Original Paper

MicroRNA-125a-5p Contributes to Hepatic Stellate Cell Activation through Targeting FIH1

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Key Words

MicroRNA • MiR-125a-5p • Hepatic stellate cells • Liver fibrosis • FIH1

Abstract

Background/Aims: Emerging evidence shows that microRNAs (miRNAs) play a crucial role in the regulation of activation, proliferation and apoptosis of hepatic stellate cells (HSCs). Previous studies have indicated that miR-125a-5p is correlated with hepatitis B virus replication and disease progression. However, little is known about the biological role and underlying mechanism of miR-125a-5p in liver fibrosis. **Methods:** We analyzed the level of miR-125a-5p in carbon tetrachloride-induced liver fibrosis and activated HSCs. We analyzed the effects of miR-125a-5p down-regulation on HSC activation and proliferation. We also analyzed the binding of miR-125a-5p to the 3'-untranslated region of factor inhibiting hypoxia-inducible factor 1 (FIH1) mRNA. **Results:** Up-regulation of miR-125a-5p was observed in the liver tissues of fibrotic mice and activated HSCs. Down-regulation of miR-125a-5p prevented the activation and proliferation of HSCs. FIH1, a negative modulator of hypoxia inducible factor 1, was confirmed to be a target of miR-125a-5p using the luciferase reporter assay. Further studies demonstrated that miR-125a-5p prompted the activation and proliferation of HSCs, at least in part, by down-regulating FIH1. **Conclusion:** Our findings shed new light on miRNAs as a promising therapeutic target in liver fibrosis.

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Introduction

Liver fibrosis, characterized by the accumulation of extracellular matrix, is a frequent outcome of the pathological response to chronic liver injury. In response to chronic liver injury, hepatic stellate cells (HSCs) are activated and differentiate into myofibroblast-like cells, and express abundant alpha smooth muscle actin (α -SMA) and extracellular matrix components [1, 2]. Therefore, the activation and proliferation of HSCs are central events in the progression of liver fibrosis. However, to date, the regulatory mechanisms of HSCs are only partially understood.

MicroRNAs (miRNAs) are endogenous, short non-coding RNAs of approximately 21–25 nucleotides in length that mediate gene expression by binding to the 3'-untranslated region (3'UTR) of mRNAs, thus leading to the degradation or translational suppression of the corresponding mRNAs [3, 4]. Emerging evidence indicates that miRNAs are implicated in liver fibrosis [5–7]. For instance, miR-19b is down-regulated in activated HSCs and in fibrotic liver tissues. Ectopic expression of miR-19b in activated HSCs can markedly decrease the expression of transforming growth factor- β receptor II through direct binding to the 3'UTR, thus preventing the activation of HSCs [8]. miRNA profiling has revealed that the miR-29 family is down-regulated in human and murine liver fibrosis compared with normal liver tissues [9]. A further study demonstrated that miR-29b attenuates liver fibrosis by inhibiting HSC activation and enhancing apoptosis by targeting the PI3K/AKT signaling pathway [10]. Our previous study indicated that miR-17-5p stimulates the progression of liver fibrosis by the activating Wnt/ β -catenin signaling pathway [11]. These studies clearly show that miRNAs play a crucial role in the regulation of activation, proliferation and apoptosis of HSCs.

miR-125a-5p has been shown to be overexpressed in many cancers [12–14]. In addition, miR-125-5p is also reported to trigger proliferation, invasion, and migration in tumor cells [15–17]. In chronic hepatitis B patients, miR-125a-5p is correlated with hepatitis B virus (HBV) replication and disease progression [18]. Our previous study indicated that serum miR-125a-5p may predict disease progression in human liver diseases [19]. However, the biological role and underlying mechanism of miR-125a-5p in liver fibrosis requires further study.

In the present study, we show that miR-125a-5p is up-regulated in the liver tissues of fibrotic mice and activated HSCs. Down-regulation of miR-125a-5p inhibited the activation and proliferation of HSCs, at least in part, by targeting factor inhibiting hypoxia-inducible factor 1 (FIH1).

Materials and Methods

Liver fibrosis model

Male C57BL/6 mice (20 – 22 g) received an intraperitoneal injection of carbon tetrachloride (CCl_4 ; 7 $\mu\text{l/g}$ /mouse) mixed with olive oil (10% CCl_4). To induce liver fibrosis, CCl_4 was given twice a week for 8 weeks. Control mice were treated with an equal volume of olive oil at the same time intervals. The mice were euthanized 48 h after the last CCl_4 administration. The experiments were performed in accordance with the principles summarized in the Animal Experimentation Ethics Committee Guide for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Wenzhou Medical University (Wenzhou, China). Some of the liver samples were fixed, embedded in paraffin, and processed for hematoxylin-eosin (HE) staining, van Gieson (VG) staining, and Sirius Red staining. Other liver samples were snap frozen in liquid nitrogen and stored at -80°C for further analysis.

Isolation of primary mouse hepatocytes

Primary mouse hepatocytes were isolated as reported previously [20]. Briefly, after liver was sequentially *in situ* perfused with magnesium- and calcium- free Hanks' balanced salt solution supplemented with 0.5 mM EDTA (Invitrogen) and Dulbecco's modified Eagle's medium (DMEM) supplemented with collagenase (Sigma), the liver became soft and was removed in DMEM supplemented with collagenase at

37°C for 15 min. Hepatocytes were isolated by centrifugation of the filtered homogenate at 40 *g* for 5 min. The pellet was washed two times with DMEM and seeded with the culture medium.

Cell transfection

Primary mouse HSCs were isolated as reported previously [21]. HSCs were maintained in DMEM containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco). Cells were cultured at 37°C in a humidified 5% CO₂ incubator. The miR-125a-5p inhibitor, miR-125a-5p mimics, miRNA negative control (miR-NC), siRNA against FIH1 (si-FIH1) and siRNA control (si-Ctrl) were obtained from GenePharma Corporation (Shanghai, China). Transfections were performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Cell proliferation assay

Cell proliferation was analyzed using a CCK-8 assay kit (Beyotime Biotechnology, Jiangsu, China). HSCs were maintained in 96-well plates for 24 h. These cells were then transfected with miR-125a-5p inhibitor or si-FIH1 using Lipofectamine 2000 (Life Technologies). Approximately 48 h later, CCK-8 solution was added to each well in the 96-well plates, and the cells were incubated for another 2 h at 37°C. Absorbance was measured at 450 nm on a microplate reader (Bio-Rad, Hercules, CA, USA).

Luciferase reporter assay

HEK293T cells were co-transfected with either luciferase reporter plasmid harboring wild-type FIH1 3'UTR (pmirGLO-FIH1-wt) or mutant FIH1 3'UTR (pmirGLO-FIH1-mut) together with miR-125a-5p mimics or miR-NC. The 3'UTR of FIH1 was cloned downstream of the firefly luciferase gene. If there was an interaction between miR-125a-5p and the 3'UTR of FIH1 mRNA, the activity of firefly luciferase was decreased. If there was no interaction, the activity of firefly luciferase was unchanged. Approximately 48 h after transfection, the cells were harvested and luciferase activity was determined by a luminometer using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Quantitative real-time PCR

To examine mRNA expression, total RNA was isolated from HSCs using the TRIzol Kit (Life Technologies). Fifty nanograms of total RNA were then reverse-transcribed to cDNA using the PrimeScript™ 1st Strand cDNA Synthesis Reagent (TaKaRa Biotechnology, Dalian, China) following the manufacturer's protocols. To examine miRNA expression, total RNA was isolated using a miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols. Gene expression was quantified by real-time PCR using the KAPA SYBR FAST qPCR Kit (KapaBiosystems, Boston, MA, USA), cDNA and gene-specific primers (Table 1). The relative abundance of mRNAs and miRNAs were normalized to GAPDH and U6 snRNA, respectively.

Western blot

The antibodies used in this experiment were as follows: collagen 1a1 (Col1A1) (Sigma-Aldrich, St Louis, MO, USA), α-SMA, FIH1, hypoxia-inducible factor 1 alpha (HIF-1α) and β-actin (Abcam, Cambridge, MA, USA). The protein samples were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The membranes were incubated with the primary antibodies overnight at 4°C and then with the secondary antibodies at 37°C for 1 h. After the membranes were washed three times with PBS-T, they were analyzed using an Odyssey infrared scanner (LI-COR Biosciences Inc., Lincoln, NE, USA).

Table 1. List of primer sequences

Gene		Sequences (5'-3')
Mouse	Forward	TGCACCACCAACTGCTTAG
GAPDH	Reverse	GGATGCAGGGATGATGTTT
Mouse	Forward	CCTGGCAAAGACGGACTCAAC
Col1A1	Reverse	GCTGAAGTCATAACCGCCACTG
Mouse	Forward	TCCCTGGAGAAGAGCTACGAACT
α-SMA	Reverse	AAGCGTTTCGTTTCCAATGGT
Mouse	Forward	AGGCATTTGCAGCCGTTT
FIH1	Reverse	CTAGGCTGAGTCAGGCAGTATC
Mouse	Forward	TCATCAGTTGCCACTTCC
HIF-1α	Reverse	CACCATCACAAAGCATCTAGG
Mouse	Forward	CTCGCTTCGGCAGCACA
U6	Reverse	AACGCTTCACGAATTTGCGT
Mouse	Forward	ACACTCCAGCTGGGTCCCTGAGACCCCTTA
miR-125a-5p	Reverse	TGGTGTCTGGAGTCG
	RT	CTCAACTGGTGTCTGGAGTCGGCAATT
		CAGTTGAGTCACAGGT

Statistical Analysis

The results are reported as the mean $\pm$ SD. Data are compared among the groups using the Student's *t*-test or one-way analysis of variance, and $P < 0.05$ was considered statistically significant. All statistical analyses were carried out using SPSS 13.0 (IBM, Armonk, NY, USA).

Results

miR-125a-5p is up-regulated in liver fibrosis and activated HSCs

miR-125a-5p has been reported to be increased in human fibrotic liver tissues [18]. To determine the level of miR-125a-5p in liver tissues from mice with CCl₄-induced liver fibrosis, the severity of liver fibrosis was analyzed by HE staining, VG staining, and Sirius Red staining (Fig. 1A-C). The results indicated that 8 weeks of CCl₄ administration resulted in obvious fibrous septa in liver tissues. Compared with the control, an increased level of miR-125a-5p was observed in liver fibrosis (Fig. 1D), consistent with previous results in humans [18, 19].

Activation and proliferation of HSCs are crucial factors leading to the progression of liver fibrosis. Therefore, we examined the level of miR-125a-5p in primary HSCs and hepatocytes isolated from mice with CCl₄-induced liver fibrosis. As shown in Fig. 1E, F, in parallel with the control, the level of miR-125a-5p was markedly increased in HSCs isolated from mice treated with CCl₄, whereas there was a slight increase in hepatocytes isolated from mice treated with

Fig. 1. Expression of miR-125a-5p is increased in liver fibrosis and activated HSCs. (A-C) HE staining, VG staining, and Sirius Red staining for the evaluation of liver fibrosis ($n = 6$). Scale bars, 100 μ m. (D) miR-125a-5p expression was detected in CCl₄-induced fibrotic liver tissues and normal liver tissues ($n = 6$). (E) miR-125a-5p expression was detected in HSCs isolated from olive oil/CCl₄-treated mice ($n = 3$). (F) miR-125a-5p expression was detected in hepatocytes isolated from olive oil/CCl₄-treated mice ($n = 3$). Data are presented as mean $\pm$ SD, * $P < 0.05$, ** $P < 0.01$.

Fig. 2. Down-regulation of miR-125a-5p prevents activation and proliferation of HSCs. (A) miR-125a-5p inhibitor decreased the mRNA expression of Col1A1 and α-SMA. (B, C) miR-125a-5p inhibitor decreased the protein expression of type1 collagen and α-SMA. (D) miR-125a-5p inhibitor decreased the proliferation of HSCs. Results were analyzed using the CCK-8 assay. Data represent the mean ± SD of three independent experiments, ** $P < 0.01$. inh, inhibitor.

CCl₄. Taken together, these results indicate that miR-125a-5p is up-regulated in liver fibrosis and activated HSCs.

Effects of miR-125a-5p down-regulation on activation and proliferation of HSCs

We next determined the effects of miR-125a-5p down-regulation on the activation of HSCs. As shown in Fig. 2A, the level of Col1A1 mRNA in HSCs transfected with the miR-125a-5p inhibitor was decreased by 67% compared with the miR-NC. There was also a 71% decrease in the level of α-SMA mRNA. Consistent with the results of mRNA levels, western blotting results indicated that the protein levels of type 1 collagen and α-SMA were notably inhibited by the miR-125a-5p inhibitor (Fig. 2B, C). We next investigated the effects of miR-125a-5p down-regulation on the proliferation of HSCs using the CCK-8 assay. Our results demonstrated that the miR-125a-5p inhibitor triggered a 57% reduction in HSC proliferation compared with the miR-NC (Fig. 2D). Collectively, our data suggest that down-regulation of miR-125a-5p suppresses the activation and proliferation of HSCs.

FIH1 is a target of miR-125a-5p

To further investigate the possible mechanism of miR-125a-5p in the progression of liver fibrosis, the prediction of miRNA target regions by TargetScan software (<http://www.targetscan.org/>) suggested that miR-125a-5p interacts with the 3'UTR of mouse FIH1 mRNA (Fig. 3H). FIH1 can negatively regulate the expression of HIF-1α, which has been reported to be involved in liver fibrosis [22]. Our results demonstrated that the level of FIH1 mRNA was decreased in liver fibrosis and activated HSCs (Fig. 3A, B). We then determined whether miR-125a-5p influenced the expression of FIH1. Similar to the miR-NC, the mRNA and protein levels of FIH1 in HSCs transfected with miR-125a-5p mimics were decreased by 69% and

Fig. 3. Interaction of miR-125a-5p with the 3'UTR of FIH1 mRNA. (A) The mRNA expression of FIH1 was detected in CCl₄-induced fibrotic liver tissues and normal liver tissues (n = 6). (B) The mRNA expression of FIH1 was detected in HSCs isolated from olive oil/CCl₄-treated mice (n = 3). (C) miR-125a-5p mimics decreased the mRNA expression of FIH1 (n = 3). (D) miR-125a-5p mimics increased the mRNA expression of HIF-1α (n = 3). (E-G) miR-125a-5p mimics decreased the protein expression of FIH1 and increased the protein expression of HIF-1α (n = 3). (H) miR-125a-5p-binding sites in the 3'UTR of FIH1 mRNA based on TargetScan software. (I) Analysis of luciferase activity in HEK293T cells transfected with pmirGLO-FIH1-wt or pmirGLO-FIH1-mut as well as those co-transfected with miR-125a-5p mimics or miR-NC (n = 3). Data represent the mean ± SD, **P < 0.01.

52%, respectively (Fig. 3C, E and F). In contrast, the mRNA and protein levels of HIF-1α were increased by 1.3-fold and 60%, respectively (Fig. 3D, E and G). These data indicated that the FIH1/HIF-1α signaling pathway was mediated by miR-125a-5p in HSCs. To assess whether miR-125a-5p directly represses FIH1 expression via the putative miR-125a-5p binding sites, the 3'UTR target sequence of FIH1 mRNA was cloned into the pmirGLO plasmid. The construct was transfected into HEK293T cells together with miR-125a-5p mimics and miR-NC. In pmirGLO-FIH1-wt, miR-125a-5p mimics led to an obvious reduction in luciferase activity compared with the miR-NC (Fig. 4I). However, in pmirGLO-FIH1-mut, miR-125a-5p mimics did not decrease luciferase activity compared with the miR-NC. In summary, our data indicate that FIH1 is a direct target of miR-125a-5p.

Down-regulation of miR-125a-5p inhibits activation and proliferation of HSCs by increasing FIH1

Hu et al. [22] reported that FIH1 is able to prevent the activation of HSCs. Thus, we examined whether FIH1 is required for the activation of HSCs induced by miR-125a-5p. As shown in Fig. 4A, B, si-FIH1 markedly decreased the mRNA and protein levels of FIH1.

Fig. 4. miR-125a-5p inhibitor prevents activation and proliferation of HSCs by up-regulating FIH1. (A) si-FIH1 decreased the mRNA expression of FIH1. (B) si-FIH1 decreased the protein expression of FIH1. (C) Down-regulation of α -SMA protein by miR-125a-5p inhibitor was attenuated by si-FIH1. (D) Down-regulation of HSC proliferation by miR-125a-5p inhibitor was attenuated by si-FIH1. Data represent the mean $\pm$ SD of three independent experiments, ** P < 0.01. inh, inhibitor.

In addition, the decrease in cell activation by the miR-125a-5p inhibitor was partially reversed in the presence of si-FIH1 (Fig. 4C). We next investigated whether FIH1 is required for the proliferation of HSCs induced by miR-125a-5p. Our results demonstrated that decreased cell proliferation by the miR-125a-5p inhibitor was also partially restored by the addition of si-FIH1 (Fig. 4D). In conclusion, our data indicate that miR-125a-5p promotes the activation and proliferation of HSCs, at least in part, by decreasing FIH1.

Discussion

In this study we present evidence to show that miR-125a-5p has a biological role in HSCs. Mechanistically, miR-125a-5p promotes the activation and proliferation of HSCs, at least in part, by reducing FIH1 expression. Our findings extend the field of miRNA research in liver fibrosis.

A number of studies have indicated that miR-125a-5p is involved in a variety of diseases [23-25]. miR-125a-5p expression can be induced by GATA binding protein 3 under inflammatory conditions, thus reducing the sensitivity of regulatory T cells by suppressing IL-6 receptor and STAT3 expression [26]. In gastric cancer, miR-125a-5p can prevent the proliferation of tumor cells and act as an independent prognostic factor [13]. In breast cancer, serum miR-125a-5p expression is a useful prognostic biomarker and provides a novel therapeutic approach by targeting HDAC4 [14]. With regard to HBV, it has been reported that miR-125a-5p can bind the viral transcript encoding the surface antigen, thus interfering with its expression and repressing viral replication [27]. Coppola et al. [18] further indicated that liver miR-125a-5p is correlated with HBV replication and disease progression in chronic hepatitis B patients. Our previous study demonstrated that serum miR-125a-5p may be a non-invasive biomarker for predicting disease progression in human liver diseases [19]. Here we report that miR-125a-5p is up-regulated in mice with liver fibrosis and activated HSCs, in line with the results obtained in humans. In addition, our results demonstrated that the addition of miR-125a-5p inhibitor hinders the activation of HSCs. Data from the proliferation assay showed that down-regulation of miR-125a-5p reduces the proliferation

of HSCs. To the best of our knowledge, this is the first report to show that miR-125a-5p is involved in HSC activation and proliferation.

Recently, a large number of studies have demonstrated that hypoxia and HIF-1 α are involved in liver fibrosis. It has been reported that exposure of primary mouse HSCs to 0.5% oxygen can induce HIF-1 α expression, thus activating abundant genes which are pivotal for HSC function, angiogenesis, and collagen synthesis [28]. *In vitro* experiments showed that HIF-1 α knockdown can inhibit HSC activation by cooperating with mitogen activated protein kinase [29]. Data from HIF-1 α -deficient mice demonstrate that the levels of type 1 collagen and α -SMA were substantially decreased compared with control mice after bile duct ligation [30]. Deng et al. [31] reported that HIF-1 α can activate HSCs by regulating autophagy. FIH1 has been shown to be involved in liver fibrosis by negatively regulating HIF-1 α expression [22]. In our study, bioinformatics analysis and luciferase reporter experiments indicated that FIH1 is a target of miR-125a-5p, which expands the repertoire of miR-125a-5p targets. Further studies revealed that miR-125a-5p promotes the activation and proliferation of HSCs by down-regulating FIH1 level. However, the functional role of miR-125a-5p *in vivo* requires further investigation.

Taken together, our findings indicate that miR-125a-5p can induce the activation and proliferation of HSCs, at least in part, by down-regulating FIH1. Possible use of this miRNA as a therapeutic target requires better knowledge of its effects on carcinogenesis.

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Disclosure Statement

The authors have stated that there is no competing interest in this study.

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