
SRF regulates pro-fibrogenic transcription in HSCs

We first examined whether SRF depletion might dampen HSC activation in vitro. We isolated primary HSCs from $Srf^{f/f}$ mice and deleted SRF by infecting these cells with adenovirus carrying Cre enzyme. As shown in Fig. S1, when the cells were harvested at day 8, they typically had a myofibroblast-like morphology and expressed large amount of α-SMA indicative of successful trans-differentiation. Compared to HSCs infected with GFP adenovirus, Cre adenovirus significantly down-regulated SRF levels. Consequently, expression of pro-fibrogenic genes, including collagen type I (*Col1a1*/*Col1a2*), collagen type III (*Col3a1*), and α-SMA (*Acta2*), was decreased at both mRNA (Fig. 1a) and protein (Fig. 1b) levels. We also tried an alternative strategy by depleting SRF with siRNA. Two separate pairs of siRNAs comparably decreased SRF expression. Concomitantly, SRF siRNAs repressed the expression of pro-fibrogenic genes (Fig. 1c, d).

We also crossed the SRF-Flox mouse strain [19] with a GFAP-Cre strain [20, 21] to specifically delete SRF in HSCs. Primary HSCs were isolated from SRF conditional knockout (CKO) mice or wild-type (WT) mice and allowed to undergo spontaneous activation. As shown in Fig. 1e, f, expression of pro-fibrogenic genes were significantly down-regulated in HSCs isolated from CKO mice compared to those from WT mice.

SRF recruits MRTF-A to activate pro-fibrogenic transcription during HSC activation

We then assessed the activity of SRF during HSC activation by examining its recruitment to pro-fibrogenic gene promoters. ChIP assays showed that SRF did not bind to the promoter regions of *Col1a1*, *Col1a2*, *Col3a1*, and *Acta2*, all of which contain at least one verified CArG box [23, 35–37],


a
Srf
Acta2
Col1a1
Col1a2
Col3a1
Relative mRNA levels
GFP
Cre
b
GFP
Cre
α-SMA
Collagen type I
SRF
β-actin
d
SCR
siSrf#1
siSrf#2
α-SMA
Collagen type I
SRF
β-actin
c
Srf
Acta2
Col1a1
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Col3a1
Relative mRNA levels
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siSrf#1
siSrf#2
e
Srf
Acta2
Col1a1
Col1a2
Col3a1
Relative mRNA levels
WT
CKO
f
WT
CKO
α-SMA
Collagen type I
SRF
β-actin

◀ **Fig. 1** SRF regulates pro-fibrogenic transcription in HSCs. **a**, **b** Primary hepatic stellate cells were isolated from $Srf^{f/f}$ mice and allowed to undergo spontaneous activation. At day 5, the cells were infected with adenovirus carrying GFP or Cre. Expression of pro-fibrogenic gene expression was measured by qPCR (**a**) and Western (**b**). **c**, **d** Primary hepatic stellate cells were isolated from C57/BL6 mice and allowed to undergo spontaneous activation. At day 5, the cells were transfected with siRNA targeting SRF or scrambled siRNA (SCR). Expression of pro-fibrogenic gene expression was measured by qPCR (**c**) and Western (**d**). **e**, **f** Primary hepatic stellate cells were isolated from WT and SRF CKO mice and allowed to undergo spontaneous activation for 7 days. Expression of pro-fibrogenic gene expression was measured by qPCR (**e**) and Western (**f**). All experiments were repeated three times

with significant affinity relative to the IgG control during the initial phase of HSC activation (Fig. 2a). At day 4 after HSC activation, SRF started to occupy the pro-fibrogenic gene promoters, and by day 8, SRF bound to the promoters with even higher affinity. By comparison, SRF did not bind to the *Lox* promoter either before or after HSC activation (Fig. 2a).

Myocardin-related transcription factor A (MRTF-A) is a co-factor for SRF and a well-documented pro-fibrogenic protein [23, 35, 38–40]. We asked whether SRF might recruit MRTF-A to activate pro-fibrogenic transcription during HSC activation. Indeed, Re-ChIP assay showed

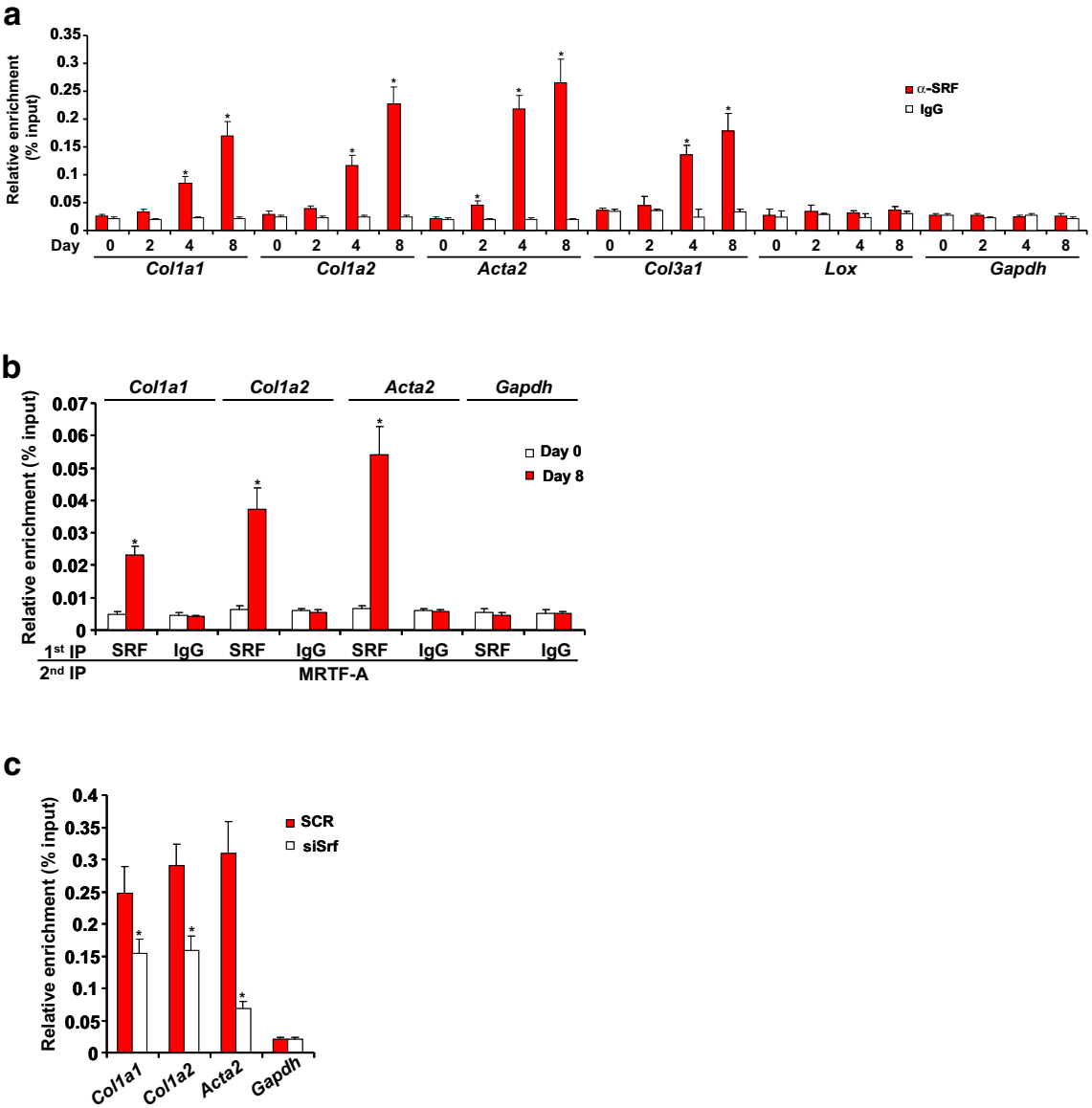


Fig. 2 SRF recruits MRTF-A to activate pro-fibrogenic transcription during HSC activation. **a** Primary hepatic stellate cells were isolated from C57/BL6 mice and allowed to undergo spontaneous activation. The cells were harvested at indicated time points and ChIP assays were performed with anti-SRF or IgG. **b** Primary hepatic stellate cells were isolated from C57/BL6 mice and allowed to undergo spontaneous activation. The cells were harvested at indicated time points and Re-ChIP assays were performed with indicated antibodies. **c** Primary hepatic stellate cells were isolated from C57/BL6 mice and allowed to undergo spontaneous activation. At day 5, the cells were transfected with siRNA targeting SRF or scrambled siRNA (SCR). ChIP assays were performed with anti-MRTF-A. All experiments were repeated three times

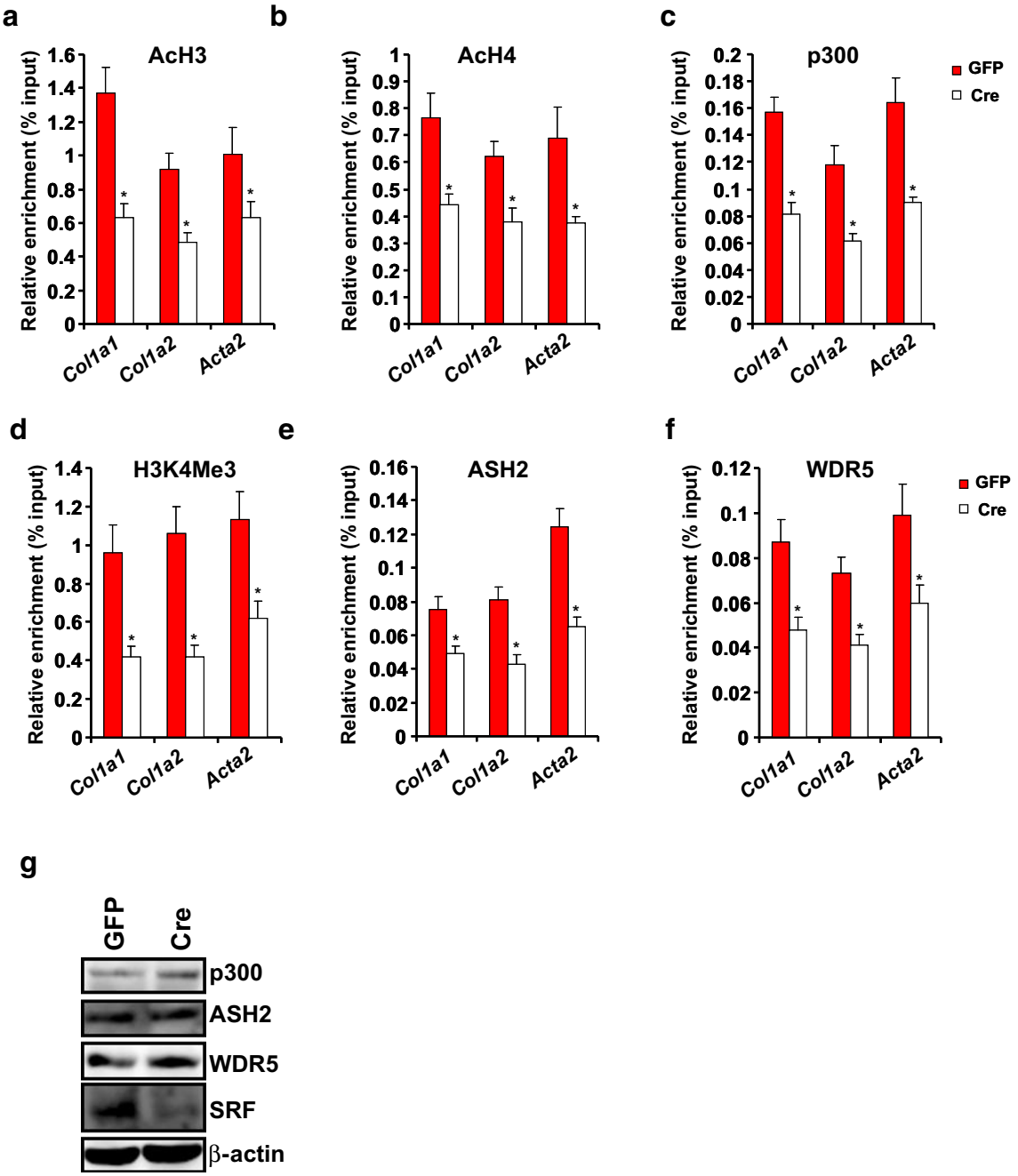


Fig. 3 SRF regulates pro-fibrogenic transcription by recruiting epigenetic co-factors. **a**–**g** Primary hepatic stellate cells were isolated from $Srf^{f/f}$ mice and allowed to undergo spontaneous activation. At day 5, the cells were infected with adenovirus carrying GFP or Cre. ChIP assays were performed with anti-acetylated histone H3 (**a**), anti-acetyl histone H4 (**b**), anti-p300 (**c**), anti-trimethylated H3K4 (**d**), anti-ASH2 (**e**), and anti-WDR5 (**f**). **g** Protein expression was examined by Western. All experiments were repeated three times

that an SRF-MRTF-A complex could be detected on the pro-fibrogenic promoters in activated HSCs as opposed to the quiescent HSCs (Fig. 2b). In addition, SRF knockdown by siRNA significantly weakened the binding of MRTF-A to target promoters (Fig. 2c). Combined, these data suggest that SRF may play a role in pro-fibrogenic transcription by recruiting MRTF-A during HSC activation.

SRF regulates pro-fibrogenic transcription by recruiting epigenetic co-factors

We have previously shown that MRTF-A recruits histone-modifying enzymes to activate the transcription of pro-fibrogenic genes, thereby promoting HSC maturation [23]. We asked whether SRF deficiency would cripple the interaction between the histone-modifying enzymes with pro-

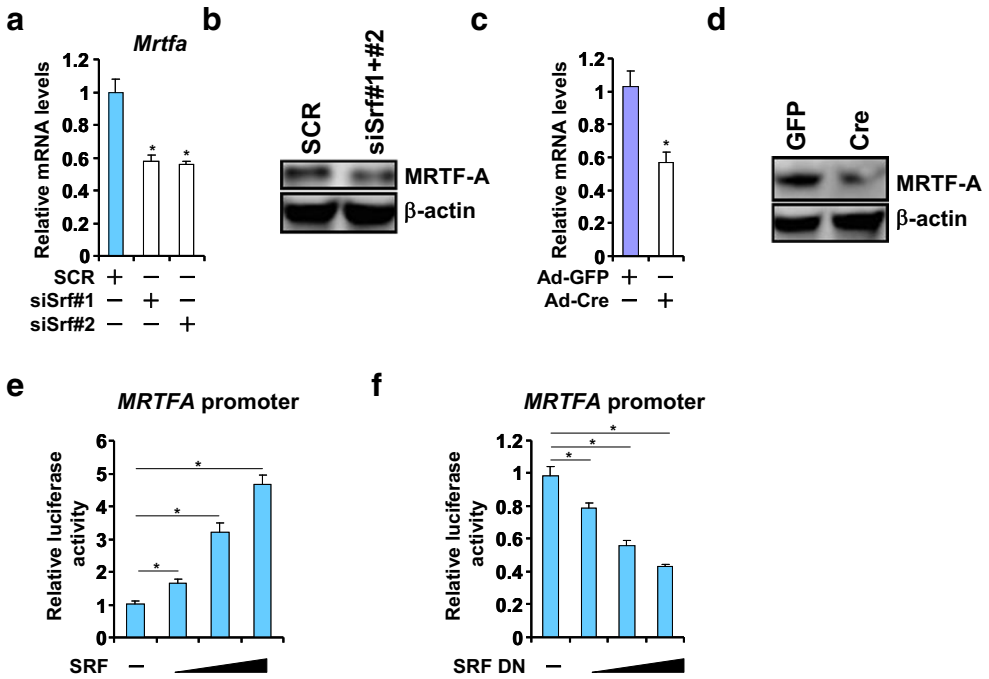


Fig. 4 SRF directly regulates MRTF-A transcription during HSC activation. **a**, **b** Primary hepatic stellate cells were isolated from C57/BL6 mice and allowed to undergo spontaneous activation. At day 5, the cells were transfected with siRNA targeting SRF or scrambled siRNA (SCR). MRTF-A expression was measured by qPCR and Western. **c**, **d** Primary hepatic stellate cells were isolated from $Srf^{f/f}$ mice and allowed to undergo spontaneous activation. At day 5, the cells were infected with adenovirus carrying GFP or Cre. MRTF-A expression was measured by qPCR and Western. **e** An MRTF-A promoter luciferase construct was transfected into HSC-T6 cells with or without increasing doses of SRF expression construct. Luciferase activities were normalized by both GFP fluorescence and protein concentration. **f** An MRTF-A promoter luciferase construct was transfected into HSC-T6 cells with or without increasing doses of dominant negative SRF expression construct. Luciferase activities were normalized by both GFP fluorescence and protein concentration. All experiments were repeated three times

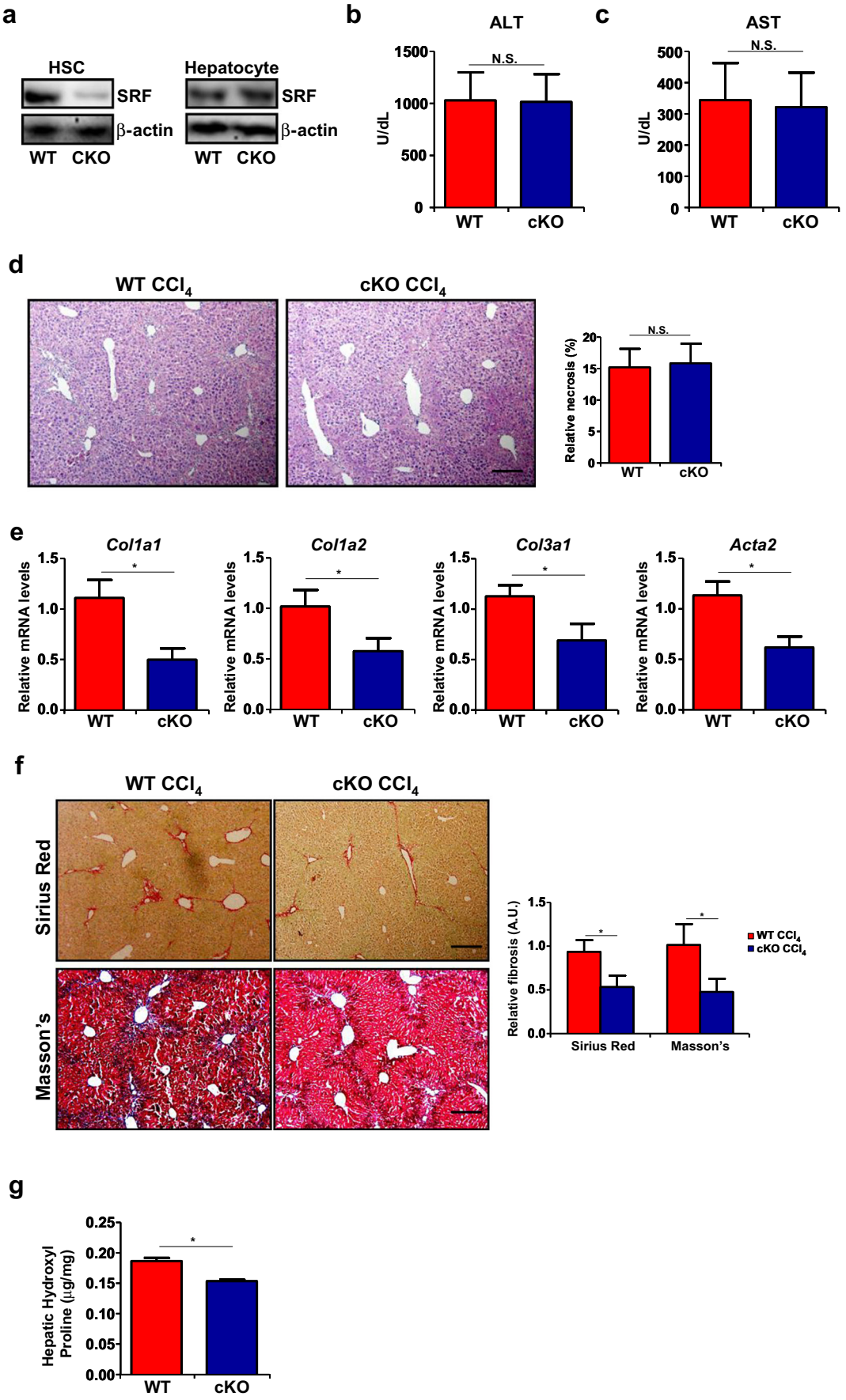
fibrogenic gene promoters. Indeed, SRF depletion via Cre adenovirus infection significantly downregulated the enrichment of acetylated H3 (Fig. 3a) and H4 (Fig. 3b) surrounding the pro-fibrogenic gene promoters. In accordance, occupancies of p300, a key acetyltransferase responsible for H3 and H4 acetylation, were reduced by SRF deletion (Fig. 3c). In addition, we also found that SRF deletion attenuated the accumulation of trimethylated H3K4 surrounding the pro-fibrogenic gene promoters (Fig. 3d). H3K4 trimethylation is catalyzed by the COMPASS complex in mammals [41]. Congruent with decreased trimethyl H3K4 levels, SRF deletion also weakened the binding of ASH2 (Fig. 3e) and WDR5 (Fig. 3f), two core components of COMPASS, to the pro-fibrogenic gene promoters. Of note, SRF deletion did not alter the expression levels of these histone-modifying proteins (Fig. 3g).

Similarly, SRF silencing by siRNA dampened the deposition of acetyl H3 (Fig.S2A), acetyl H4 (Fig.S2B), and trimethyl H3K4 (Fig.S2C) surrounding the pro-fibrogenic gene promoters. The decrease in histone modifications was probably due to the weakened recruitment of histone-modifying enzymes such as p300 (Fig.S2D), ASH2 (Fig.S2E), and WDR5 (Fig.S2F). Taken together, these data suggest that SRF might contribute to pro-fibrogenic transcription by recruiting epigenetic co-factors to influence locus-specific histone modifications.

SRF directly regulates MRTF-A transcription during HSC activation

Of interest, we found that SRF depletion by siRNA in HSCs led to a decrease in MRTF-A expression at both mRNA (Fig. 4a) and protein (Fig. 4b) levels. Similarly, Cre-mediated SRF ablation in HSCs also reduced MRTF-A expression (Fig. 4c, d), indicating that SRF might directly regulate MRTF-A transcription to promote HSC activation.

We next transfected into immortalized rat HSC cells (HSC-T6) an MRTF-A promoter-luciferase construct with or without an SRF expression construct (Fig. 4e). SRF over-expression dose-dependently activated the MRTF-A promoter activity. On the contrary, over-expression of a dominant negative (DN) SRF construct repressed the MRTF-A promoter activity in a dose-dependent manner (Fig. 4f). These data collectively suggest that SRF may regulate HSC phenotype by directly activating MRTF-A transcription.


a
HSC
Hepatocyte
SRF
β-actin
WT CKO
WT CKO
b
ALT
U/dL
N.S.
WT cKO
c
AST
U/dL
N.S.
WT cKO
d
WT CCl4
cKO CCl4
Relative necrosis (%)
N.S.
WT cKO
e
Col1a1
Col1a2
Col3a1
Acta2
Relative mRNA levels
WT cKO
f
WT CCl4
cKO CCl4
Sirius Red
Masson's
Relative fibrosis (A.U.)
WT CCl4
cKO CCl4
Sirius Red
Masson's
g
Hepatic Hydroxyl Proline (μg/mg)
WT cKO

◀ **Fig. 5** SRF ablation attenuates CCl_4-induced liver fibrosis in mice. **a** Primary hepatocytes and HSCs were isolated from WT and SRF CKO mice. SRF expression was examined by Western blotting. **b**–**g** WT and SRF CKO mice were injected with CCl_4 to induce liver fibrosis as described in "Materials and methods." **b** Plasma ALT levels. **c** Plasma AST levels. **d** H&E staining. **e** Hepatic expression of pro-fibrogenic genes was examined by qPCR. **f** Picrosirius red and Masson's trichrome stainings. **g** Hepatic hydroxyproline levels. $N = 8$~9 mice for each group. Error bars represent SD (*$p < 0.05$, two-tailed t test)

SRF ablation attenuates liver fibrosis in mice

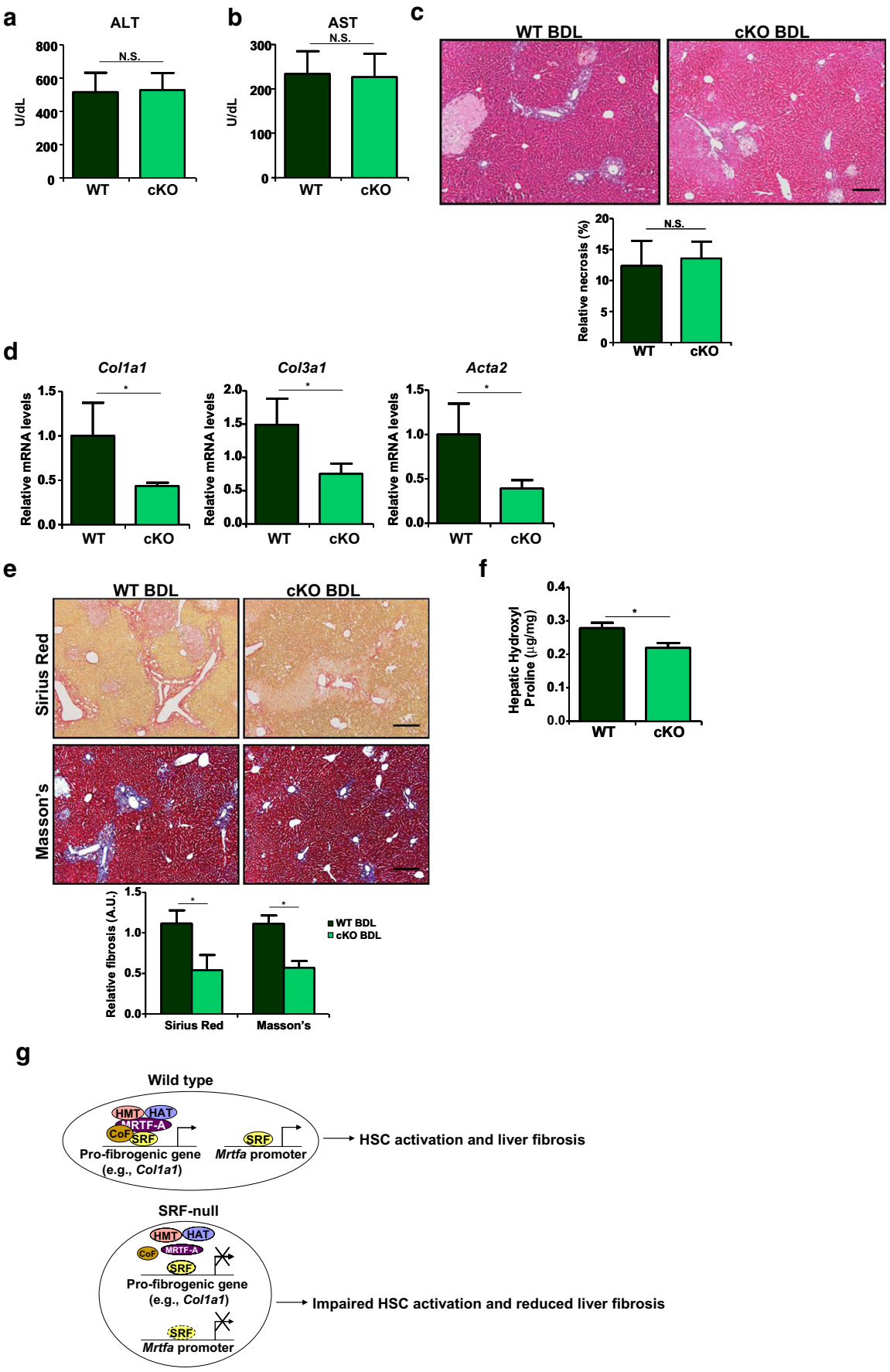
In order to probe the role of SRF in HSC activation and liver fibrosis in vivo, we exploited two classic animal models of liver fibrosis. Western blotting showed that SRF expression was decreased in primary HSCs, but not hepatocytes, isolated from CKO ($Srf^{f/f}$; *GFAP*-Cre) mice compared to those isolated from WT ($Srf^{f/f}$) mice (Fig. 5a). Both the CKO and the WT mice were subjected to weekly CCl_4 injection for 4 weeks. CCl_4 injection in WT and CKO mice inflicted comparable liver damages, as evidenced by plasma ALT (Fig. 5b) and AST (Fig. 5c) levels and by H&E staining of liver sections (Fig. 5d). Several lines of evidence indicate that liver fibrosis was attenuated in CKO mice compared to WT mice. First, qPCR measurements showed that expression levels of pro-fibrogenic genes were collectively downregulated in the CKO livers (Fig. 5e). Second, both picrosirius red staining and Masson's trichrome staining showed that liver fibrosis was less severe in CKO mice than in WT mice (Fig. 5f). Finally, hepatic hydroxyproline quantification confirmed that fibrillar collagen levels were decreased in CKO livers (Fig. 5g).

Next, we exploited an alternative mouse model of liver fibrosis in which the mice were subjected to bile duct ligation (BDL) and sacrificed 2 weeks after the surgery. Both the WT mice and the CKO mice displayed similar levels of liver injury induced by the BDL procedure as gauged by plasma ALT (Fig. 6a) and AST (Fig. 6b) as well as hepatic necrotic areas identified by H&E staining (Fig. 6c). Quantitative PCR showed that SRF deficiency in HSCs reduced the expression of pro-fibrogenic genes (Fig. 6d). In accordance, picrosirius red and Masson's trichrome stainings indicate that liver fibrosis was less widespread in CKO livers than in WT livers (Fig. 6e). Consistently, fewer fibrillar collagens were detected by hepatic hydroxyproline quantification (Fig. 6f). Together, these data support an essential for HSC-specific SRF in liver fibrosis.

Discussion

Recent investigations strongly argue for a role for SRF in liver fibrosis. Here we provide direct evidence that links SRF deficiency in hepatic stellate cells to attenuated liver fibrosis in mice. Herrmann et al. previously have shown that SRF expression is upregulated by TGF-β in activated HSCs [15]. Moreover, SRF levels are found to be higher in the livers of Long-Evans Cinnamon rats that develop hepatitis and hepatocellular carcinoma, both of which are preceded or followed by liver fibrosis [42]. Of note, ablation of SRF in HSCs did not appear to affect either hepatotoxic substance (CCl_4)-induced liver injury or cholestatic liver injury (BDL). These observations contrast a previous report wherein hepatocyte-specific deletion of SRF leads to partial lethality and postnatal growth retardation owing to increased apoptosis of hepatocyte [43], indicating that SRF may play cell type-dependent, distinctive roles in liver injury and liver fibrosis. It alludes to one of main weaknesses of the present study. We focused on the role of HSC-specific SRF in liver fibrosis because HSCs are thought to be the predominant source of myofibroblasts in the liver. Other cell types, including endothelial cells [44] and portal fibroblast cells [45], also contribute to this process. In addition, the specificity of the Cre driver (GFAP) used in this study to delete SRF in HSCs has been called into question recently [5]. The potential effect of SRF deletion on HSC development cannot be ignored since SRF is absent the entire time even when HSCs are quiescent. Clearly, further studies are warranted to determine the role of SRF in liver fibrosis.

We and others have previously shown that MRTF-A is a key regulator of liver fibrosis [23, 38, 46]. Here we show that SRF recruited MRTF-A to the pro-fibrogenic gene promoters. Moreover, SRF deficiencies are synonymous with a repressed chromatin structure surrounding the promoter regions of the genes involved in fibrogenesis. The ability to engage the epigenetic machinery is considered a paradigm in SRF-dependent regulation of smooth muscle phenotypic modulation [47]. More recently, Rosen and colleagues have performed extensive ChIP-seq analysis to correlate specific chromatin structure with activation of genes key to adipogenesis, which consequently leads to the identification of SRF as a novel transcription factor that bridge epigenetic factors to locus-specific gene transcription [48]. It is possible that SRF may regulate HSC trans-differentiation by the same virtue. Alternatively, SRF may rely on MRTF-A to recruit various histone-modifying enzymes to activate transcription because MRTF-A has been found to make extensive dialogs with the epigenetic machinery [25, 49–53]. We note that there is a caveat regarding this model because SRF can directly regulate MRTF-A transcription (Fig. 3). The observation that MRTF-A recruitment was impaired in the absence of SRF might be due to decreased MRTF-A expression (Fig. 2). In the same vein, attenuation of histone-modifying enzymes on the SRF target promoters in SRF-deficient cells may also be attributed to lower MRTF-A levels (Fig. 4). We propose that SRF regulates pro-fibrogenic transcription via several inter-dependent mechanisms, by recruiting co-factors (e.g., MRTF-A), by directly


a
ALT
U/dL
N.S.
WT
cKO
b
AST
N.S.
c
WT BDL
cKO BDL
Relative necrosis (%)
d
Col1a1
Col3a1
Acta2
Relative mRNA levels
e
WT BDL
cKO BDL
Sirius Red
Masson's
Relative fibrosis (A.U.)
f
Hepatic Hydroxyl Proline (μg/mg)
g
Wild type
HMT
HAT
MRTF-A
CoF
SRF
Pro-fibrogenic gene (e.g., Col1a1)
Mrtfa promoter
HSC activation and liver fibrosis
SRF-null
Impaired HSC activation and reduced liver fibrosis

◀ **Fig. 6** SRF ablation attenuates BDL-induced liver fibrosis in mice. **a**–**f** WT and SRF CKO mice were subjected to BDL to induce liver fibrosis as described in "Materials and methods." **a** Plasma ALT levels. **b** Plasma AST levels. **c** H&E staining. **d** Hepatic expression of pro-fibrogenic genes was examined by qPCR. **e** $N = 6$ mice for each group. Error bars represent SD (*$p < 0.05$, two-tailed t test). **g** A schematic model. In wild-type HSCs, SRF recruits MRTF-A and possibly other co-factors (CoF), which in turn may help bring various histone-modifying enzymes to remodel the chromatin surrounding and activate the transcription of the pro-fibrogenic gene promoters. SRF may also directly activate MRTF-A transcription and maintain MRTF-A levels in HSCs. In SRF null HSCs, transcription of both the pro-fibrogenic genes and MRTF-A is disrupted leading to impaired HSC activation and attenuation of liver fibrosis

controlling the availability (expression) of co-factors (e.g., MRTF-A), and by engaging histone-modifying enzymes. In addition to MRTF-A, other SRF co-factors have been found to bridge SRF to the epigenetic machinery. BRG1, for instance, has been shown to act as a co-factor for SRF-dependent transcription of smooth muscle-specific genes by forming a complex with both SRF and MRTF-A [51]. We have reported previously that BRG1 can interact with and recruit several different histone-modifying enzymes to regulate transcription [54–58]. SRF is absolutely required for the integrity of this complex; without SRF, the stability of these binding factors are affected so that pro-fibrogenic transcription is essentially shut down (Fig. 6g). The lingering issues regarding this model as highlighted above must be resolved to clarify the epigenetic mechanism whereby SRF regulates liver fibrosis.

An interesting finding in the present study is that SRF may directly regulate MRTF-A transcription in activated HSCs. Although the activation of MRTF-A is thought to be determined predominantly by its sub-cellular localization, MRTF-A expression levels are sensitive to various cues attributable to both transcriptional and post-transcriptional regulation. We have previously shown that during HSC activation, MRTF-A proteins are upregulated via a post-transcriptional mechanism whereby the histone deacetylase HDAC4 represses miR-206 to stabilize MRTF-A messages [46]. Our data suggest that multiple mechanisms contribute to the maintenance of MRTF-A levels in HSCs to sustain fibrogenesis. It remains to be determined whether SRF may directly bind to the MRTF-A promoter and activate its transcription.

In summary, we provide evidence to directly link HSC-specific SRF to liver fibrosis both in vitro and in vivo. Future studies exploiting additional mouse models and transcriptomic/epigenomic techniques will hopefully solidify the role for SRF as a key regulator of fibrogenesis and pave the way for targeting SRF in the intervention of liver fibrosis.

Funding information This work was supported, in part, by grants from the National Natural Science Foundation of China (81725001, 81700554, 81770487), from the Nanjing Municipal Administration of Health and Human Services (YKK17061), from the Fundamental Research Funds for Central Universities (021414380323), and from the Haihan Province R&D Fund Key Project (ZDYF2018102).

Compliance with ethical standards

All the animal protocols were reviewed and approved by the intramural Ethics Committee on Humane Treatment of Experimental Animals.

Conflict of interest The authors declare that they have no competing interests.

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