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ORIGINAL ARTICLE

MicroRNA-744/transforming growth factor β1 relationship regulates liver cirrhosis

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Abstract

Background MicroRNAs have added a new dimension to our understanding of liver cirrhosis (LC) and associated processes like the activation of hepatic stellate cells (HSCs).

Methods Serum samples were collected from 40 LC patients and 30 healthy donors. CCl_4-induced LC mouse model in vivo and in vitro human HSC LX-2 and murine HSC JS-1 cells were researched.

Results The levels of serum microRNA (miR)-744 is inversely correlated with the severity of LC and is a reliable biomarker of LC. In CCl_4-induced LC model, the abundance of miR-744 was reduced in both sera and livers compared with sham controls. Importantly, increasing miR-744 abundance with synthetic miR-744 Agomir alleviated liver fibrosis, a critical component of LC, while reducing miR-744 with Antagomir exacerbated it. To elucidate molecular mechanism underlying the suppressive role of miR-744 in LC, we observed that miR-744 and transforming growth factor β1 (TGFβ1) are inversely correlated in LC patients' sera as well as sera/livers from CCl_4-induced LC mice. We demonstrated that miR-744 Agomir downregulated the expression of TGFβ1 and further confirmed that TGFβ1 mRNA was a *bona fide* miR-744 target in HSCs. Moreover, miR-744 Agomir reduced the degree of F-actin formation and cell proliferation while miR-744 Antagomir promoted these events, suggesting that miR-744 is a negative regulator of HSC activation.

Conclusions MiR-744-led suppression in HSC activation is most likely through TGFβ1 because exogenous TGFβ1 nearly negated miR-744 Agomir's action. This study suggests that reduction of miR-744 is a reliable biomarker for LC and miR-744/TGFβ1 relationship is a key regulator of LC.

Keywords Liver cirrhosis · miRNA · TGFβ1 · Hepatic stellate cells · miR-744

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Abbreviations

LC	Liver cirrhosis
HSC	Hepatic stellate cell
ECM	Extracellular matrix
miRNA	MicroRNA
UTR	Untranslated region
TGFβ	Transforming growth factor β
EMT	Epithelial-to-mesenchymal transition
CHB	Chronic hepatitis B
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
SUTCM	Shanghai University of Traditional Chinese Medicine
CCl_4	Carbon tetrachloride
PBS	Phosphate buffered saline
ANOVA	Analysis of variance
ROC	Receiver–operator characteristic

Introduction

Although liver cirrhosis (LC) is associated with high morbidity and mortality, there is only limited number of therapeutic options. LC is a result of pathological response to initial liver injuries of various causes [1]. Upon liver damage, the hepatic stellate cells (HSCs) are activated, resulting in the build-up of excessive amount of extracellular matrix (ECM) proteins which lead to the fibrotic liver with impaired organ function [2]. Activation of HSCs is considered as the main contributor of LC.

MicroRNAs (miRNAs) are 20–22 nucleotide noncoding RNAs that silence gene expression by base-pairing mostly with 3′-untranslated region (3′-UTR) of target mRNAs [3]. Recent evidences implicate that miRNAs are involved in the initiation, promotion and progression of human liver cirrhosis [4]. For example, miR-27 and miR-29 can slow down liver fibrosis by interfering with retinoid X receptor α and transforming growth factor β (TGFβ)/Smad3 signaling, respectively [5], while loss of miR-200a facilitates the progression of liver fibrosis through the regulation of epithelial-to-mesenchymal transition (EMT) [6]. By comparing miRNAs differentially expressed in sera between healthy donors and chronic hepatitis B (CHB) patients, we previously identified miR-744 as the only miRNA significantly downregulated in CHB patients [7]. Therefore, it is imperative to investigate whether miR-744 plays a role in the development of LC.

Activation of HSCs, which is defined as the conversion of quiescent vascular pericyte-like cells to myofibroblastic cells, plays a key role in the development of LC. The activation of HSCs is characterized by enhanced F-actin formation, cell proliferation, motility and the ability to deposit ECM [8]. Experimental evidences show that TGFβ1 potently activates HSCs by at least two mechanisms [9]. One of them is that TGFβ1 stimulates HSC proliferation through Smad signaling pathway [10]. The other is that TGF-β1 facilitates ECM deposition by regulating the expression of ECM components including fibrillar collagens and fibronectin, ECM-degrading protease inhibitors plasminogen activator inhibitor-1 and tissue inhibitors of metalloproteinase [11]. Importantly, overexpression of TGFβ1 is frequently detected in the blood and liver of LC patients. Given the critical role of HSC activation in LC, TGFβ1 has been recognized as a major modulator of LC [12].

The objective of this study is to investigate the importance of miR-744 in LC. Using sera from LC patients and healthy donors, we demonstrated that level of serum miR-744 was not only consistently lower in LC patients than health donors but also inversely correlated with the severity of LC (Child–Pugh stages). Reduction of miR-744 in LC patients is most likely to be associated with LC because the abundance of miR-744 was also greatly decreased in sera and livers of CCl_4-induced experimental LC mice. Increasing miR-744 abundance with miR-744 Agomir lessened liver fibrosis while decreasing miR-744 abundance with miR-744 Antagomir exacerbated it, indicating that reduction of miR-744 plays a critical role in LC progression. To define the role of miR-744 in LC, we revealed that levels of miR-744 and TGFβ1 were inversely correlated in the sera of LC patients and also both sera and livers of experimental LC mice. In HSCs, miR-744 Agomir downregulated TGFβ1 expression and disrupted TGFβ signaling. In fact, TGFβ1 mRNA is a *bona fide* target of miR-744 in HSCs. To functionally link miR-744/TGFβ1 relationship to HSC activation, we showed that miR-744 Agomir suppressed F-actin formation and cell proliferation of HSCs while miR-744 Antagomir augmented these cellular events. However, exogenous TGFβ1 nearly negated miR-744 Agomir-led suppression in F-actin formation and cell proliferation. We conclude that miR-744/TGFβ1 relationship is an important regulator of LC progression and development.

Materials and methods

Cell lines and reagents

Immortalized human HSC LX-2 and murine HSC JS-1 cells were kindly provided by Dr. S. Friedman, Mount Sinai Medical School, New York. Information on antibodies, miRNA agomir/Antagomir and other reagents is included in Supplementary Materials.

Study subjects

Serum samples were collected from 40 LC patients who were treated in Shuguang Hospital (Shanghai, China) and 30 healthy donors who had annual physical examination at Shuguang Hospital. The diagnostic criteria for LC were based on the published clinical guidelines [13]. The clinical parameters of these patients are included in Table 1. This study was approved by the Institutional Review Board of Shuguang Hospital.

Serum sample collection and qRT-PCR

Serum samples prepared from freshly-drawn blood were aliquoted and stored at − 80 °C until use. RNA was isolated from 200 µl of serum using miRserum&plasma kit (Qiagen, Valencia, CA) and further subjected to qRT-PCR to quantitate miR-744 and TGFβ1 mRNA. Synthetic *C. elegans* (cel)-miR-39 was spiked during the total RNA isolation and

Table 1 Clinical characteristics of LC patients and health donors

	Liver cirrhosis	Health control	*p*
Individuals (*n*)	40	30	
Gender (*n*)			
Male	21	9	
Female	19	21	
Age (year)	50.23 ± 9.2	48.34 ± 7.7	< 0.01
ALT (IU/L)	112.3 ± 190.1	16.2 ± 7.8	< 0.01
AST (IU/L)	110.2 ± 180.2	17.3 ± 4.5	< 0.01
GGT (IU/L)	66.6 ± 54.6	16.9 ± 6.2	< 0.01
ALP (IU/L)	120.5 ± 85.2	63.5 ± 14.6	< 0.01
TBIL (μmol/L)	66.2 ± 45.2	14.5 ± 4.1	< 0.01
HBV DNA (+)	17	0	
Bile acid (μmol/L)	28.3 ± 20.1	4 ± 1.4	< 0.01
HBV status (*n*)			
HBsAg+	30	0	
HBsAg−	10	30	
Albumin	30.5 ± 7.9	43.6 ± 3.1	< 0.01
Child–Pugh grade			
A	12		
B	12		
C	16		

Bile acid was given as medians (range)

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *GGT* g-glutamyltransferase, *ALP* alkaline phosphatase, *TBIL* total bilirubinand, *HBsAg* hepatitis B surface antigen

used for normalization. To measure the amount of miR-744 in HSCs and liver tissues, RNA was extracted using miRNeasy isolation kit (Qiagen) and miR-16 was used as an internal standard. To measure the amount of TGFβ1 mRNA in HSCs and liver tissues, RNA was extracted using Trizol (Life Technologies, Carlsbad, CA) and GAPDH mRNA was used as an internal standard. TaqMan miR RNA Assay kits (Life Technologies) were used to quantitate miRNAs. Information on the primers used for qRT-PCR is included in Supplementary Materials.

CCl_4-induced experimental LC mouse model

Male C57 mice were obtained from Shanghai University of Traditional Chinese Medicine (SUTCM). LC model was generated by *i.p.* injecting 6-week-old mice with carbon tetrachloride (CCl_4; 0.6 mL/Kg body weight) three times a week for 2 weeks. To determine the effect of miR-744 Agomir or Antagomir on liver fibrosis, CCl_4-induced LC mice were randomly divided into 4 groups: control Agomir, miR-744 Agomir, control Antagomir and miR-744 Antagomir. Agomirs or Antagomirs were injected at a dose of 80 mg/Kg body weight through tail vein three times a week for 2 weeks. All procedures performed in the animal study were approved by the Institutional Animal Care and Use Committee of SUTCM.

Detection of TGFβ1 and collagen IV

TGFβ1 in serum or medium was measured using Human or mouse TGFβ1 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN). To determine the effect of miR-744 on TGFβ1 secretion, LX-2 cells were first transfected with miR-744 or control Agomir for 48 h and then starved in serum-free medium for another 72–96 h, after which media were collected for quantitation of TGFβ1. Collagen IV in serum was measured using Human Collagen IV Quantikine ELISA Kit (BioTNT, Shanghai, China).

Determination of actin reorganization

Cells seeded in 96-well plates were transfected with Agomir or Antagomir for 72–96 h and then fixed with 4% paraformaldehyde. Fixed cells were permeabilized with 0.5% Triton X-100 followed by incubation with FITC-labeled phalloidin (Life Technologies). F-actin was visualized by ArrayScan VTI HCS Reader (Thermo Scientific, Waltham, MA) and quantitated using Cell Health Profiling BioApplication Software (Thermo Scientific).

Cell proliferation and flow cytometry

Cell proliferation was assayed using Cell-Light™ EdU Apollo®488 In Vitro Imaging Kit (Ribobio, Guangzhou, China). Briefly, cells seeded in 96-well plates were transfected with Agomir or Antagomir for 72–96 h and then subjected to EdU labeling for 2 h according to manufacturer's protocol. EdU incorporation was visualized by ArrayScan VTI HCS Reader and quantitated using Cell Health Profiling BioApplication Software. To determine the status of cell cycle, cells were fixed in 100% ethanol and then resuspended in PBS containing 1% of fetal calf serum and 20 μg/mL of propidium iodide followed by flow cytometry analysis using FACSCanto II flow cytometer (BD Biosciences, Bedford, MA). The data were analyzed using the BD FACSDiva Software.

TGFβ1 mRNA 3′-UTR-containing luciferase reporter plasmid and luciferase assay

Sequence containing TGF-β1 3′-UTR was subcloned into pmiR plasmid (Life Technologies). To generate mutated miR-744 targeting site in TGFβ1 3′-UTR (Mut), site-directed mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). To determine luciferase activity, LX-2 cells were transfected with empty pmiR, TGF-β1 3′-UTR-containing

pmiR or the mutated version for 2 d followed by measuring luciferase activity. pGK-RLuc plasmid (Promega, Madison, WI) was co-transfected to normalize transfection efficiency. Luciferase activities were determined using Dual-Luciferase Reporter Assay System (Promega).

In situ hybridization and immunostaining

In situ RNA hybridization was performed using Basescope technology (Advanced Cell Diagnostics, Hayward, California) following the manufacturer's protocol with minor modifications.

Double Immunofluorescence Analysis

To assess which cells uptake antagomiR744 and agomir744 in vivo experiments, double immunofluorescence staining, which was performed on 7 µm thick frozen slides, was performed to detect the co-expressions of α-SMA (green) and Cy3-labeled miR-744 Agomir or Antagomir (red). Nuclei were stained with 49,6-diamidino-2-phenylindole. Images were acquired using a laser scanning confocal microscope.

Statistical analysis

The experimental data are presented as the mean ± standard deviation (SD). All statistical analyses were performed using ANOVA or a two-tailed Student's *t* test. The correlation between miR-744 and TGFβ1 or Collage type IV was determined by Spearman correlation test. Clinicopathological correlations were analyzed using Fisher's exact test. Differences were considered statistically significant when $p < 0.05$.

Results

MiR-744 is downregulated in the sera of LC patients

With limited number of samples, we previously identified miR-744 as the only miRNA significantly reduced in the sera of patients with CHB [7]. We expanded this study by examining miR-744 level in a cohort consisting sera from 40 LC patients and 30 healthy donors. QRT-PCR showed that miR-744 was significantly reduced in LC patients when compared to healthy donors (Fig. 1a). When we further analyzed the level of miR-744 in sera of LC patients with varying prognosis stages (Child–Pugh score A, B, and C), miR-744 was found to be the lowest in LC patients with Child–Pugh C and the highest in those with Child–Pugh A LC patients (Fig. 1b), indicating that reduction of serum miR-744 correlates with the severity of LC patients.

To assess the potential of serum miR-744 as LC biomarker, we generated a receiver-operator characteristic (ROC) curve to analyze the difference in the abundance of serum miR-744 between LC patients and healthy donors. In this analysis, the area under the curve for serum miR-744 was 0.82 (95% CI 0.64–1.00), representing 87.5% and 100% in the sensitivity and specificity, respectively (Fig. 1c). These results suggest that the level of serum

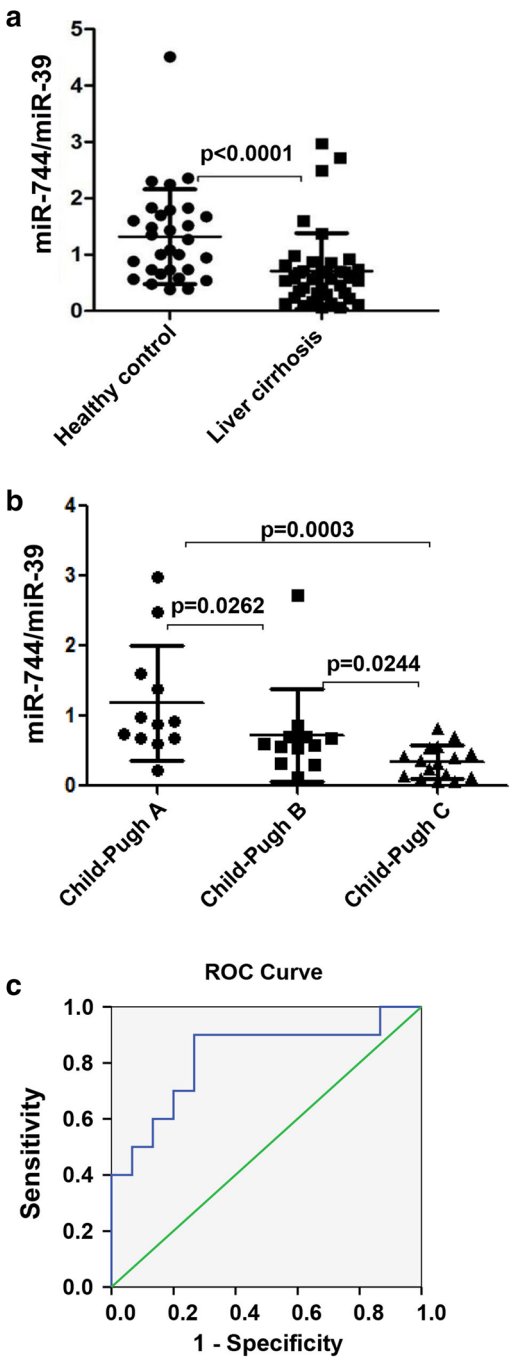


Fig. 1 MiR-744 is a reliable biomarker of LC. **a** Serum miR-744 in LC patients and healthy donors. **b** Serum miR-744 in LC patients staged as Child–Pugh A, B and C. **c** ROC curve of serum miR-744: AUC (the areas under the ROC curve) is 0.82 (95% CI 0.637–1.003)

miR-744 can be used as a biomarker to distinguish LC patients from the healthy individuals.

MiR-744 alleviates liver fibrosis

The observation that miR-744 was downregulated in LC patients prompted us to further explore the connection between miR744 and LC. We set up an experimental LC model by treating C57 mice with CCl_4 for 2 weeks.

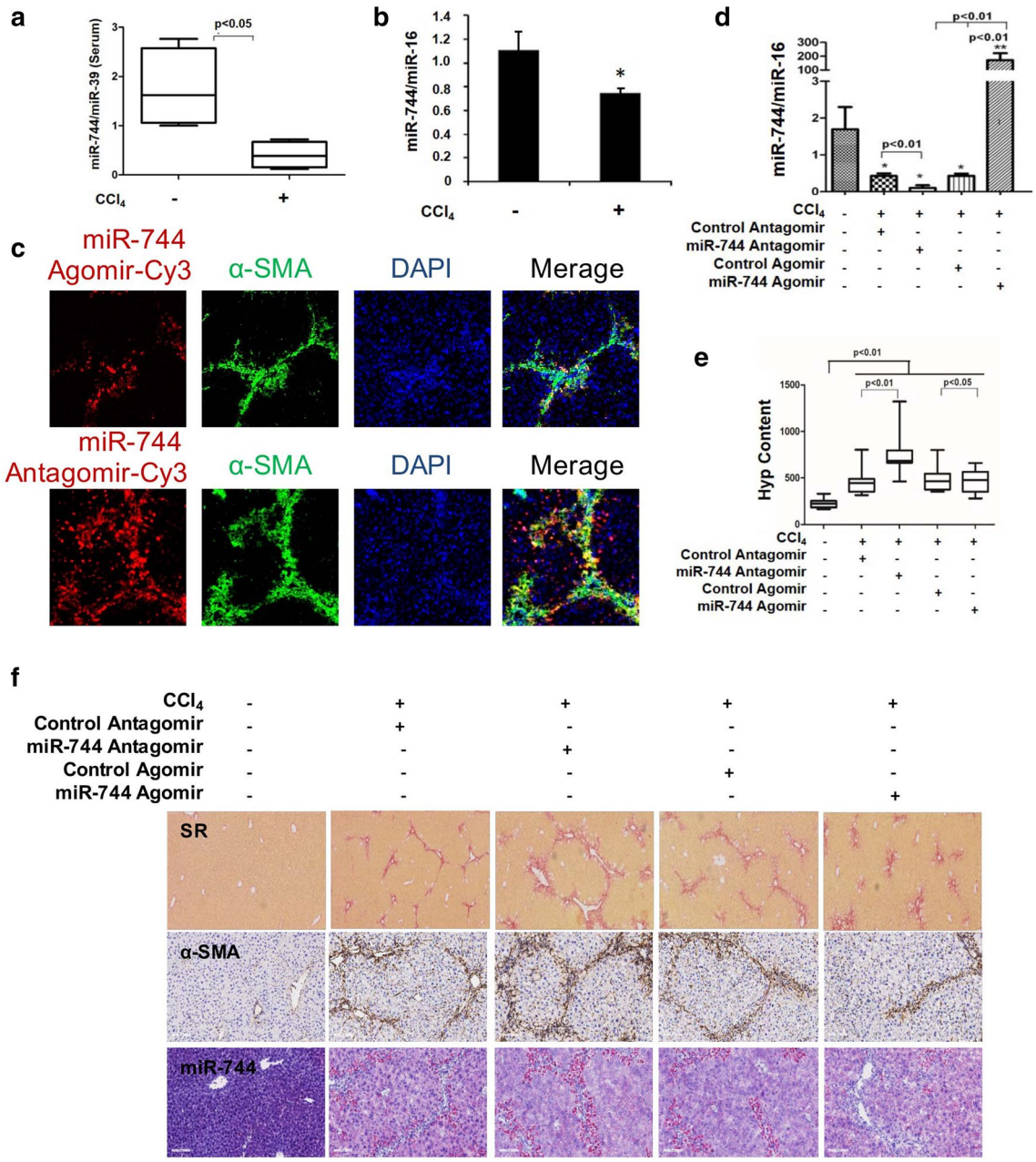


Fig. 2 Modulating abundance of \miR-744 impacts the extent of liver fibrosis. **a** Level of Serum miR-744 in sham control and CCl_4-induced LC mice. **b** Level of miR-744 in livers of sham control and CCl4-induced LC mice. $*p<0.05$ vs sham control. **c** Immunofluorescence analyses of α-SMA (green) and Cy3-labeled miR-744 Agomir or Antagomir (red). Nuclei were counterstained with DAPI (blue) (200×). **d** Level of miR-744 in livers of CCl_4-induced LC mice administrated with control Antagomir, miR-744 Antagomir, control Agomir or miR-744 Agomir. **e** HYP content in livers of CCl_4-induced LC mice administrated with control Antagomir, miR-744 Antagomir, control Agomir or miR-744 Agomir. **f** Sirius red stain, in situ hybridization of miR-744 and α-SMA immumohistochemical staining in livers of CCl_4-induced LC mice administrated with control Antagomir, miR-744 Antagomir, control Agomir or miR-744 Agomir

QRT-PCR showed that the abundance of miR-744 was reduced approximately 80% in sera and 40% in livers of CCl_4-induced LC mice when compared with the sham controls (Fig. 2a, b). These results apparently support the notion that reduction of miR-744 is functionally associated with LC progression.

We next investigated how modulating the abundance of miR-744 impacted LC progression by *i.v.* administering miR-744 Agomir, Antagomir or the respective controls to CCl_4-induced LC mice 3 times a week for 2 weeks. To assess which cells uptake miR-744 Agomir and Antagomir in vivo experiments, double immunofluorescence staining was performed to detect the co-expressions of α-SMA and Cy3-labeled miR-744 Agomir or Antagomir. Immunofluorescence staining showed that Cy3 was expressed in α-SMA $^{(+)}$ positive cells after the mice were injected with Cy3-labeled miR-744 Agomir or Antagomir, it demonstrated that HSCs uptake miR-744 Agomir or Antagomir (Fig. 2c). In situ hybridization showed that miR-744 was mainly located in HSCs (Fig. 2f). qRT-PCR showed that administration of miR-744 Antagomir, but not control Antagomir, resulted in further reduction in miR-744 abundance in the livers of CCl_4-treated mice which had already endured with severe miR-744 reduction compared with the sham mice (Fig. 2d). In contrast, the amount of miR-744 increased more than 100-fold in mice receiving miR-744 Agomir (Fig. 2d), confirming the effectiveness of synthesized miR-744 Antagomir or Agomir to modulate miR-744 abundance in livers. To evaluate miR-744 Agomir and Antagomir on liver fibrosis, we first examined hydroxyproline (Hyp) content, Sirius staining and the expression of α-SMA in livers of CCl_4-induced mice. Level of Hyp was more than doubled in LC mice compared with sham mice (Fig. 2e, f). Administering miR-744 Antagomor led to further increase in Hyp content and more significant Sirius staining and the expression of α-SMA (Fig. 2e, f). However, mice receiving miR-744 Agomir displayed moderate decrease in both Hyp content and Sirius staining and the expression of α-SMA compared to the Agomir control (Fig. 2e, f). These results suggest that modulating miR-744 abundance can impact the degree of liver fibrosis and are thus consistent with the notion that reduction of miR-744 plays a causal role in LC progression.

Level of miR-744 is inversely correlated with TGFβ1 in LC patients and experimental LC model

The observation that miR-744 deters LC progression prompted us to elucidate the underlying molecular mechanism. Searching available miRNA interactome (RNA–RNA crosslinking immunoprecipitation) datasets, we found that TGFβ1 is one of the miR-744 targets in various cell types [14–16]. Because of the prominent role of TGFβ1 in LC,

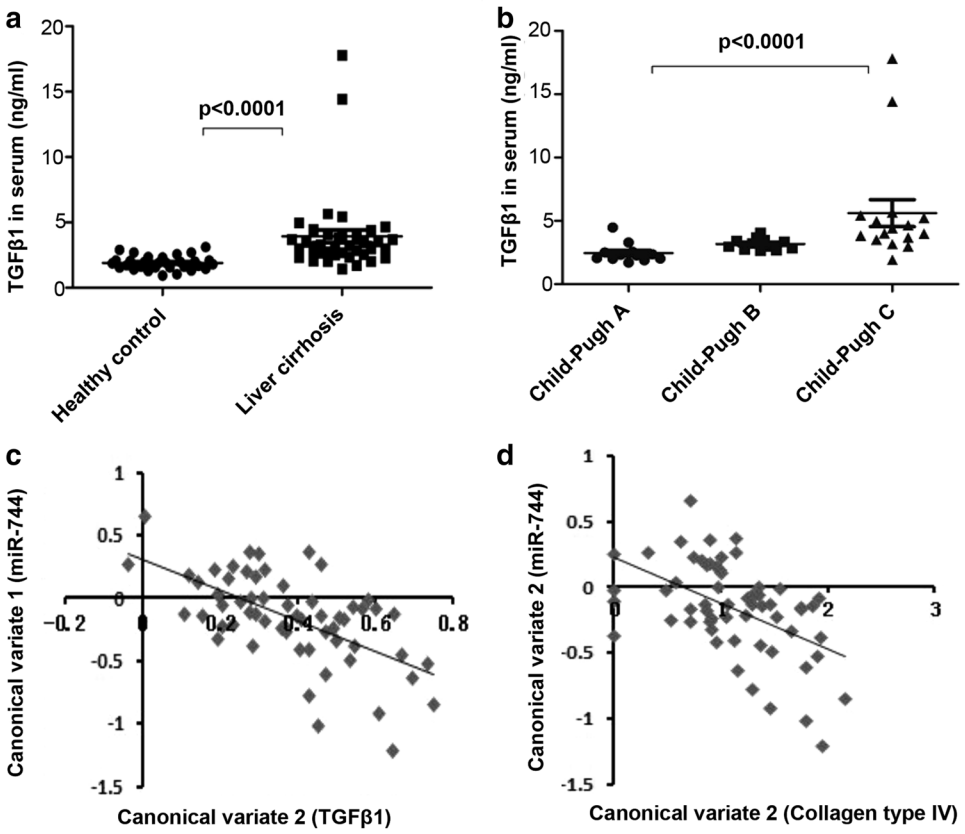


Fig. 3 TGF-β1 is elevated and inversely correlated with miR-744 in LC. **a** Serum TGFβ1 in LC patients and healthy donors. **b** Serum TGFβ1 in LC patients staged as Child–Pugh A, B and C. **c** Pearson correlation coefficient analysis of miR-744/TGFβ1 in LC patients' sera. $R=0.54$, $p<1.00\times10^{-4}$. **d** Pearson correlation coefficient analysis of miR-744/Collagen IV in LC patients' sera. $R=0.44$, $p<1.00\times10^{-4}$

we initially investigated the potential association between miR-744 and TGFβ1 in LC patients by analyzing the amount of TGFβ1 in sera of both LC patients and healthy donors. Level of serum TGFβ1 was not only higher in LC patients than healthy donors (Fig. 3a) but also increased along the disease severity (highest in Child–Pugh C and lowest in Child–Pugh A) (Fig. 3b). Pearson correlation coefficient analysis revealed that serum miR-744 and TGFβ1 were in a strong inverse correlation ($R=0.54$, $p<1.00\times10^{-4}$; Fig. 3c). As the expression of collagen IV is known to be positively regulated by TGFβ and often elevated in LC patients' sera, we further analyzed the correlation between miR-744 and collagen IV. Pearson correlation coefficient analysis again showed a negative correlation between miR-744 and collagen IV ($R=0.44$, $p<1.00\times10^{-4}$; Fig. 3d).

We next measured the level of TGFβ1 in sera and livers of CCl_4-induced LC mice and sham controls. Amount of TGFβ1 was more than threefold higher in the sera of LC mice over those of the shams (Fig. 4a). Similarly, both the levels of TGFβ1 mRNA and protein were greater in the livers of CCl_4-induced LC mice when compared with shams (Fig. 4b, c). Treatment of miR-744 Antagomir further increased TGFβ1 expression in the livers of CCl_4-induced LC mice while miR-744 Agomir downregulated the elevated level of TGFβ1 in these mice (Fig. 4d, e). Using α-SMA as the indicator of TGFβ signaling activity, we observed that miR-744 Antagomir increased the amount of α-SMA in livers while miR-744 Agomir decreased α-SMA (Fig.S1). These results indicate that miR-744 and TGFβ1 are functionally linked during LC progression.

TGF-β1 is a bona fide miR-744 target in HSCs

The ability of miR-744 to modulate TGFβ1 abundance in experimental LC model led us to further determine the effect of miR-744 on TGFβ1 expression in HSCs. We introduced miR-744 Agomir into LX-2 and JS-1 cells followed by qRT-PCR and western blot to, respectively, detect TGFβ1 mRNA and protein. MiR-744 Agomir, but not the control, downregulated both TGFβ1 mRNA and protein in both

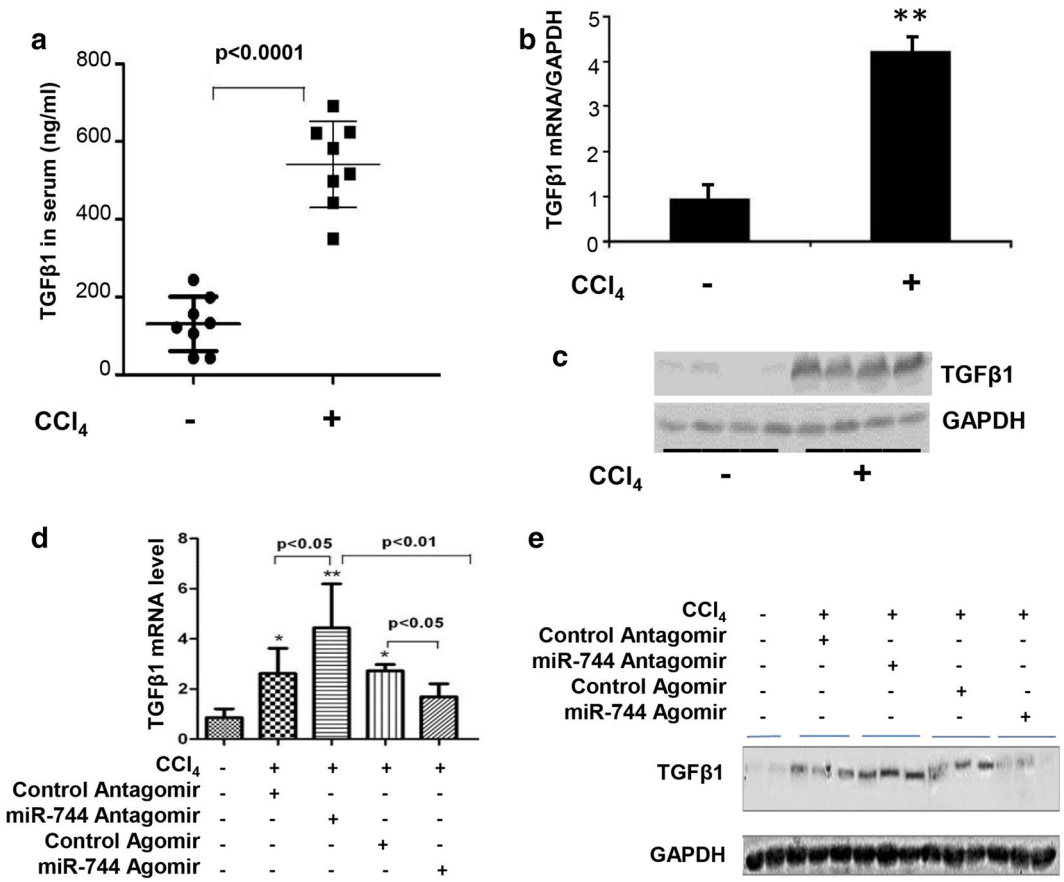
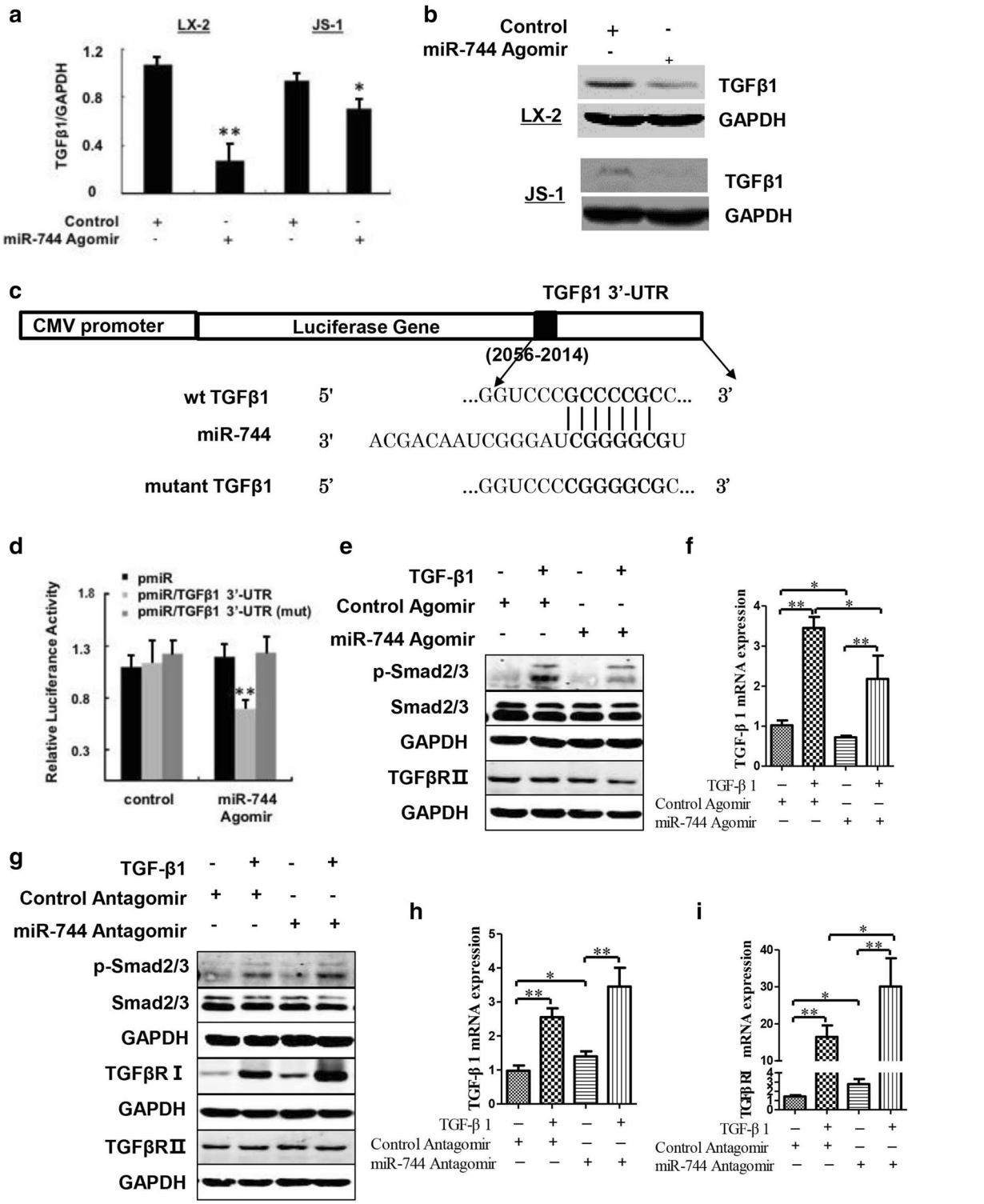


Fig. 4 TGF-β1 and miR-744 are functionally linked in liver fibrosis. **a** Serum TGFβ1 in sham control and CCl_4-induced LC mice. **b** TGFβ1 mRNA in livers of sham control and CCl_4-induced LC mice. $^{**}p<0.01$ vs sham control. **c** TGFβ1 protein in livers of sham control and CCl_4-induced LC mice. **d**, **e** Livers of sham control and CCl_4-induced LC mice administrated with control Antagomir, miR-744 Antagomir, control Agomir or miR-744 Agomir were analyzed for level of TGFβ1 mRNA (**d**) and amount of TGFβ1 protein (**e**)



HSC lines (Fig. 5a, b). Since 3′-UTR of TGFβ1 contains potential miR-744 targeting site (Fig. 5c), we examined whether TGFβ1 mRNA is a *bona fide* target of miR-744 in HSCs by generating a plasmid containing luciferase reporter plasmid that contains 3′-UTR of TGFβ1 mRNA (Fig. 5c). While control did not significantly alter luciferase activity in LX2 cells transfected with this plasmid, miR-744 Agomir reduced approximately 50% of luciferase activity (Fig. 5d). To ascertain that miR-744 targets TGFβ1 mRNA through its putative pairing site at nucleotides 2062–2068 (predicted by TargetScan) (Fig. 5c), we performed G/C → C/G mutations in this site (Fig. 5c). Transfection experiment with the

◂**Fig. 5** TGFβ1 is a *bona fide* target of miR-744 in HSCs. **a** Effect of control or miR-744 Agomir on TGFβ1 mRNA level in HSCs. $^{**}p<0.05$; $^{*}p<0.05$ vs control. **b** Effect of control or miR-744 Agomir on the amount of TGFβ1 protein in HSCs. **c** Diagram of miR-744 binding site in TGFβ1 3′-UTR and the mutation generated at this site. Alphabetical numbers are the relative nucleotide position in TGFβ1 mRNA (based on GeneEntry NM_000660 from NCBI). **d** pmiR containing TGFβ1 3′-UTR with or without mutation in miR-744-pairing site was transfected into LX-2 cells pretreated with control or miR-744 Agomir for 2 days. Cells were lysed and cell lysates were analyzed for luciferase activity. $^{**}p<0.05$ vs pmiR. **e** Western blot of p-smad2/3, smad2/3, and TGFβRII with or without miR-744 Agomir in LX-2 cells. **f** Effect of control or miR-744 Agomir on the amount of TGFβ1 mRNA in LX-2 cells. **g** Western blot of p-smad2/3, smad2/3, TGFβRI and TGFβRII with or without miR-744 Antagomir in LX-2 cells. **h** Effect of control or miR-744 Antagomir on the amount of TGFβ1 mRNA in LX-2 cells. **i** Effect of control or miR-744 Antagomir on the amount of TGFβRI mRNA in LX-2 cells. $^{*}p<0.05$, $^{**}p<0.01$

mutant construct showed that mutation in this site abolished the ability of miR-744 to reduce luciferase activity (Fig. 5d). Taken together, these results suggest that TGFβ1 mRNA is a *bona fide* miR-744 target in HSCs.

MiR-744 inhibits the activation of HSCs through the suppression of TGFβ1

The ability of miR-744 to suppress TGFβ1 expression in HSCs indicates that miR-744 has the potential to disrupt TGFβ signaling in HSCs. To test this possibility, we examined the effect of miR-744 Agomir on the levels of α-SMA, Col-I, p-smad2/3, smad2/3, TGFβRI and TGFβRII. MiR-744 Agomir, but not the control, downregulated the expression of p-smad2/3 (Fig. 5e, f). In contrast, MiR-744 Antagomir, but not the control, upregulated the expression of TGFβ1, p-smad2/3 and TGFβRI (Fig. 5g–i) while the expression of TGFβRII was not significantly changed before and after treated with miR-744 Agomir and Antagomir (Fig. 5e, g). Western blot and qRT-PCR showed that miR-744 Agomir reduced the levels of α-SMA and Col-I (Fig. 6a–c), miR-744 Antagomir increased the levels of α-SMA and Col-I (Fig. 6e), confirming that miR-744 is capable of impairing TGFβ signaling in HSCs.

Because HSC activation is accompanied by the increased formation of F-actin and cell proliferation, we analyzed the effect of miR-744 on the amount of intracellular F-actin in LX-2 cells by immunofluorescence staining with FITC-labeled phalloidin. Intensity of F-actin staining was less in LX-2 cells transfected with miR-744 Agomir when compared to those transfected with control (Fig. 6d). In contrast, miR-744 Antagomir enhanced the intensity of F-actin staining in LX-2 cells over the control (Fig. 6f). Since miR-744-caused reduction in F-actin staining was fully restored by exogenous TGFβ1 (Fig. 6d), these results suggest that miR-744 inhibits actin polymerization by diminishing TGFβ1 expression in HSCs.

We subsequently assessed the effect of miR-744 on cell proliferation in LX-2 cells. EdU assay showed that miR-744 Agomir inhibited cell proliferation while miR-744 Antagomir enhanced cell proliferation (Fig. 6g, h). However, miR-744 Agomir-led inhibition in cell proliferation was nearly restored by exogenous TGFβ1 (Fig. 6g). In a parallel experiment, we performed flow cytometry to analyze the status of cell cycle. MiR-744 Agomir led to a decrease in the percentage of cells in G0/G1 but an increase in the percentage of cells in both S and G2/M phases (Fig. 6i, j). These results suggest that miR-744 deters cell proliferation by arresting cell cycle at G2/M.

Discusion

Accumulating evidences have indicated the importance of miRNA-mediated regulation in LC [4, 17]. For example, loss of miR-200a leads to the progression of liver fibrosis by facilitating EMT [6]. Dysregulation of miR-29 is often detected in LC patients [18, 19]. Further in vitro study shows that miR-29 suppresses liver fibrosis by inhibiting the production of fibrillar collagen in HSCs [19]. In fact, miR-29 is also involved in the expression of other fibrosis-associated genes including Adam metalloproteinases (mainly type 12 and 9), ECM forming components (fibrillin-1 and follistatin-1) and different integrin chains [20]. In a previous effort to identify miRNAs differentially expressed in the circulation of LC patients, we identified miR-744 as the only circulating miRNA consistently being present in a lower abundance in LC patients when compared with healthy donors [7]. Here, we show that circulating miR-744 is not only a reliable LC biomarker (Fig. 1a, c) but also a promising diagnosis/prognosis indicator because the extent of its reduction correlates well with the severity of LC (Fig. 1b). The reduction of miR-744 is apparently LC-associated because the abundance of miR-744 is also significantly reduced in sera and livers of CCl_4-induced experimental LC mice (Fig. 2a, b, d). Together with miR-221 and miR-376c, circulating miR-744 has been reported to be a potential biomarker for gastric cancer [21]. Analysis of multiple myeloma patients' sera reveals that low circulating miR-744 level is associated with shorter overall survival and remission of myeloma patients [22]. An early study on circulating miRNA profiling showed that the level of miR-744 is one of the most constant in all tested healthy mouse strains [23], suggesting that the level of serum miR-744 may also be relatively stable in healthy people. If this is the case, we reason that alteration of circulating miR-744 may be used as a reliable biomarker for monitoring various diseases including LC.

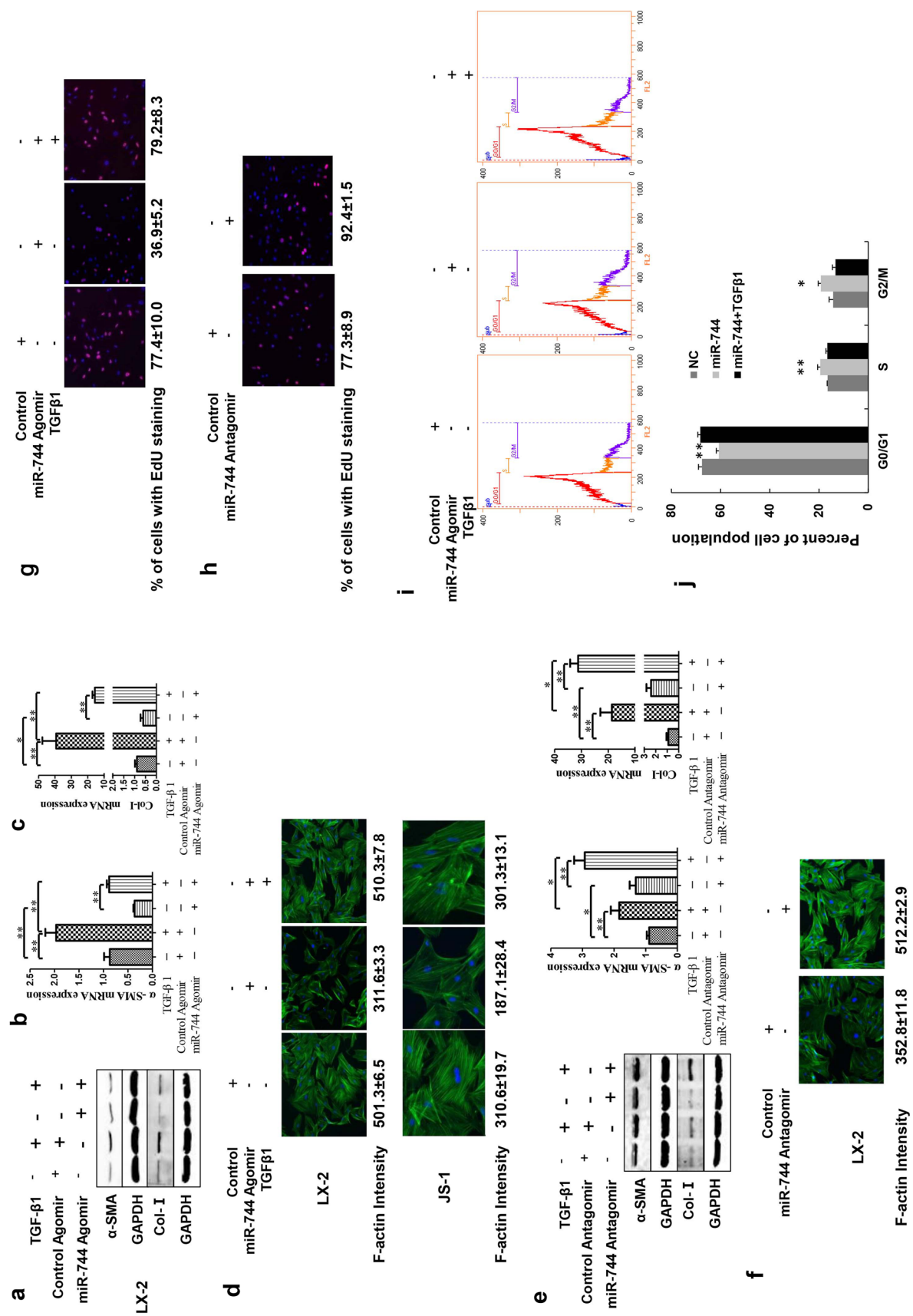

a
TGF-β1
Control Agomir
miR-744 Agomir
α-SMA
GAPDH
Col-I
GAPDH
LX-2
b
α -SMA mRNA expression
TGF-β 1
Control Agomir
miR-744 Agomir
c
Col-I mRNA expression
d
Control
miR-744 Agomir
TGFβ1
LX-2
F-actin Intensity
501.3±6.5
311.6±3.3
510.3±7.8
JS-1
F-actin Intensity
310.6±19.7
187.1±28.4
301.3±13.1
e
TGF-β1
Control Antagomir
miR-744 Antagomir
α-SMA
GAPDH
Col-I
GAPDH
α -SMA mRNA expression
Control Antagomir
miR-744 Antagomir
Col-I mRNA expression
f
Control
miR-744 Antagomir
LX-2
F-actin Intensity
352.8±11.8
512.2±2.9
g
Control
miR-744 Agomir
TGFβ1
% of cells with EdU staining
77.4±10.0
36.9±5.2
79.2±8.3
h
Control
miR-744 Antagomir
% of cells with EdU staining
77.3±8.9
92.4±1.5
i
Control
miR-744 Agomir
TGFβ1
G0/G1
S
G2/M
j
NC
miR-744
miR-744+TGFβ1
Percent of cell population
G0/G1
S
G2/M

Fig. 6 MiR-744 modulates actin reorganization in HSCs and cell proliferation of HSCs. **a** Western blot of α-SMA and Col-I in LX-2 cells treated with control or miR-744 Agomir. **b** QRT-PCR of α-SMA in LX-2 cells treated with control or miR-744 Agomir. **c** QRT-PCR of Col-I in LX-2 cells treated with control or miR-744 Agomir. **d** F-actin staining of LX-2 and JS-1 cells treated with control Agomir, miR-744 Agomir or miR-744 Agomir + TGFβ1. **e** Western blot and qRT-PCR of α-SMA and Col-I in LX-2 cells treated with control or miR-744 Agomir. **f** F-actin staining of LX-2 and JS-1 cells treated with control or miR-744 Antagomir. (**G**) EDU labeling of LX-2 cells treated with control Agomir, miR-744 Agomir or miR-744 Agomir + TGFβ1. **h** EDU labeling of LX-2 cells treated with control or miR-744 Antagomir. **i**, **j** Flow cytometry to analyze cell cycle of LX-2 cells treated with control Agomir, miR-744 Agomir and miR-744 Antagomir + TGFβ1. $^{**}p < 0.01$ vs control

The level of TGFβ1 has been reported to be higher in LC patients' blood [24]. With sera from LC patients and healthy donors, we showed that serum TGFβ1 is not only higher in LC patients (Fig. 3a) but also correlates with the severity of LC (Fig. 3b). Elevated serum level of TGFβ1 in LC patients is most likely to be associated with LC because we also detected higher level of TGFβ1 in sera and livers of CCl_4-induced experimental LC mice (Fig. 4a–c). Since level of TGFβ1 is inversely correlated with miR-744 in LC patients' sera (Fig. 3c), we considered the possibility that miR-744 and TGFβ1 may be functionally associated with LC. In fact, we observed that miR-744 was able to down-regulate TGFβ1 expression in HSCs (Fig. 5a, b, f h). Moreover, we presented evidences that TGFβ1 mRNA is a *bona fide* target of miR-744 in HSCs (Fig. 5c, d). This finding is consistent with several available miRNA interactome datasets demonstrating TGFβ1 as a miR-744 target [14–16]. One of the examples is that miR-744 can post-transcriptionally regulate TGFβ1 expression in proximal tubular cells [25].

The role of TGFβ1 in liver fibrosis and subsequent development of LC has been well established. In patients with chronic hepatitis, chronic damages lead to the activation of HSCs, resulting in the release of TGFβ1 and other profibrogenic factors. TGFβ1 then further activates HSCs, reflected by increased cell proliferation, cytoskeleton reorganization and accumulation of ECM such as collagens and ECM-degrading enzyme inhibitors [26]. In this study, we show that miR-744 Agomir is capable of inhibiting cytoskeleton reorganization (formation of F-actin) and proliferation of HSCs while miR-744 Antagomir further increases the extent of F-actin formation and cell proliferation in HSCs (Fig. 6). Since miR-744 Agomir effectively inhibits TGFβ signaling (Fig. 6) and exogenous TGFβ1 reverses miR-744-led inhibition in F-actin accumulation and proliferation (Fig. 6), we reason that miR-744 negatively regulates HSC activation and liver fibrosis by diminishing TGFβ1 expression in HSCs.

Several miRNAs have been shown to inhibit HSC activation and liver fibrosis [27]. For example, miR-16 and miR-15b suppress HSC activation by simultaneously reducing Bcl-2 and increasing caspases 3/8/9 expression [28]. MiR-150 blocks HSC activation by reducing collagen I/IV [29]. Transgenic mice with the overexpression of miR-483-5p/3p are resistant to CCl4-induced liver fibrosis and LC [30]. However, whether the administration of miRNA Agomir/Antagomir can modulate the status of liver fibrosis is unclear. Here, we show that tail vein injection of miR-744 Antagomir or Agomir effectively modulates the abundance of miR-744 in liver of CCl_4-induced experimental LC mice (Fig. 2d). MiR-744 Antagomir exacerbates liver fibrosis while miR-744 Agomir soothes the development of liver fibrosis (Fig. 2e, f). Our study thus demonstrates the feasibility of using synthetic miRNAs such as miR-744 Agomir to suppress LC progression and development.

Our mechanistic study was performed with established HSC lines that may not fully simulate clinical setting. However, the consistency seen in the in vivo model and the inverse correlation between miR-744 and TGFβ1 in LC patients' sera support the causal role of miR-744 reduction in LC established by our experimental studies. Although the mechanisms do miR744 levels decrease during LC progression was unclear, and we will perform in vivo and in vitro experiments in our further investigations to determine by what kind of mechanisms do miR744 levels decrease. Our studies conclude that miR-744/TGFβ1 functional relationship regulates the development of LC.

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Author contributions SR, SH and PL designed research. SR, JC, XL, WF and XZ performed the experiments and analyzed the data. YM, HZ, MS and CL contributed experimental materials or provided helpful suggestions. SR, JC, SH and PL wrote the manuscript.

Compliance with ethical standards

Conflict of interest Shuang Ren, Jiamei Chen, Qinglan Wang, Xuewei Li, Ying Xu, Xiao Zhang, Yongping Mu, Hua Zhang, Shuang Huang, Ping Liu have no conflict of interest to declare.

Ethics approval Ethics approval all procedures performed in studies involving human participants and animals were in accordance with the ethical standards of Shuguang Hospital affiliated to Shanghai University of Traditional Chinese Medicine. The entire study was approved by Shuguang Hospital affiliated to Shanghai University of Traditional Chinese Medicine.

Informed consent Informed consent was collected from patients to approve utilization of their samples for research purposes.

References

1. Jiao J, Friedman SL, Aloman C. Hepatic fibrosis. Curr Opin Gastroenterol 2009;25:223–229

2. Friedman SL. Mechanisms of hepatic fibrogenesis. Gastroenterology 2008;134:1655–1669
3. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–297
4. Schueller F, Roy S, Vucur M, Trautwein C, Luedde T, Roderburg C. The role of miRNAs in the pathophysiology of liver diseases and toxicity. Int J Mol Sci 2018;19:261
5. Ji J, Zhang J, Huang G, Qian J, Wang X, Mei S. Over-expressed microRNA-27a and 27b influence fat accumulation and cell proliferation during rat hepatic stellate cell activation. FEBS Lett 2009;583:759–766
6. Sun X, He Y, Ma TT, Huang C, Zhang L, Li J. Participation of miR-200a in TGF-beta1-mediated hepatic stellate cell activation. Mol Cell Biochem 2014;388:11–23
7. Zhang H, Li QY, Guo ZZ, Guan Y, Du J, Lu YY, et al. Serum levels of microRNAs can specifically predict liver injury of chronic hepatitis B. World J Gastroenterol 2012;18:5188–5196
8. Iredale JP. Hepatic stellate cell behavior during resolution of liver injury. Semin Liver Dis 2001;21:427–436
9. Gressner AM, Weiskirchen R, Breitkopf K, Dooley S. Roles of TGF-beta in hepatic fibrosis. Front Biosci 2002;7:d793–d807
10. Shen H, Huang G, Hadi M, Choy P, Zhang M, Minuk GY, et al. Transforming growth factor-beta1 downregulation of Smad1 gene expression in rat hepatic stellate cells. Am J Physiol Gastrointest Liver Physiol 2003;285:G539–G546
11. Cheng K, Yang N, Mahato RI. TGF-beta1 gene silencing for treating liver fibrosis. Mol Pharm 2009;6:772–779
12. Qi Z, Atsuchi N, Ooshima A, Takeshita A, Ueno H. Blockade of type beta transforming growth factor signaling prevents liver fibrosis and dysfunction in the rat. Proc Natl Acad Sci USA 1999;96:2345–2349
13. Chinese Society of Hepatology and Chinese Society of Infectious Diseases CMA. The guideline of prevention and treatment for chronic hepatitis B (2010 version). J Clin Hepatol 2011;27:I–XVI
14. Yi Y, Zhao Y, Li C, Zhang L, Huang H, Li Y, et al. RAID v2.0: an updated resource of RNA-associated interactions across organisms. Nucleic Acids Res 2017;45:D115–D118
15. Blin K, Dieterich C, Wurmus R, Rajewsky N, Landthaler M, Akalin A. DoRiNA 2.0–upgrading the doRiNA database of RNA interactions in post-transcriptional regulation. Nucleic Acids Res 2015;43:D160–D167
16. Bhattacharya A, Ziebarth JD, Cui Y. PolymiRTS Database 3.0: linking polymorphisms in microRNAs and their target sites with human diseases and biological pathways. Nucleic Acids Res 2014;42:D86–D91
17. Xin X, Zhang Y, Liu X, Xin H, Cao Y, Geng M. MicroRNA in hepatic fibrosis and cirrhosis. Front Biosci (Landmark Ed). 2014;19:1418–1424
18. Kwiecinski M, Noetel A, Elfimova N, Trebicka J, Schievenbusch S, Strack I, et al. Hepatocyte growth factor (HGF) inhibits collagen I and IV synthesis in hepatic stellate cells by miRNA-29 induction. PLoS One 2011;6:e24568
19. Roderburg C, Urban GW, Bettermann K, Vucur M, Zimmermann H, Schmidt S, et al. Micro-RNA profiling reveals a role for miR-29 in human and murine liver fibrosis. Hepatology 2011;53:209–218
20. Cushing L, Kuang PP, Qian J, Shao F, Wu J, Little F, et al. miR-29 is a major regulator of genes associated with pulmonary fibrosis. Am J Respir Cell Mol Biol 2011;45:287–294
21. Song MY, Pan KF, Su HJ, Zhang L, Ma JL, Li JY, et al. Identification of serum microRNAs as novel non-invasive biomarkers for early detection of gastric cancer. PLoS One 2012;7:e33608
22. Kubiczkova L, Kryukov F, Slaby O, Dementyeva E, Jarkovsky J, Nekvindova J, et al. Circulating serum microRNAs as novel diagnostic and prognostic biomarkers for multiple myeloma and monoclonal gammopathy of undetermined significance. Haematologica 2014;99:511–518
23. Mi QS, Weiland M, Qi RQ, Gao XH, Poisson LM, Zhou L. Identification of mouse serum miRNA endogenous references by global gene expression profiles. PLoS One 2012;7:e31278
24. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. N Engl J Med 2000;342:1350–1358
25. Martin J, Jenkins RH, Bennagi R, Krupa A, Phillips AO, Bowen T, et al. Post-transcriptional regulation of Transforming Growth Factor Beta-1 by microRNA-744. PLoS One 2011;6:e25044
26. Matsuzaki K, Murata M, Yoshida K, Sekimoto G, Uemura Y, Sakaida N, et al. Chronic inflammation associated with hepatitis C virus infection perturbs hepatic transforming growth factor beta signaling, promoting cirrhosis and hepatocellular carcinoma. Hepatology 2007;46:48–57
27. Zhang CY, Yuan WG, He P, Lei JH, Wang CX. Liver fibrosis and hepatic stellate cells: etiology, pathological hallmarks and therapeutic targets. World J Gastroenterol 2016;22:10512–10522
28. Guo CJ, Pan Q, Li DG, Sun H, Liu BW. miR-15b and miR-16 are implicated in activation of the rat hepatic stellate cell: an essential role for apoptosis. J Hepatol 2009;50:766–778
29. Zheng J, Lin Z, Dong P, Lu Z, Gao S, Chen X, et al. Activation of hepatic stellate cells is suppressed by microRNA-150. Int J Mol Med 2013;32:17–24
30. Li F, Ma N, Zhao R, Wu G, Zhang Y, Qiao Y, et al. Overexpression of miR-483-5p/3p cooperate to inhibit mouse liver fibrosis by suppressing the TGF-beta stimulated HSCs in transgenic mice. J Cell Mol Med 2014;18:966–974