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Stat3 signaling activation crosslinking of TGF-β1 in hepatic stellate cell exacerbates liver injury and fibrosis

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ABSTRACT

Background/Aims: The role of signal transducer and activator of transcription 3 (Stat3) in liver fibrosis is still controversial. Since hepatic stellate cells (HSCs) and transforming growth factor-β1 (TGF-β1) are central to the fibrogenesis, our goal was to clarify the mechanism of Stat3 crosslinking of TGF-β1 signaling.
Methods: Stat3, TGF-β1 mRNA and protein expressions were examined in liver tissues of chronic hepatitis B (CHB) patients and diethylinitrosamine (DEN)-induced rat fibrosis model. The effect of Stat3 activation or suppression on TGF-β1 signaling in HSCs was tested in vitro and in vivo.
Results: Stat3 expression as well as TGF-β1 was increased in CHB patients and DEN-induced fibrosis rat model. This was strongly correlated with increase in fibrosis staging. TGF-β1, a mediator of fibrosis, was enhanced by Stat3, but suppressed by siRNA-mediated RNA knockdown of Stat3 (siStat3) or Janus kinase 2 inhibitor (AG490) both in vivo and in vitro. Stat3 crosslinking TGF-β1 signaling plays an important role in HSC activation and increasing fibrosis related products. TGF-β1 could not achieve profibrogenic cytokine and anti-apoptosis characteristics without Stat3 activation in HSCs.
Conclusion: We provide a novel role of Stat3 cooperating TGF-β1 in activation and anti-apoptotic effect of HSCs. Stat3 worsens liver fibrosis through the up-regulation of TGF-β1 and fibrotic product expression.
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1. Introduction

Liver fibrosis and its end stage cirrhosis represent the final pathway of chronic liver diseases [1]. Progression of liver fibrosis is a dynamic process, characterized by accumulation exceeding degradation of extracellular matrix (ECM). Over the past two decades, sinusoidal resident hepatic stellate cells (HSCs) have been recognized as the major source of ECM. HSC activation and trans-differentiation into myofibroblasts are believed to be the key events in liver fibrogenesis [2].

Transforming growth factor-β1 (TGF-β1) is considered a pivotal profibrogenic cytokine in the liver [2] and in vitro, TGF-β1 participates in every step of HSC activation. TGF-β1 triggers phenotypical HSC transdifferentiation from quiescence to activation by paracrine and autocrine action, and directly induces collagen I (Col 1) expression and α-smooth muscle actin (α-SMA) stress fiber organization [3,4]. TGF-β1 signals through transmembrane receptors consisting of type I (TGF-βRI) and II (TGF-βRII) heterodimers to initiate downstream signaling via Smad proteins, which modulate the transcription of target genes, including those encoding ECM components, such as procollagen-I and -III [5,6].

Signal transducer and activator of transcription 3 (Stat3) is a transcription factor that is phosphorylated by Janus tyrosine kinases (Jak) in response to cytokine activation which then dimerizes and moves into the nucleus to activate transcription of cytokine-responsive genes. Cytokines that activate Stat3 include growth hormone (GH), IL-6 family cytokines, and G-CSF. Stat3 induces progression through the cell cycle, prevents apoptosis and may be associated with cancer development. Stat3 activation is detected virtually in all rodent models of liver injury and in human liver diseases. However, the role of Stat3 pathway in liver fibrogenesis appears to be controversial. Due to the hepatoprotective and proliferative functions of Stat3, Stat3 in hepatocytes plays a protective role in preventing liver fibrosis [7–11]. Interestingly, Stat3 plays a counteraction role in HSCs. Inhibition of the Stat3 pathway induces apoptosis of HSC [12]. Mice devoid of Stat3 signaling in HSCs ($GFAPStat3^{-/-}$ mice) are less susceptible to fibrosis [13]. Stat3 pathway mediates the promotional role of leptin in liver fibrosis in mouse model and is involved in leptin inhibition of SREBP-1c expression in HSCs in vivo and in vitro [14]. Importantly, Ogata first revealed that TGF-β1 is a target gene of Stat3 and Stat3 enhances hepatic fibrosis through

Abbreviations: ECM, extracellular matrix; HSCs, hepatic stellate cells; TGF-β1, transforming growth factor-β1; Col 1, collagen I; α-SMA, α-smooth muscle actin; Stat3, signal transducer and activator of transcription 3; Jak, Janus tyrosine kinase; GH, growth hormone; CHB, chronic hepatitis B; DEN, diethylinitrosamine; HBV, hepatitis B virus; AF, advanced fibrosis; EF, early fibrosis; Vim, vimentin; IH, immunohistochemistry; IF, immunofluorescence; DAPI, 4′6-diamidino-2-phenylindole; qPCR, quantitative polymerase chain reaction; WB, western blotting; Fn 1, fibronectin 1

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upregulation of TGF-β1 in hepatocytes and in liver fibrosis mice model [15]. Kinjyo identified two Stat3-binding sites in the promoter region of TGF-β1 and demonstrated that Stat3 activates the promoter activity in T cells [16].

Given that to date no studies have defined clearly the impact of Stat3 on TGF-β1 expression, we investigated possible molecular mechanisms of Stat3 dependent TGF-β1 expression in HSCs. This hypothesis was also tested in liver tissues of chronic hepatitis B (CHB) patients and diethylinitrosamine (DEN) induced liver fibrosis rat model. We also examined whether TGF-β1/Smad signaling is activated by IL-6/Stat3.

2. Materials and methods

2.1. Experimental animal models

Seventy-five male fibrosis Wistar rats model was induced by watering of 0.05 g/l diethylinitrosamine (DEN) daily. Thirty rats were administered selective Janus kinase (Jak) 2 inhibitor tyrphostin AG490 (1 mg/kg/d) intraperitoneally in first week. Early fibrosis (EF) rats were sacrificed 4 weeks after DEN watering and advanced fibrosis (AF) rats were sacrificed 8 weeks. Rats were then separated into 5 groups (Control: $n = 15$; EF: $n = 15$; EF + AG490: DEN 4wk plus AG490 1wk, $n = 15$; AF: $n = 15$; AF + AG490: DEN 8wk plus AG490 1wk, $n = 15$). These studies were approved by the Ethical Committee of the Shanghai First People's Hospital (Shanghai, China).

2.2. Cell culture and lentivirus

Primary HSCs were isolated from livers of Wistar rats and cultured on plastic dishes in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA), supplemented with 4 mmol/l L-glutamine, 10%FCS, and penicillin (100 IU/ml)/streptomycin (100 mg/ml). Primary HSCs were treated with TGF-β1 (Miltenyi Biotec, Auburn, CA) at 2 ng/ml or IL-6 at 10 ng/ml, respectively. Lentivirus encoding siRNA gene of Stat3 for transduction (siStat3) and the control virus showed green fluorescent protein (GFP) expression in the cells (Microbix, Ontario, Canada).

2.3. Patients and liver samples

All human liver tissues were obtained through percutaneous biopsy of liver of patients at Shanghai First People's Hospital which included

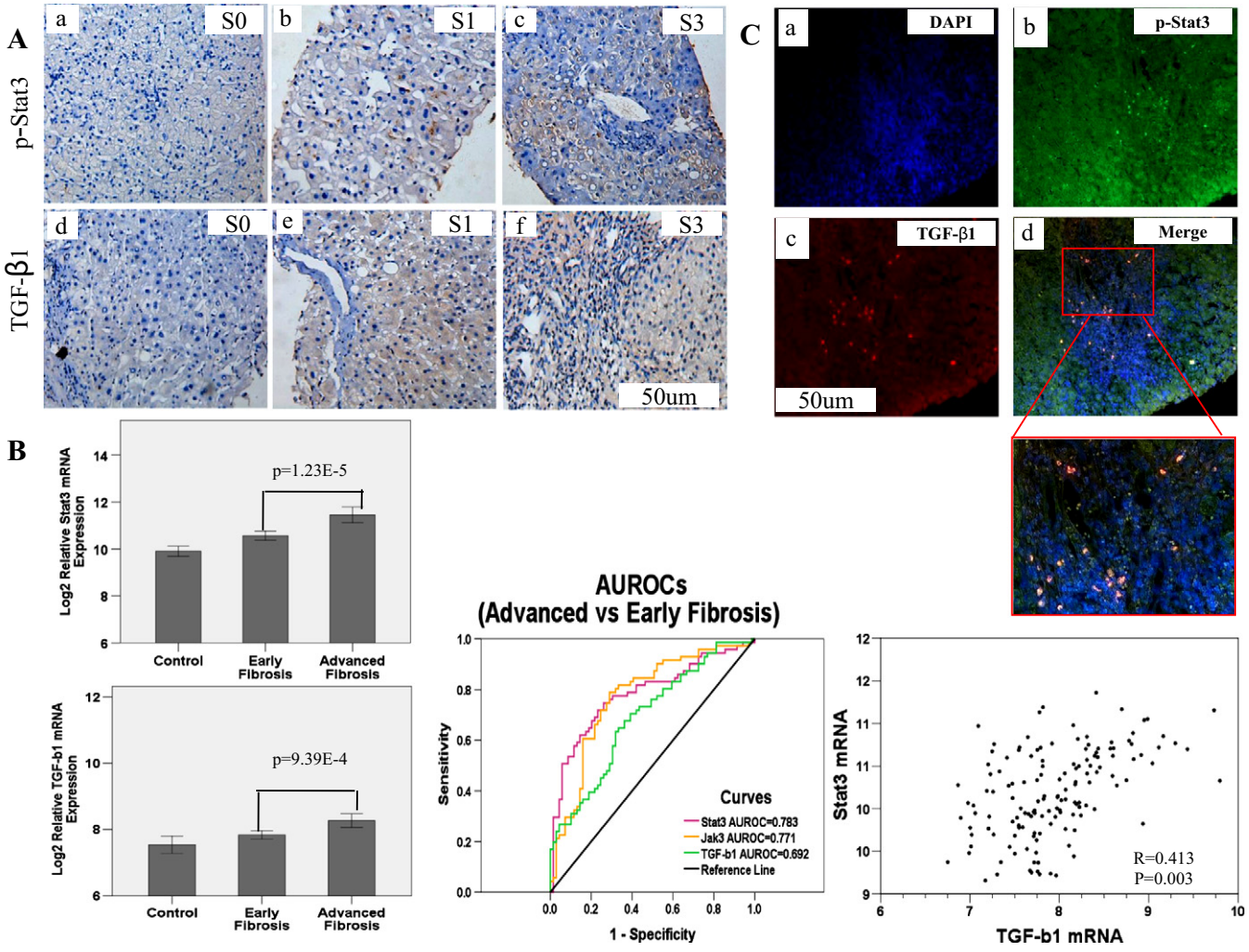

Fig. 1. Stat3 and TGF-β1 expression in liver fibrosis of CHB patients. Stat3, TGF-β1 mRNA and protein expression were examined in liver tissues of 60 CHB patients by use of IH/IF staining and qPCR. Patients were divided to 3 groups: Control, $n = 10$; early fibrosis (EF, liver fibrotic stages: S0–S2), $n = 30$; advanced fibrosis (AF, liver fibrotic stages: S3–S4), $n = 20$. IH, IF and qPCR were performed three times. (A) IH-stained liver tissues were taken from CHB patients with liver fibrotic stage of S0, S1, and S3 (×200 magnification). (a), (b), and (c) show p-Stat3 expression; (d), (e), and (f) show TGF-β1 expression. (B) Stat3 and TGF-β1 mRNA expressions were statistically up-regulated in AF compared to EF or Control patients (Stat3: AF comparing to EF, $p = 1.25E-05$; TGF-β1: AF comparing to EF, $p = 9.39E-4$). The correlation coefficient of Stat3 and TGF-β1 mRNA was 0.413 ($p = 0.003$). AUROCs of Stat3 and TGF-β1 mRNA diagnosing AF stages were 0.783 and 0.692. (C) IF of (a) DAPI (blue), (b) p-Stat3 (green) and (c) TGF-β1 (red) in the liver of a CHB patient (liver fibrotic stages: S3). Nuclei were stained with DAPI. (d) A small portion of p-Stat3 positive cells of portal area were found to co-express TGF-β1 (see red frame).

128 CHB patients (Suppl. Table 1) for microarray and 60 CHB patients for qPCR (Suppl. Table 2). The procedure for human sample collection was approved by the Ethics Committee of Shanghai First People's Hospital.

2.4. Immunohistochemistry (IH) and immunofluorescence (IF) staining

Formaldehyde-fixed, paraffin-embedded sections of liver tissue were subjected to hematoxylin and eosin (H&E) staining and IH following routine protocols as described. Antibodies were as follows: TGF-β1 (Santa Cruz Biotechnology) and p-Stat3 (Cell Signaling). IF double staining of human or rat liver tissue was incubated with human anti-p-Stat3 and anti-TGF-β1 (Invitrogen). IF staining of α-SMA and vimentin (Vim) was used to identify activated HSCs. Briefly, cell slides were incubated with anti-vim (Abcam, United Kingdom) and anti-α-SMA (Boster, China) primary antibodies. 4′6-Diamidino-2-phenylindole (DAPI) was applied to show the nucleus. Representative images were captured with an Olympus IX70.

2.5. Quantitative polymerase chain reaction (qPCR) analysis

The qPCR was performed using SYBR Green PCR Kit (Applied Biosystems, Foster City, CA) and ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Another qPCR was performed using M-MLV1 PCR Kit (Promega) and TRI REAGENT® RNA System (Molecular Research Center). The messenger RNA (mRNA) level of specific genes was normalized against β-actin. Primers used are listed in Suppl. Table 3.

2.6. Western blotting (WB)

Western blotting was performed using antibodies as follows: p-Smad3 (Epitomics/Biomol); β-actin, survivin, ChK1 and Bcl2 (Sigma-Aldrich); α-SMA (Dako); E-Cad, TGF-β1, Caspase3 and GAPDH (Santa Cruz Biotechnology); and p-Stat3, Stat3, p-Smad2, p-Smad1, and Cleaved-Caspase3 (Cell Signaling). The secondary antibody was an anti-rabbit antibody (BD Bioscience, 1:4000).

2.7. Cell apoptosis assays

Cells were washed with PBS and re-suspended in 500 μl binding buffer containing 2 μl Annexin V-FITC. After incubation, the samples were analyzed using flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA).

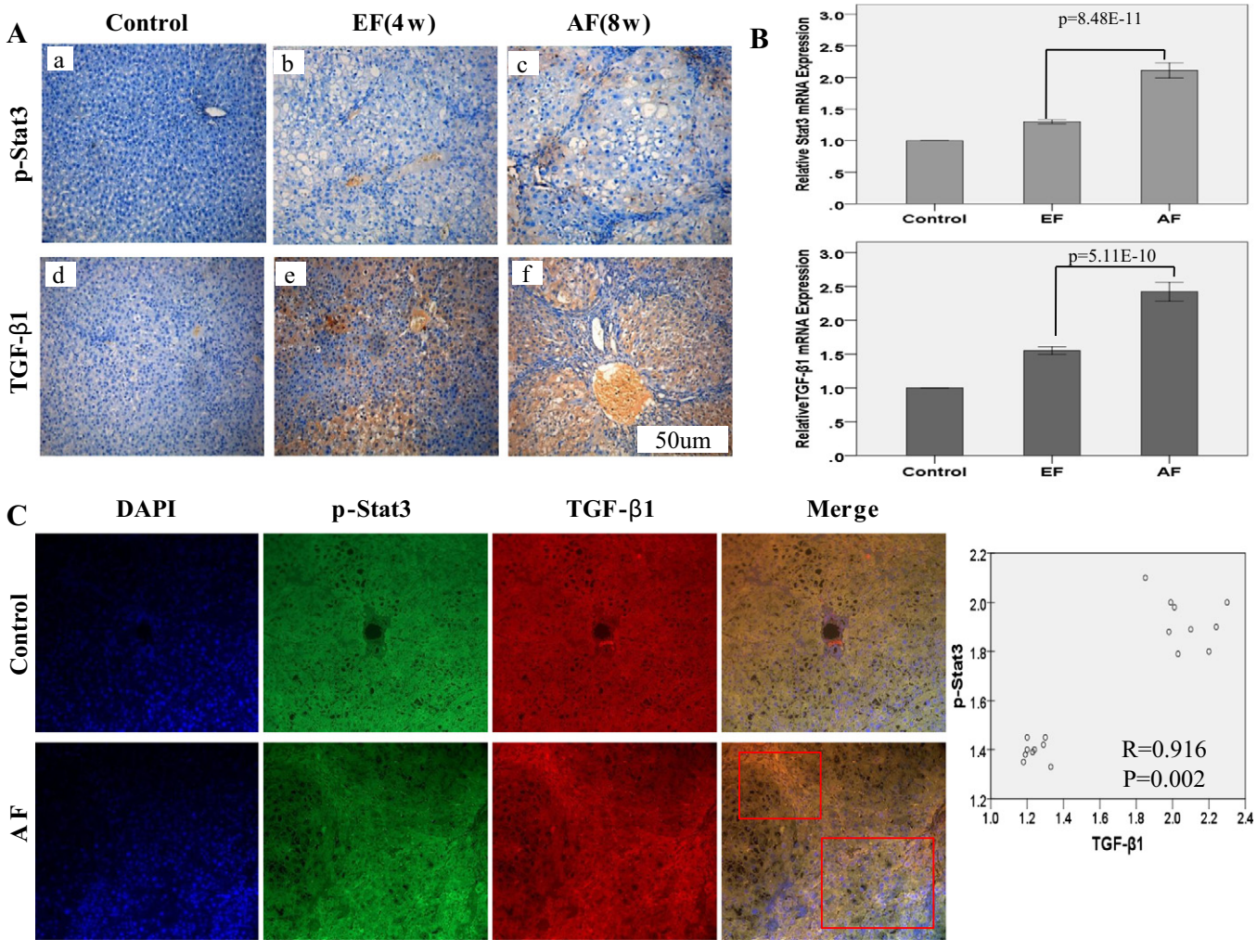

Fig. 2. Stat3 and TGF-β1 expression in DEN-induced liver fibrosis rats. Stat3 and TGF-β1 mRNA and protein expression were examined in liver tissues of 45 DEN-induced fibrosis rats (EF: $n = 15$; AF: $n = 15$; Control: $n = 15$) by use of IH/IF staining and qPCR. IH, IF and qPCR were performed three times. (A) IH-stained liver tissues were taken from rats (×200 magnification). (a), (b), and (c) show us p-Stat3 expression; (d), (e), and (f) show us TGF-β1 expression. (B) Stat3 and TGF-β1 mRNA expressions were statistically up-regulated in AF than EF or Control rats [Stat3: AF (2.11 ± 0.14) comparing to EF (1.30 ± 0.39), $p = 8.48E-11$; TGF-β1: AF (2.42 ± 0.17 comparing to EF (1.55 ± 0.18), $p = 5.11E-10$]. The correlation coefficient of Stat3 and TGF-β1 mRNA was 0.916 ($p = 0.002$). (C) IF of (a) DAPI (blue), (b) p-Stat3 (green) and (c) TGF-β1 (red) in the liver of control rat and AF rat. Nuclei were stained with DAPI. A small portion of p-Stat3 positive cells were found to co-express TGF-β1 (see red frame of AF rat liver).

2.8. Cell cycle analysis

Cells were harvested after 48 h and fixed in 70% ice-cold ethanol overnight. The cells were then washed with PBS, and stained with propidium iodide (50 mg/ml) in PBS supplemented with RNase (50 mg/ml) in the dark at room temperature for 30 min. Tests were performed in triplicate for each sample, and analyses of cell cycle distribution were performed by flow cytometer in accordance with the manufacturer's guidelines (FACS, BD Bioscience, USA).

2.9. Statistical analysis

Results are expressed as mean ± SE and the experiment was repeated three times. Data were analyzed using ANOVA test. A p value <0.05 was considered statistically significant.

3. Results

3.1. Up-regulation of Stat3 correlates with high expression of TGF-β1 in human advanced liver fibrosis

Total genomic profiles of liver tissues were compared in 7 controls and 121 CHB patients (Fibrosis Scheuer score: S0–S4). Expression of TGF-β1 and Stat3 mRNA was significantly higher in advanced fibrosis (AF, S3-S4) patients than in early fibrosis (EF, S0–S2) patients with microarray (Suppl. Fig. 1). To explore the influence of Stat3 on the human liver fibrosis, 60 liver samples of CHB patients (Control: 10; EF: 30; AF: 20) were collected. As expected, p-Stat3 and TGF-β1 positive cells around hepatic portal area preferentially existed in AF livers (Fig. 1A-c, f) and only a few of them were detected in EF livers (Fig. 1A-b, e). Stat3 mRNA were scarcely expressed in normal and EF livers but highly expressed in AF livers along with up-regulated TGF-β1 (Fig. 1B). Furthermore, Stat3 levels were positively correlated with TGF-β1 expression in AF livers ($r = 0.413$, $p < 0.01$) and AUROCs of Stat3 and TGF-β1 for diagnosing of AF stages were 0.783 and 0.692 (Fig. 1B), which is further proof to the effect of Stat3 and TGF-β1 on liver fibrogenesis. As illustrated in Fig. 1C, a small portion of p-Stat3 positive cells of portal area were found to co-express TGF-β1 in the AF liver of a CHB patient (Scheuer score: S3). These data suggested the importance of Stat3 in the generation of liver fibrosis in humans.

3.2. Hepatic p-Stat3 positive tissues exhibit high TGF-β1 expression and aggravate fibrosis characteristics in DEN-induced fibrosis rat model

To explore the role of Stat3 in fibrogenesis, we examined the Stat3 status in the livers of DEN-induced fibrosis rats (EF: $n = 15$; AF: $n = 15$;

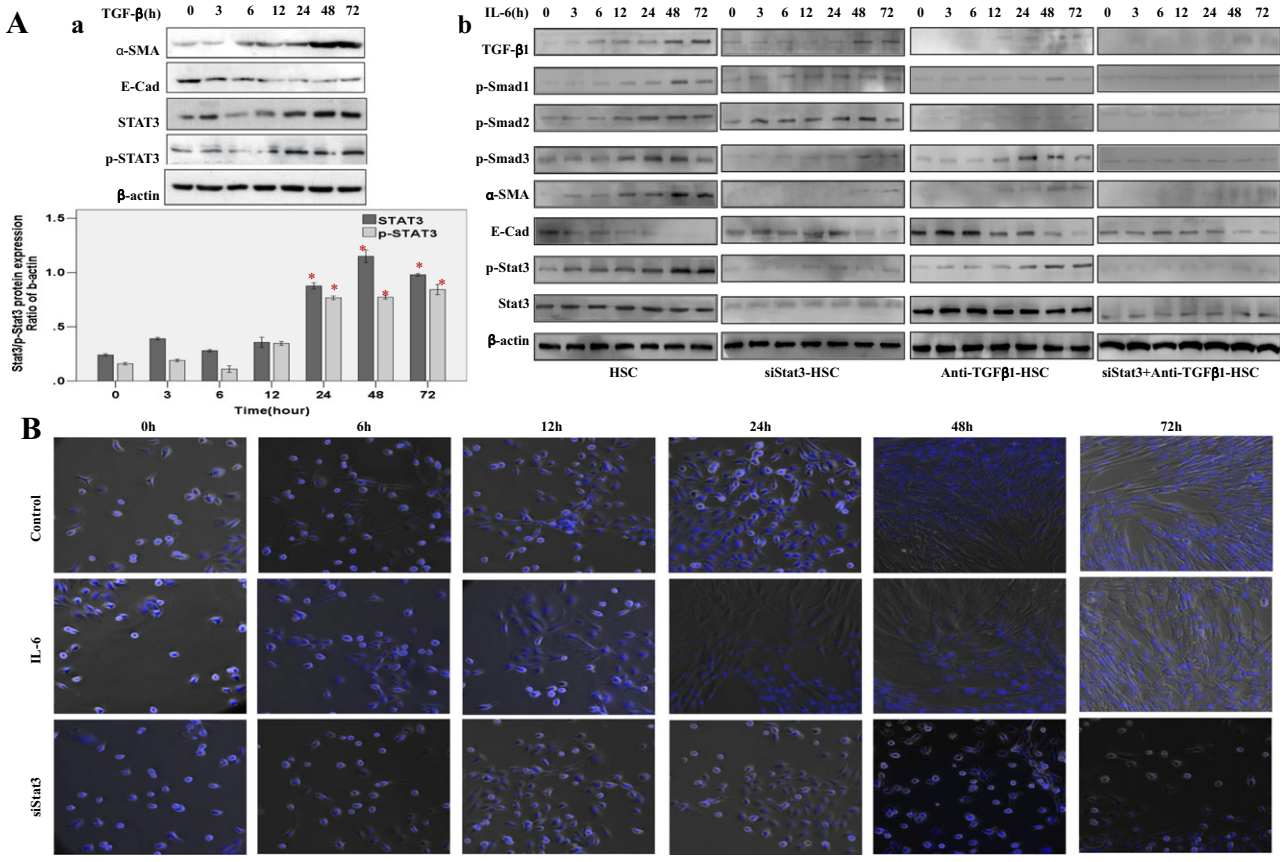

Fig. 3. IL-6/Stat3 and TGF-β1 interaction and IL-6/siStat3 influence activation of HSC. Rat primary HSCs were isolated and cultured for 0, 3, 6, 12, 24, 48, and 72 h. HSCs treated with TGF-β1 at 2 ng/ml or IL-6 at 10 ng/ml, respectively. Lentivirus encoding siRNA gene of Stat3 for transduction (siStat3) and the control virus showed green fluorescent protein (GFP) expression in the cells. WB graphs represent at least three independent experiments. (A) WB of HSCs treated with TGF-β1 for 0, 3, 6, 12, 24, 48, and 72 h were examined. The values represent ratio of β-actin. *, compared to HSCs cultured for 0 h, $p < 0.05$. (a) a-SMA, E-Cad, STAT3, p-STAT3 and β-actin from HSC protein extracts treated with TGF-β1. STAT3/p-STAT3 levels were significantly higher in HSCs treated with TGF-β1 at 24 h, 48 h, 72 h than 0 h [p-value of STAT3/p-STAT3: 24 h (7.68E − 14/0.002), 48 h (5.47E − 16/0.029), 72 h (9.62E − 15/0.008)]. (b) TGF-β1, p-Smad1/2/3, a-SMA, E-Cad, Stat3, p-Stat3 and β-actin levels were detected in 4 groups of HSCs (HSC, siStat3-HSC, anti-TGF-β1-HSC and siStat3 + anti-TGF-β1-HSC) treated with IL-6. ①TGF-β1 levels in group HSC were significantly up-regulated at 12 h, 24 h, 48 h, and 72 h compared to 0 h [p-values: 12 h (0.046), 24 h (0.038), 48 h (2.45E − 6), 72 h (1.38E − 7)]. TGF-β1 levels in group siStat3-HSC were significantly up-regulated at 48 h, 72 h compared to 0 h [p-values: 48 h (5.08E − 4), 72 h (6.77E − 5)]. ②p-Stat3 levels significantly increased at 3 h, 6 h, 12 h, 24 h, 48 h, and 72 h compared to 0 h in the HSC group [p-values: 3 h (0.049), 6 h (0.047), 12 h (0.036), 24 h (0.022), 48 h (1.42E − 2), and 72 h (6.30E − 5)]. But p-Stat3 levels significantly increased only at 24 h, 48 h and 72 h compared to 0 h in the anti-TGF-β1-HSC group [p-values: 24 h (0.036), 48 h (2.11E − 2), and 72 h (1.07E − 2)]. (B) IF of GFP revealed morphology and number of quiescent/activating HSCs treated with IL-6 or siStat3 and untreated HSCs (Control) at 0 h, 6 h, 12 h, 24 h, 48 h and 72 h.

Control: $n = 15$). The increasing level of p-Stat3 was in concomitance with TGF-β1 in liver tissues stained by IH while liver fibrosis grades aggravated (Fig. 2A). Notably, expression of Stat3 mRNA also increased during liver fibrosis aggravation and closely correlated with the up-regulation of TGF-β1 (Fig. 2B). More importantly, positive p-Stat3 tissues of hepatic portal area in DEN-treated AF rats were found to co-express TGF-β1, indicating that Stat3 may acquire the feature of fibrotic promoter during fibrogenesis (Fig. 2C). Considering the consistent results of human fibrogenesis, we speculated that the activated Stat3 may undergo fibrotic transformation in AF liver, where the unique TGF-β1 exposure may play an important role.

3.3. Stat3 can induce TGF-β1 up-regulation and plays an important role in HSC activation

These findings prompted us to investigate whether there is a relationship between TGF-β1 signaling and Stat3 activation and to study the functional importance of this toward their production in activated HSCs. Rat primary HSCs responded to TGF-β1 treatment by expressing statistically higher levels of Stat3/p-Stat3 at 24, 48 and 72 h comparing to 0 h ($p < 0.05$, Fig. 3Aa). Then we analyzed TGF-β1, p-Smad1/2/3, Stat3/p-Stat3 levels in 4 groups of HSCs incubated with IL-6 [HSC, Stat3 knock-down HSC (siStat3-HSC), HSC treated with blocking antibodies of the TGF-β1 (anti-TGF-β1-HSC) and HSC treated both with siStat3 and blocking antibodies of the TGF-β1 (siStat3 + anti-TGF-β1-HSC)] at 0, 3, 6, 12, 24, 48 and 72 h. First, we observed up-regulation of TGF-β1 upon Stat3/p-Stat3 challenge in HSCs prior to IL-6 induction after 12 h (Fig. 3Ab). Secondly, up-regulation of TGF-β1 was only observed in siStat3-HSCs prior to IL-6 induction after 48 h because of cell activation by themselves (Fig. 3Ab). Thirdly, Stat3/p-Stat3 levels increased among 3, 6, 12, 24, 48 and 72 h, TGF-β1 and p-Smad2/3 levels significantly increased among 12, 24, 48 and 72 h in HSCs. As inhibiting TGF-β1 pathway, p-Stat3 levels were lower in anti-TGF-β1-HSC than HSC (Fig. 3Ab). It is possible that some, but not all, of Stat3 phosphorylation are affected by TGF-β1. All of the above data showed that incubation of HSC with IL-6 increased TGF-β1 protein levels. The results led us to propose that p-Stat3 mediates TGF-β1 up-regulation. Regardless activation of TGF-β1 or Stat3, α-SMA levels gradually increased and

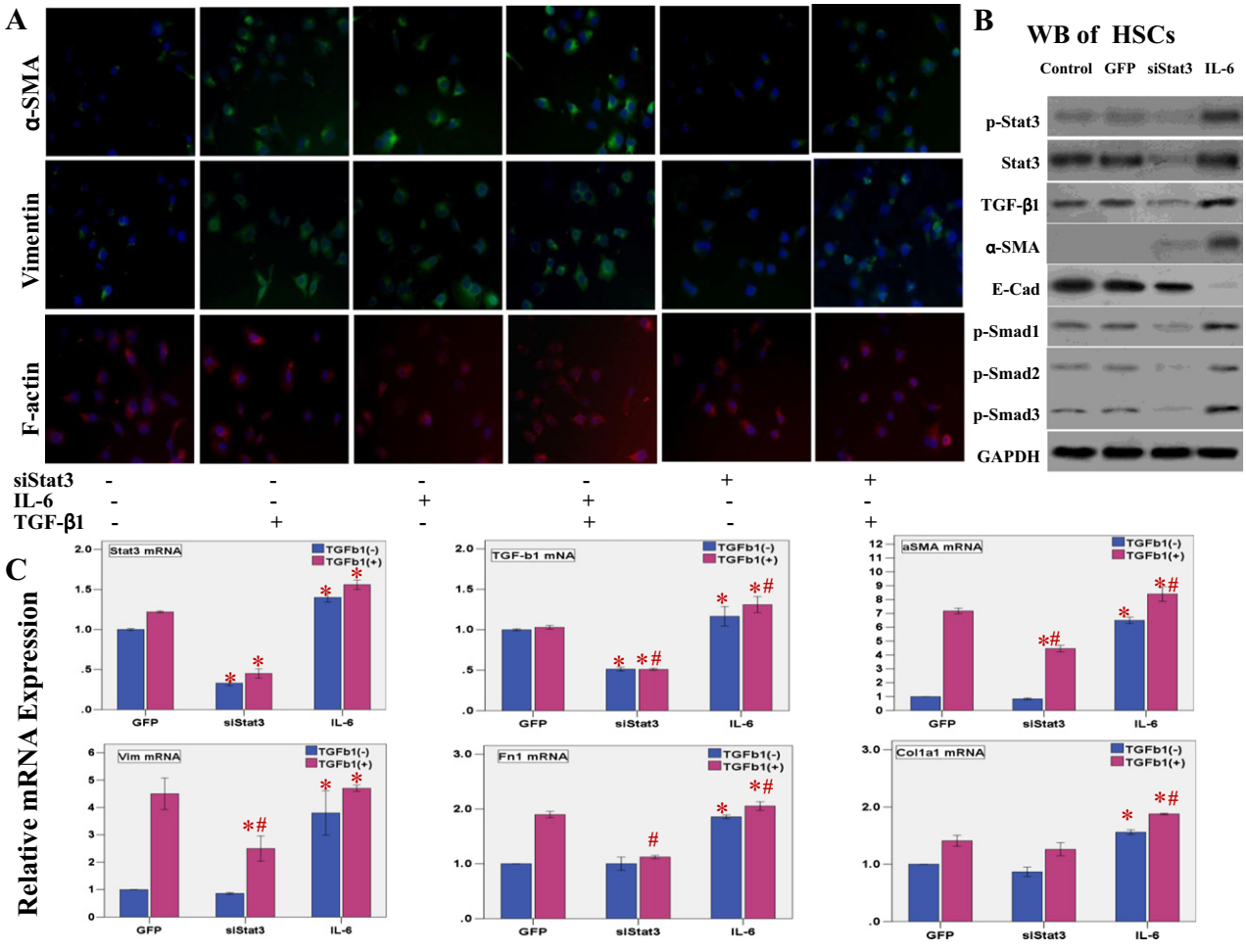

Fig. 4. Alteration of fibrosis related products induced by IL-6/siStat3 or TGF-β1 in rat primary HSCs. Rat primary HSCs were isolated and cultured for 48 h (A) and 12 h (B and C). HSCs treated with TGF-β1 at 2 ng/ml, IL-6 at 10 ng/ml, siStat3, GFP respectively. All graphs represented 3 independent experiments. (A) IF of α-SMA and Vimentin (green) and F-actin (red) in HSCs treated with TGF-β1, IL-6, or siStat3. Nuclei were stained with GFP (blue). (B) WB analyses of Stat3, p-Stat3, TGF-β1, a-SMA, E-Cad, p-Smad1/2/3, and GAPDH from HSC protein extracts treated with GFP, siStat3, IL-6 and untreated Control. (C) Relative mRNA expression of Stat3, TGF-β1, α-SMA, Vim, Fn1 and Col 1a1 in HSCs treated with IL-6/siStat3, TGF-β1 or not. *, compared to GFP HSCs with TGF-β1(−), $p < 0.05$; #, compared to GFP HSCs with TGF-β1(+), $p < 0.05$. HSCs barely treated with IL-6, Stat3, TGF-β1, α-SMA, Vim, Fn1, and Col1a1 mRNA statistically up-regulated compared to untreated GFP (p-values: 6.05E − 06, 0.016, 2.09E − 09, 1.47E − 04, 2.99E − 06, 5.69E − 06). HSCs only treated with siStat3, Stat3 and TGF-β1 mRNA statistically down-regulated compared to GFP (p-values: 2.87E − 06, 6.96E − 05). HSCs treated with IL-6 plus TGF-β1, Stat3, TGF-β1, a-SMA, Vim, Fn1, and Col1a1 mRNA statistically up-regulated compared to untreated GFP (p-values: 2.84E − 06, 2.61E − 04, 7.04E − 08, 1.39E − 05, 7.79E − 08, 1.57E − 06). HSCs treated with siStat3 plus TGF-β1, Stat3, TGF-β1, α-SMA, and Vim mRNA statistically different compared to untreated GFP (p-values: 3.16E − 06, 1.95E − 05, 6.33E − 06, 2.48E − 04). HSCs treated with TGF-β1 plus siStat3, TGF-β1, α-SMA, Vim, and Fn1 mRNA statistically down-regulated compared to TGF-β1 treated GFP (p-values: 1.61E − 05, 3.62E − 05, 6.00E − 03, 1.51E − 06). And HSCs treated with TGF-β1 plus IL-6, TGF-β1, α-SMA, Fn1, and Col1a1 mRNA statistically up-regulated compared to TGF-β1 treated GFP (p-values: 0.001, 0.003, 0.010, 2.53E − 04).

E-Cad levels decreased. Most importantly, α-SMA and E-Cad expression had an identical changing trend in HSCs accompanied with TGF-β1 or Stat3 alteration (Fig. 3Aa and Ab). To further consolidate this result, IF analysis revealed that rat primary HSCs treated with IL-6 activated more excessively and earlier than controls in 24 h (Fig. 3B). On the contrary, rat primary HSCs treated with siStat3 maintained their inactivity at 24, 48 and 72 h (Fig. 3B). These results imply that the Stat3-mediated signaling pathway could induce TGF-β1 up-regulation and is pivotal in HSC activation.

3.4. The increasing fibrosis related products effect of Stat3 crosslinking TGF-β1 signaling can be abolished in rat primary HSCs treated with siStat3

We next intervened with IL-6, siStat3 or TGF-β1 in rat primary HSCs to clarify the change of fibrosis related products. IF analysis illustrated that cytoplasmic α-SMA and Vim were dramatically increased in IL-6 or TGF-β1 treated cells compared to untreated cells, and they reached the maximum extent in IL-6 plus TGF-β1 treated cells (Fig. 4A). Interestingly, cytoplasmic α-SMA and Vim were negatively expressed with siStat3 treatment and the effect was unchangeable with TGF-β1 plus siStat3 treatment (Fig. 4A). This implies that TGF-β1-mediated fibrosis related products exportation might be, at least partially, involved in HSCs upon Stat3 intervention. To further test the hypothesis, expressions of fibrosis related products were studied in rat primary HSCs treated with siStat3, IL-6 and TGF-β1 respectively at 12 h. After induction of IL-6 with HSCs, the levels of α-SMA and p-Smad1/2/3 were significantly higher and E-Cad were significantly lower in them than in control cells and siStat3 treated cells ($p < 0.05$, Fig. 4B). After induction of IL-6 or TGF-β1 or IL-6 plus TGF-β1 with cells, the mRNA expression of 4 fibrosis related genes was significantly higher than untreated control cells ($p < 0.05$; Fig. 4C). Of note, while IL-6 plus TGF-β1 treatment significantly enhanced the expression of 3 genes than TGF-β1 treated cells (α-SMA, Fn 1 and Col 1a1: $p < 0.05$; Fig. 4C). Such effects were not seen in siStat3 treated cells (Fig. 4C). Most importantly, inhibition of Stat3 counterworked the profibrotic effect in TGF-β1 plus siStat3 treated cells compared to TGF-β1 treated cells (α-SMA, Vim and Fn 1, $p < 0.05$, Fig. 4C). Therefore TGF-β1 could not achieve profibrogenic cytokine effect without Stat3 activation in HSCs. We also showed that siStat3 does not affect the mRNA levels of TGF-β1 in HSCs, but siStat3 attenuates the pro-fibrogenic effect of TGF-β1 on the expression of fibrotic genes at 48 h (Suppl. Fig. 2). It indicated that Stat3 activation mediates TGF-β1 signaling (Stat3 appears to be required not only upstream but also downstream TGF-β1).

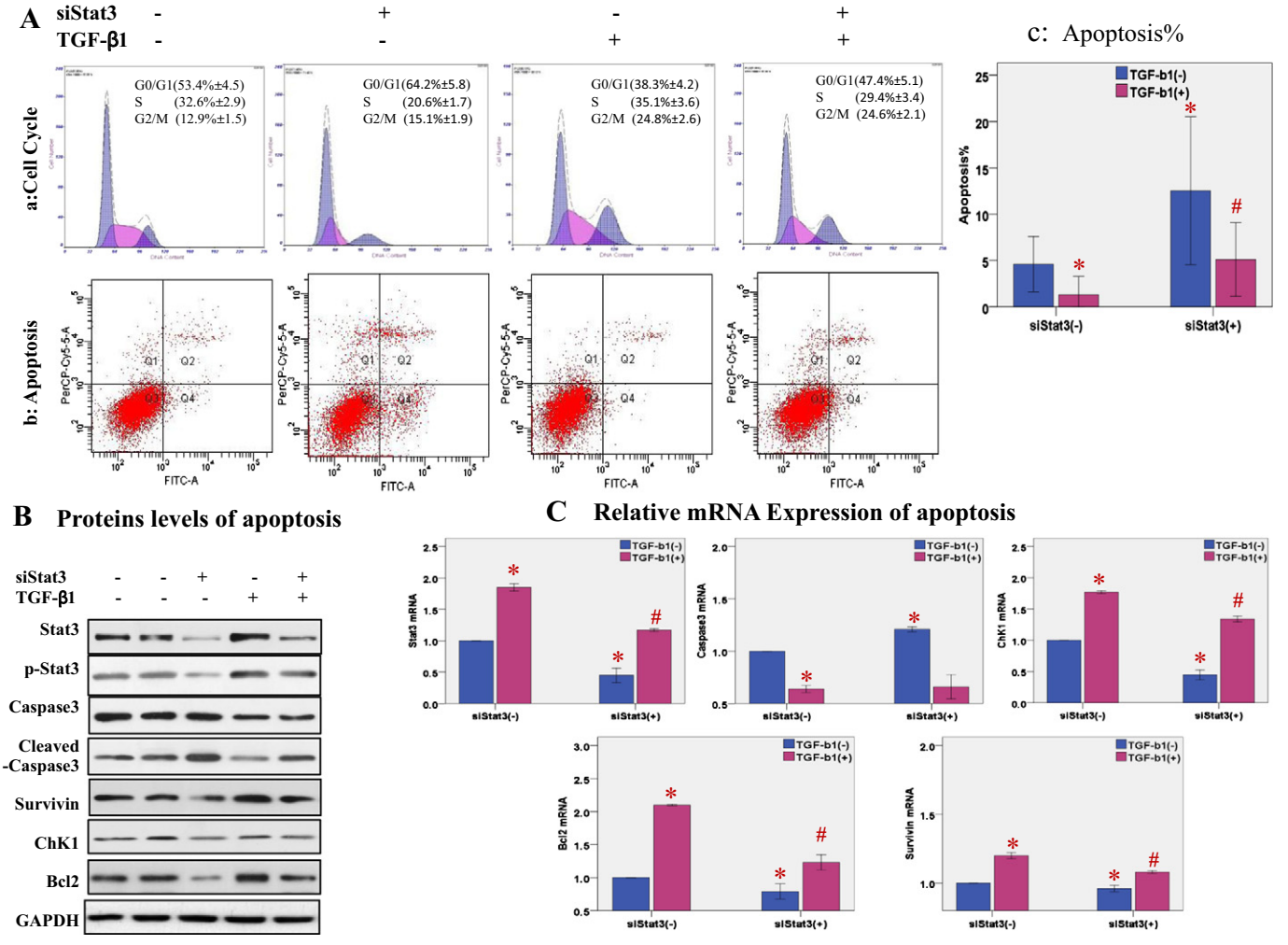

Fig. 5. Effect of siStat3 or TGF-β1 on apoptosis of HSCs. Rat primary HSCs were isolated and cultured for 24 h. Cell apoptosis studies as flow cytometry was done. Apoptosis factors of HSCs treated by siStat3 or TGF-β1 were studied by WB and qPCR. Graphs represent at least three independent experiments. *, comparison of siStats (−) HSCs with TGF-β1(−) $p < 0.05$; #, comparison of siStats (−) HSCs with TGF-β1(+) $p < 0.05$. (A) Maps of cell cycle (a) and apoptosis (b) on rat primary HSCs treated with siStat3 or TGF-β1 were analyzed. Percentages of cells in G0/G1, S, G2/M phase of the cell cycle were shown (a). Apoptotic rate of HSCs treated with siStat3/TGF-β1 was shown (c). (B) WB analyses of Stat3, p-Stat3, caspase3, cleaved-caspase3, survivin, ChK1, Bcl2 and GAPDH from HSC protein extracts treated with GFP, siStat3, TGF-β1 and untreated Control. (C) Relative mRNA expression of Stat3, caspase3, survivin, ChK1, and Bcl2 in HSCs treated with siStat3 or TGF-β1.

3.5. Treatment with siStat3 counters the promotion of rat primary HSC apoptosis by TGF-β1

To explain Stat3 effect on cell proliferation or apoptosis of rat primary HSCs, apoptosis studies and expression of apoptosis related factors were examined. Our data found that cells exhibited robust proliferation ability and apoptosis was significantly reduced when HSCs were treated with TGF-β1 (Fig. 5Ab). The percentage of cells in the G0/G1 phase of the cell cycle decreased and the G2/M phase increased in TGF-β1 treated HSCs compared to untreated HSCs (p-values: G0/G1: 4.90E − 04; G2/M: 1.31E − 03; Fig. 5Aa). The percentage of apoptotic cells decreased extremely in TGF-β1 treated HSC compared to untreated HSCs (p = 0.01E − 04, Fig. 5Ac). Anti-apoptotic proteins and mRNA (Bcl2 and Survivin) were significantly higher and pro-apoptotic protein and mRNA (caspase3) were apparently lower in TGF-β1 treated HSCs than in untreated cells [p-values of qPCR: Bcl2 (0.012), Survivin (0.023), Caspase (1.67E − 03), Fig. 5B/C]. While apoptosis dramatically increased (Fig. 5Ab) and percentage of cells in the G0/G1 phase of the cell cycle significantly increased and S phase decreased in siStat3 treated HSCs compared to untreated, TGF-β1 and TGF-β1 plus siStat3 treated HSCs (p-values: G0/G1: 2.04E − 02, 1.97E − 03, 3.88E − 02; S: 0.007, 4.10E − 03, 0.046, Fig. 5Aa). The percentage of apoptosis cells increased in siStat3 treated HSC compared to untreated cells (p = 0.016, Fig. 5Ac). Anti-apoptotic proteins and mRNA (Bcl2 and Survivin) were lower and pro-apoptotic protein and mRNA (caspase3) were higher in siStat3 treated HSCs than untreated ones [p-values of qPCR: Bcl2 (0.026), Survivin (0.022), Caspase (5.37E − 5), Fig. 5B/C]. The results showed that siStat3 had a pro-apoptotic function and TGF-β1 had an anti-apoptotic function. Importantly, apoptosis increased (Fig. 5Ab) and percentage of cells in G0/G1 phase of the cell cycle increased in siStat3 plus TGF-β1 treated HSCs compared to TGF-β1 treated HSCs (p-values: G0/G1: 0.019, Fig. 5Aa). Percentage of apoptotic cells increased in siStat3 plus TGF-β1 treated HSC compared to TGF-β1 treated cells (p = 0.021, Fig. 5Ac). Anti-apoptotic proteins and mRNA (Bcl2 and Survivin) were lower in siStat3 plus TGF-β1 treated HSC compared TGF-β1 treated cells [p-values of qPCR: Bcl2 (1.15E − 04), Survivin (0.001), Fig. 5B/C]. So siStat3 could counteract the anti-apoptotic effect of TGF-β1 in HSCs. These results imply that TGF-β1 maybe could not achieve suppressing apoptosis characteristics without Stat3 participation in rat primary HSCs.

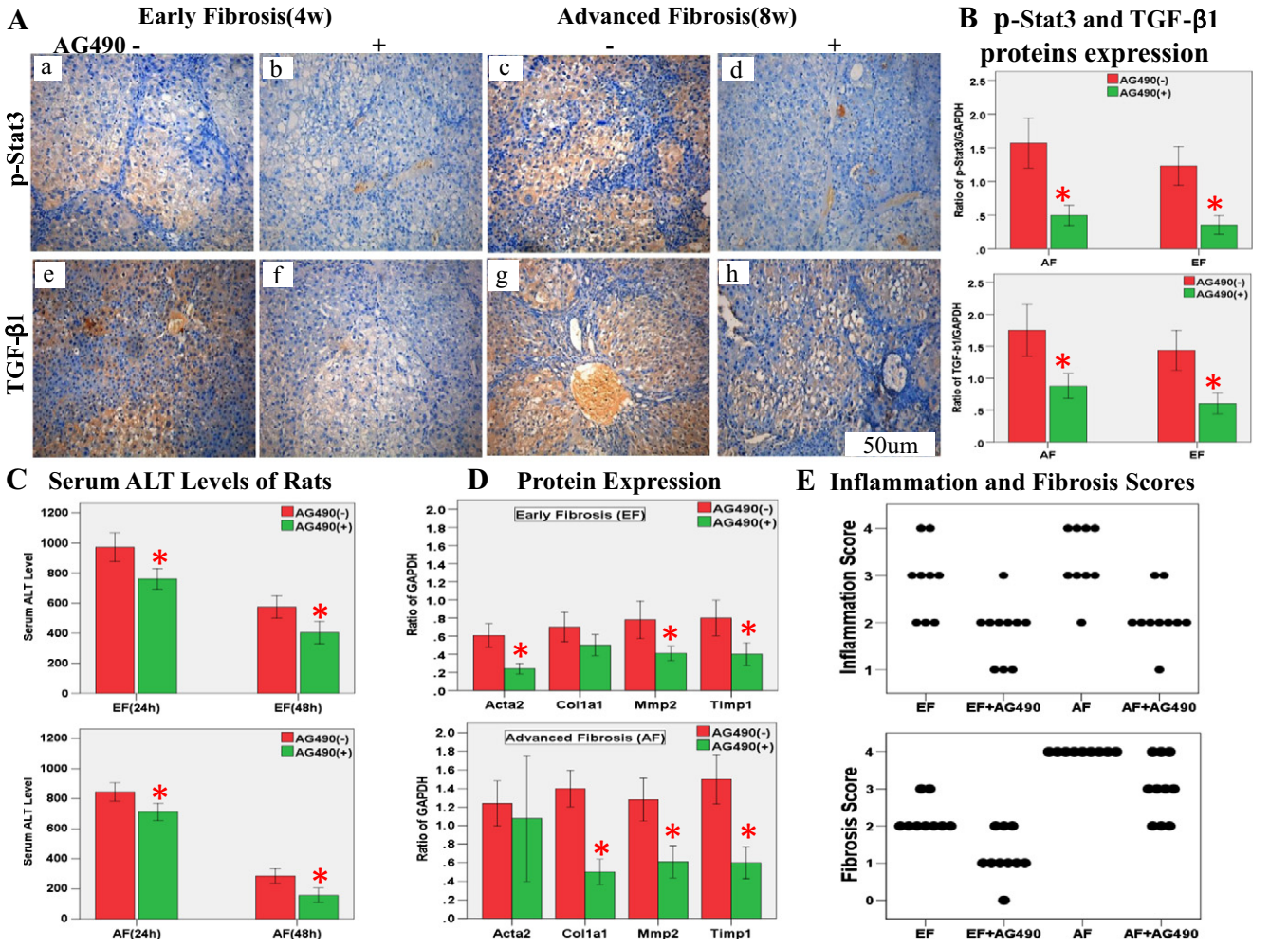

Fig. 6. AG490 treatment attenuates DEN-induced liver fibrosis of rats. TGF-β1 and p-Stat3 protein levels were examined in liver tissues of 60 DEN-induced liver fibrosis rats treated with AG490 or not. Serum ALT levels, liver fibrosis products, inflammation and fibrosis scores were evaluated. Rats were divided into 4 groups: EF (DEN 4 weeks), n = 15; EF + AG490 (DEN 4 weeks + AG490 1 week), n = 15; AF (DEN 8 weeks), n = 15; and AF + AG490 (DEN 8 weeks + AG490 1 week), n = 15. WB and IH experiments performed 3 times. *, treatment of AG490(+) rats compared to AG490(−) rats, $p < 0.05$. (A) IH-stained liver tissues were taken from rats (×200 magnification). (a), (b), (c), and (d) show p-Stat3 expression; (e), (f), (g), and (h) show TGF-β1 expression. (B) Protein levels of p-Stat3 and TGF-β1 were shown. (C) Serum ALT levels were examined at 24 h and 48 h after DEN treatment of rats. (D) Protein levels of Col1a1, Acta2, Mmp2 and Timp1 were shown. (E) Inflammation and fibrosis scores of AG490 treated and untreated rats. Fibrosis scores are based on the percentage of liver area positively stained by Sirius Red and H&E.

3.6. Suppressing Stat3 could attenuate chronic liver injury and fibrosis in DEN induced fibrosis rats by use of AG490

AG490 was used to inhibit Stat3 activation. To evaluate whether suppressing Stat3 has an effect on liver fibrogenesis crosslinking of TGF-β1, Wister rats were separated into 4 groups (EF: $n = 15$; EF + AG490: $n = 15$; AF: $n = 15$; AF + AG490: $n = 15$). Liver fibrosis and inflammation were demonstrated by H&E and Masson staining (data not shown). IH analysis (Fig. 6A) showed that the promotional role of Stat3 and TGF-β1 in liver fibrogenesis (EF and AF) was strongly inhibited by interrupting Stat3 pathway (EF + AG490 and AF + AG490) in such model. To further confirm the results, levels of p-Stat3, TGF-β1 and fibrosis related proteins (Col 1a1, Acta2, Mmp2 and Timp1) were directly examined by WB analysis. The p-Stat3 and TGF-β1 levels were significantly higher in AF rats than in the EF ones; while they were dramatically decreased in AG490 treated EF or AF rats than untreated ones [p-value: p-Stat3 (AF) 0.006; p-Stat3 (EF) 0.005; TGF-β1 (AF) 0.018; TGF-β1 (EF) 0.009; Fig. 6B]. ALT levels were significantly lower in AG490 treated EF or AF rats compared to the untreated ones at 48 h [p-value: EF (24 h) 0.002; EF (48 h) 0.005; AF (24 h) 0.006; AF (48 h) 0.002; Fig. 6C). The levels of Acta2, Mmp2 and Timp1 were significantly decreased in AG490 treated EF rats (p-value: 0.007; 0.029; 0.027; Fig. 6D). The levels of Col1a1, Mmp2 and Timp1 were also significantly decreased in AG490 treated AF rats (p-value: 0.002; 0.010; 0.005; Fig. 6D). Liver inflammation scores revealed less vacuolated cells and significantly improved portal inflammation in the AG490 treated EF and AF rats compared to untreated ones [p-value: EF (0.015); AF (0.042); Fig. 6E]. The levels of fibrosis scores were also significantly reduced in AG490 treated AF rats [p-value: AF (0.021); Fig. 6E]. These results suggested that Stat3 signaling might mediate the promotional role of TGF-β1 in liver fibrogenesis, especially in advanced fibrotic stages.

Finally we included the schematic diagram illustrating the hypothetical model that activation of the TGF-β1 pathway by IL-6/Stat3 leads to aggravating fibrosis (Fig. 7).

4. Discussion

Three main kinds of cytokines have been identified as being involved in Stat3 activation in HSCs. Leptin or IL-6 stimulates the activation of Stat3 and increases collagen mRNA expression in HSCs and subsequently accelerates liver fibrosis [17]. While GH mediated suppression of fibrosis may be due to inhibition of Stat3 and TGF-β1 production in Ito cells [15]. However, cross-talking of Stat3 and TGF-β1 signaling in HSCs and fibrogenesis has not been explained clearly. It is also an issue to understand which type of cells plays a major role in Stat3-mediated TGF-β1 production and fibrosis. A complex interplay between hepatic cell types takes place during hepatic fibrogenesis [18]. Activated HSCs secrete inflammatory chemokines, express cell adhesion molecules, and modulate the activation of lymphocytes [19]. Damaged hepatocytes and inflammatory cells stimulate fibrogenic actions and activate HSCs to secrete collagen [20, 21]. Therefore, a vicious circle in which fibrogenic and target cells stimulate each other is likely to occur.

We demonstrate the consistency of up-regulation of Stat3 and TGF-β1 mRNA in advanced fibrosis stages of CHB patients and DEN-induced liver fibrosis rat model. Rat model of DEN inducing liver fibrosis was selected in our study. This kind of rat model can imitate entire natural history of chronic liver disease from liver fibrosis, cirrhosis to carcinogenesis. DEN is a toxic chemical causing oxidative injury on liver and subsequently the oxidative damage hepatocytes that can activate Stat3 in HSCs. So DEN induced liver fibrosis rats can be used to better understand the mechanisms of Stat3. IH and IF staining of liver tissues identified p-Stat3 and TGF-β1 highly positive expression in parenchymal and nonparenchymal cells, especially associated with advanced fibrosis stages. AG490 treatment significantly attenuates DEN-induced chronic liver injury and fibrosis, associated with and dependent on suppressing Stat3. These results showed that activation of Stat3 crosslinking of TGF-β1 exacerbates liver injury and fibrosis both in CHB patients and in DEN-induced liver fibrosis rats. It may be concluded that TGF-β1 and Stat3 reciprocally regulate liver damages, fibrosis, and their expression in human and rat liver fibrosis.

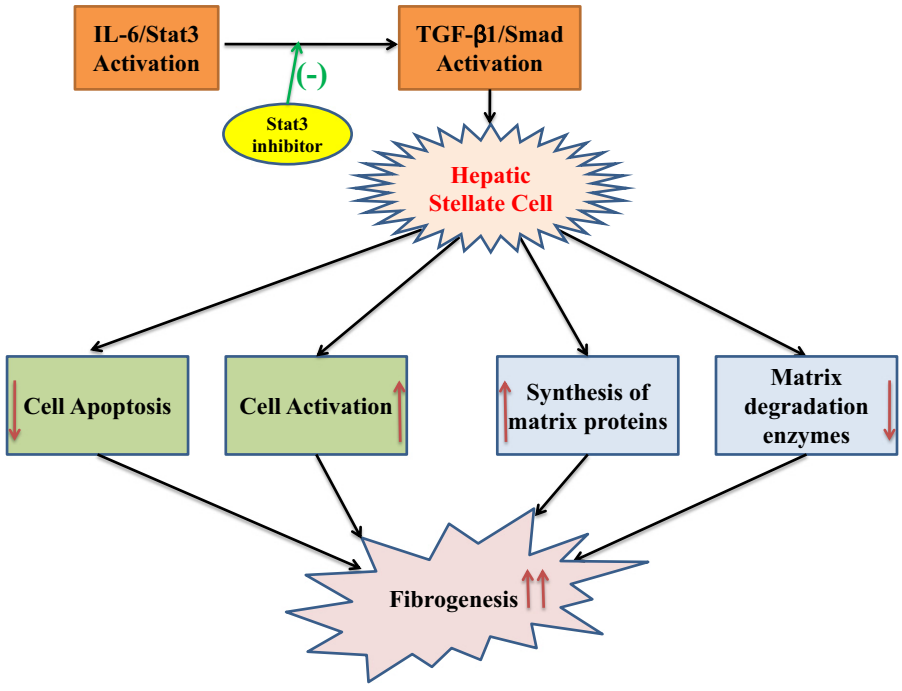

Fig. 7. Schematic diagram of TGF-β1 pathway crosslinking with Stat3 in HSCs exacerbating liver fibrosis. Hypothetical model illustrates that activation of the TGF-β1 pathway by IL-6/Stat3 inhibits HSC apoptosis, increases HSC activation and aggravates fibrosis.

Our finding of a direct induction of TGF-β1 by active Stat3 may provide another mechanism for the contribution of Stat3 to fibrogenesis. We also confirmed that IL-6-mediated up-regulation of TGF-β1 mRNA levels was suppressed by siStat3 in HSCs. Therefore, hyper-activation of Stat3 in HSCs may contribute toward enhanced production of TGF-β1. The increasing fibrosis related product effect of Stat3 crosslinking TGF-β1 signaling can be abolished in siStat3 treated HSCs. Morphological studies proved that HSCs lacking Stat3 remain inactivated, even if they were secondly added TGF-β1 treatment. These data suggest that Stat3 directly up-regulates TGF-β1 expression at the transcriptional levels in HSCs. So our study adds a new target, TGF-β1, to the list of Stat3-regulated genes involved in fibrogenesis.

Stat3 in HSCs appear to have a deteriorating role in liver fibrosis [12–14]. We used siStat3 to demonstrate that suppressing Stat3 promotes apoptosis of HSCs. In non-stimulated HSCs, Stat3 is in its inactive form and locates in the cytoplasm of hepatocytes. The binding of Stat3-related cytokines to their receptors activates the receptor-associated Jak, which phosphorylates Stat3. The resultant p-Stat3 dimers translocate to the nuclei and function as transcriptional factors for downstream genes including bcl-2, caspase3 and survivin that play important roles in promoting HSC survival and an anti-apoptotic effect. And the results illustrated that siStat3, having pro-apoptotic function, could withstand the anti-apoptotic effect of TGF-β1 in HSCs. Our results also imply that TGF-β1 could not achieve anti-apoptosis characteristics without Stat3 participation in HSCs.

Although the study of Wang has an opinion that activation of hepatic Stat3 in the early stage of liver injury is likely to play an important role in protecting against hepatocyte death and DNA damage, thereby acting as a tumor suppressor to prevent liver tumorigenesis [22]. Our results in vitro indicated that HSCs treated with IL-6 activate TGF-β1 and have pro-fibrotic function earliest in 6 h of cell incubation. Stat3 cross-talking to TGF-β1 also aggravated the fibrogenesis at early or mild stage of liver fibrosis both in CHB patients and in DEN treated rats. So we chose AG490 as an anti-fibrotic drug at an early stage but not late stage. Early administration of AG490 is proved efficient in reversing hepatitis and liver fibrosis in DEN induced liver fibrosis rats. We assumed that suppression of Stat3 activation in the early stage of liver fibrosis could be useful for preventing the progress of fibrotic disease.

In conclusion, we provide a novel role for Stat3 cooperating in the activation of TGF-β1 and in the anti-apoptotic effect of HSCs. Stat3 enhances liver fibrosis through the up-regulation of TGF-β1 and fibrotic product expression. This study provides the basis for the therapeutic potential Stat3 antagonists in the treatment of liver fibrosis.

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Conflict of interests

The authors have declared that no conflict of interest exists.

Acknowledgments

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