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# miR-181b Promotes hepatic stellate cells proliferation by targeting p27 and is elevated in the serum of cirrhosis patients

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ABSTRACT

MicroRNAs, as a kind of negative gene regulators, were demonstrated to be involved in many types of diseases. In this study, we found that transforming growth factor-beta 1 could induce the expression of miR-181a and miR-181b, and miR-181b increased in the much higher folds than miR-181a. Because of the important role of transforming growth factor-beta 1 in HSC activation and liver cirrhosis, we investigate the effect of miR-181a and miR-181b on HSC proliferation. The results showed that miR-181b could promote HSC-T6 cell proliferation by regulating cell cycle. Further study showed p27, the cell cycle regulator, was the direct target of miR-181b in HSC-T6 cell. But miR-181a had no effects on HSC-T6 cell proliferation and cell cycle, and did not target p27. Interestingly, miR-181b is elevated significantly in serum of liver cirrhosis cases comparing to that of normal persons, whereas miR-181a expression was in the similar level with that of normal persons. These results suggested that miR-181b could be induced by TGF-β1 and promote the growth of HSCs by directly targeting p27. The elevation of miR-181b in serum suggested that it may be potential diagnostic biomarkers for cirrhosis. As for miR-181a, it may work in TGF-β1 pathway by a currently unknown mechanism.



## 1. Introduction

Liver fibrosis occurs in many types of chronic liver disease and is characterized by the accumulation of extracellular matrix proteins, including collagen. Advanced liver fibrosis results in cirrhosis, liver failure, and portal hypertension. The cellular and molecular mechanisms of liver fibrosis have been the focus of intensive research. Hepatic stellate cells (HSCs) have long been considered to be critically important mediators of liver fibrosis [1]. In normal liver tissue, HSCs are nonparenchymal, quiescent cells whose main functions are to store vitamin A and to maintain a normal basement membrane. However, numerous in vivo and in vitro studies have indicated that HSCs undergo an "activation" process in response to liver injury, in which they lose their vitamin A stores, become proliferative and fibrogenic, and adopt a contractile myofibroblast-like phenotype [2]. Despite continuing research efforts, some characteristics of HSC regulation remain poorly understood, and there are no currently available drugs that inhibit liver fibrosis. As such, further studies on liver fibrosis and cirrhosis are needed. In this context, the current study explores the underlying mechanisms of HSC activation, which may prove helpful in developing new therapies for fibrotic diseases of many organs, including the liver.

MicroRNAs (miRNAs) are small noncoding RNAs (21–25 nucleotides long), which usually negatively modulate gene expression at the post-transcriptional level by the complete or incomplete complementary binding to target sequences within the 3′-untranslated region (3′-UTR) of mRNA [3]. There is an accumulating body of evidence that miRNAs are involved in a variety of biological processes, such as cell proliferation, differentiation, apoptosis, and tumorigenesis [4]. Aberrant and/or absent expression of miRNAs are often associated with pathophysiological disorders; indeed, the recent literature suggests that miRNAs may be important mediators of liver disease, and more specifically HSC activation [5]. In this study, we found that microRNA-181a and microRNA-181b (miR-181a and miR-181b, respectively) levels increased significantly in HSC-T6 cells treated with transforming growth factor-beta 1 (TGF-β1). Further experiments revealed that miR-181b promoted HSC-T6 growth by directly downregulating the expression of p27.

*Abbreviations:* HSC, hepatic stellate cells; miRNA, microRNA; 3′-UTR, 3′-untranslated region; TGF-β1, transforming growth factor-beta 1; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; α-SMA, alpha smooth muscle actin.

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Moreover, we found that serum levels of miR-181b were elevated in patients with cirrhosis. This finding indicates miR-181b may be a potential diagnostic serum marker for liver cirrhosis.

## 2. Material and methods

### 2.1. Clinical samples and cell lines

The serum of 22 patients with cirrhosis and 17 healthy controls were collected at Xinhua Hospital, Shanghai Jiaotong University, Shanghai, China. All human materials were obtained between 2010 and 2011 according to consent regulation and the Ethical Review Committee approval of Xinhua Hospital, Shanghai Jiaotong University School of Medicine.

The HSC-T6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), in a humidified incubator at 37 °C with 5% $CO_2$.

### 2.2. Activation of HSC-T6 cells induced by TGF-β1

HSC-T6 cells were plated in a 6-well plate at 30% confluence. Initially, cells were cultured with DMEM/F12 containing 10% FBS for 6 h. The medium was then replaced with DMEM/F12 without FBS to starve the cells for 12 h. The cells were then cultured with DMEM/F12 that contained 0, 2, or 5 ng/ml TGF-β1 (without FBS) for 24 or 48 h. After TGF-β1 treatment, the cells were collected for isolating total RNA using Trizol (Invitrogen, Carlsbad, CA, USA) or isolating protein with RIPA buffer (Millipore, Billerica, MA, USA). RNA samples were used to detect the expression of miR-181a, miR-181b and U6B, and protein extracts were used to detect the expression of alpha smooth muscle actin (α-SMA, Sigma–Aldrich, St. Louis, MO, USA), which is a marker of activated HSCs.

### 2.3. miRNA transfection

One day before transfection, HSC-T6 cells were seeded in a 6-well plate to ensure that they would be 30–50% confluent at the time of transfection. The synthetic mimics of miR-181a, miR-181b and GMR-miRTM (negative control; GenePharma, Shanghai, China) were transfected using lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA). The final concentration of microRNA mimics used for transfection was 50 nM. Transfected cells were collected for different assays at indicated time.

### 2.4. p27 3′-UTR construction and luciferase assay

The wild-type and mutant miR-181 binding sequence at the 3′-UTR of human p27 were synthesized, denatured/renatured and then cloned into the pRGL-4.51 luciferase vector (Promega, Madison, WI, USA). The sense sequences were 5′-TGCCTCTAAAAGCGTTGGAT GTT TGGGAGTTTTGAATGTT-3′ (wild-type), and 5′-TGCCTCTAAATCGCA ACGTACATTGGGAGTTTACTTACAT-3′ (mutant). Luciferase assays were performed as previously described [6]. In brief, the constructed p27 3′-UTR luciferase vector and miR-181a or miR-181b were co-transfected into HSC-T6 cells using Lipofectamine 2000 (Invitrogen), after 24 h the cells were collected and luciferase activity was analyzed. The mutant p27 3′-UTR was used as negative control.

### 2.5. Detection of miR-181a and miR-181b by real-time PCR

Real time PCR was used to analyze the relative expression levels of miR-181a and miR-181b induced by TGF-β1 in HSC-T6 cells, as well as the expression of miR-181a and miR-181b in human serum. Total RNA was extracted from serum and HSC-T6 cells using a virVana PARIS and miRVana miRNA isolation kit (Ambion, Austin, TX, USA). RNA samples were reverse-transcribed into cDNA with miRNA-specific primers using a TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA). The expression of miR-181a and miR-181b was detected by the TaqMan MicroRNA Assay kit (Applied Biosystems). The reverse transcription reaction was performed for 30 min at 16 °C, then 30 min at 42 °C, and 5 min at 85 °C. A lightcycler® 480 Real-Time PCR System (Roche, Basel, Switzerland) was used for PCR reaction, Realtime PCR condition was 2 min at 95 °C, 15 s at 95 °C, and 1 min at 60 °C for 40 cycles. All reactions were run in triplicate. Relative quantification was used to calculate relative gene expression levels, using u6 as an internal control.

### 2.6. Analysis of alpha smooth muscle actin expression by western-blot

Protein expression level of α-SMA was determined by Western blotting. Briefly, 20 μg of protein was loaded onto a 12% SDS–polyacrylamide gel and transferred into nitrocellulose membranes (Invitrogen) using a semi-dry blotting system (Invitrogen). Membrane blocking was carried out in 1× TBST, containing 5% nonfat dry milk and 0.2% Tween 20, for 30 min at room temperature. The membranes were then incubated overnight with the primary antibodies in blocking buffer (1× TBST, 3% BSA, 0.2% Tween 20) at 4 °C. Primary antibodies were used at a dilution of 1:1000. The membranes were washed three times for 10 min with 1× TBST and then incubated with secondary antibodies at a final dilution of 1:2000. After final washes with 1× TBS, 0.2% Tween 20, the signals were detected using ECL chemiluminescence reagents (Pierce, Rockford, IL, USA). To confirm that the same amount of protein was investigated in each sample, the expression of α-tubulin was used as a control.

### 2.7. Growth curve and cell cycle

Five hours after transfection with miR-181a, miR-181b or negative control microRNA mimics, the cells were transferred into 96-well plates, at a density of 5000 cells per well for 0, 48 and 96 h. The cells numbers were counted in triplicate using the Cell Count Kit-8 (Dojindo, Kumamoto, Japan), according to the manufacturer's protocol. The cell cycle phase was determined by FACS analysis with propidium iodide (PI) staining. Briefly, harvested cells were fixed with 70% ethanol at 4 °C. Fixed cells were then stained with 50 μg/mL PI at 37 °C for 30 min and simultaneously treated with 10 μg/mL RNase, in order to digest RNA in the cells. The cells were submitted to FACS assay.

### 2.8. Statistical analysis

*P*-values were generated by the Student's *t*-test for real-time PCR and cell proliferation assays. All experiments were performed in triplicate.

## 3. Results

### 3.1. TGF-β1 induced expression of miR-181a and miR-181b in HSC-T6 cells

Persistent activation of HSCs has been recognized as one of the most important factors that causes liver fibrosis. TGF-β1 is one of the most important molecules which promotes liver fibrosis and induces the activation of HSCs. Here, we stimulated the HSC line, HSC-T6 [7], with different concentrations of TGF-β1 and analyzed

the change of miR-181a and miR-181b expression. The results show that expression of the HSC activation marker, α-SMA, was increased by TGF-β1 treatment, which indicated that TGF-β1 induced the activation of HSC-T6 cells (Fig. 1A). In treated HSC-T6 cells, miR-181b levels increased by more than 200 times, while miR-181a also increased significantly, but with a relatively lower level (~5-folds) compared with miR-181b (Fig. 1B) These results suggested that miR-181a and miR-181b, especially miR-181b, may play a vital role in the process of liver fibrosis and cirrhosis.

### 3.2. miR-181b promoted growth of HSC-T6 cells

We assessed whether the expression of miR-181a or miR-181b affected cellular proliferation of HSC-T6 cells. In cells transfected with 50 nM miR-181b, there was a significant increase in proliferation compared to cells transfected with control miRNA (Fig. 2A). However, miR-181a had no effect on the growth of HSC-T6 cells. These observations indicated a role for miR-181b in the growth regulation of HSCs.

### 3.3. miR-181b increased S phase of HSC-T6 cells

It is known that abnormal cell proliferation is related to the altered cell cycle. Cell cycle analysis was conducted in HSC-T6 cells transfected with miR-181a and miR-181b mimics. The proportion of cells in S phase was higher in miR-181b transfected cells, than that in control miRNA transfected cells ($P < 0.05$). These data suggested that miR-181b could promote cell proliferation by increasing the population of cells in S phase. In addition, we checked the effect of miR-181a on the cell cycle and no obvious differences were observed between miR-181a-tansfected HSC-T6 cells and control cells (Fig. 2B–E).

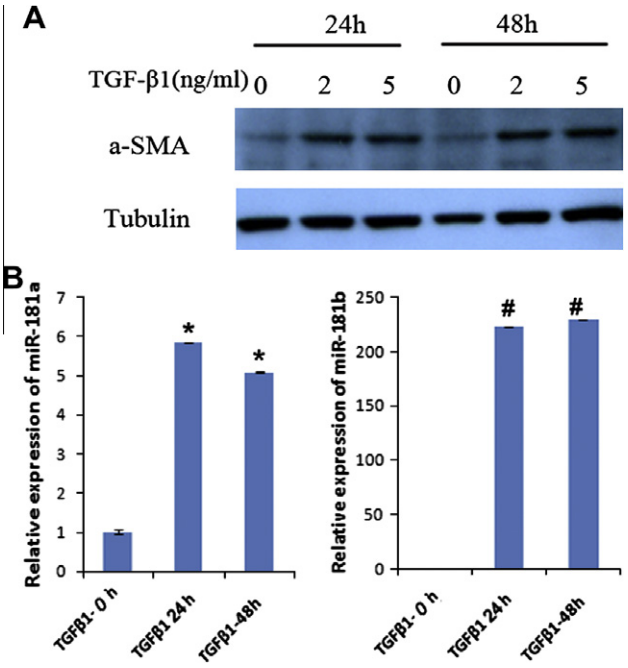


**Fig. 1.** TGF-β1 stimulated activation of HSC-T6 cells and induced the expression of miR-181a and miR-181b. (A) α-SMA is an activation marker of HSC cells. α-SMA protein expression was induced by TGF-β1 treatment for 24 and 48 h at concentration of 2 and 5 ng/mL in HSC-T6 cells. (B) TGF-β1 treatment increased the levels of miR-181a and miR-181b in HSC-T6 cells. Especially, miR-181b was increased by TGF-β 1 in the very high folds ($^{*}P < 0.05$, $^{\#}P < 0.01$).

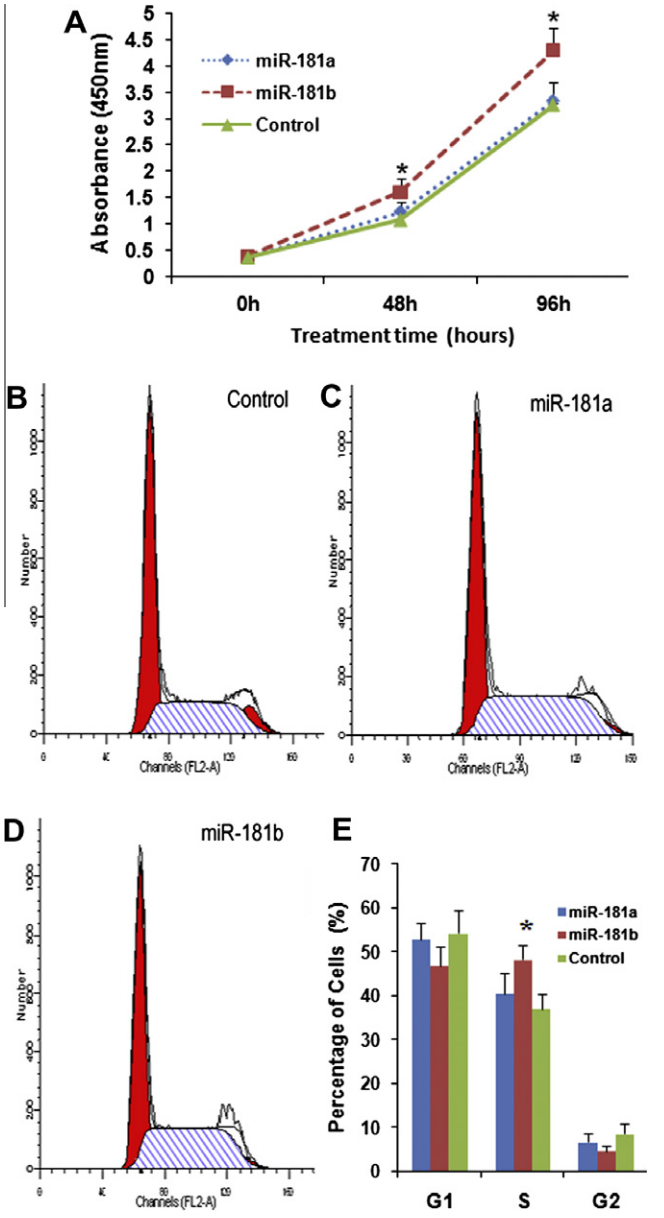


**Fig. 2.** miR-181b promoted growth of HSC-T6 cells and increased the proportion of S phase cells. (A) The proliferation of HSC-T6 cells was increased significantly by the administration of miR-181b but not miR-181a mimics. FACS was used for cell cycle analysis of control (B), miR-181a-treated (C) and miR-181b-treated (D) HSC-T6 cells. Quantification of FACS analysis (E) showed that the proportion of HSC-T6 cells in S phase was increased by introduction of miR-181b ($^{*}P < 0.05$).

### 3.4. P27 was a direct target of miR-181b

We next investigated the mechanism by which miR-181b promoted HSC proliferation. Bioinformatic analysis (microRNA.org) was performed to search for miR-181 targets. CDNK1B (encoding p27), a gene that is critical in the cell cycle regulation [8], was predicted as a putative target of miR-181a and miR-181b. To investigate if miR-181 could regulate p27 directly, mRNA of the p27 3'-UTR encompassed the predicted binding site with or without a mutation was inserted into downstream of a luciferase open reading frame in a pGL4.51 reporter vector. Fig. 3A showed that in the wild-type p27 3′-UTR reporter, a significant reduction of luciferase activity in the miR-181b transfected cells was observed compared with mutant p27 3′-UTR transfected cells ($P < 0.05$). Next, we pursued the ability of miR-181b to regulate the expression of

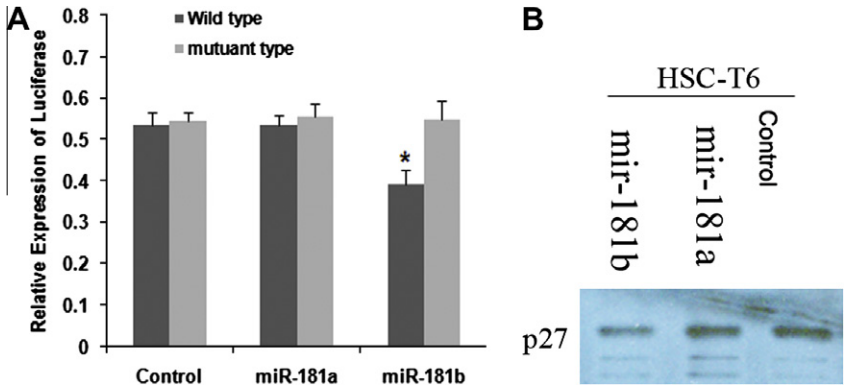


**Fig. 3.** miR-181b targeted p27 directly. (A) Wild-type p27 3′-UTR reporter had a significant reduction in luciferase activity in the miR-181b transfected cells compared with mutant type p27 3′-UTR transfected cells (*$P < 0.05$). (B) Western-blot analysis indicated that miR-181b overexpression in HSCT-T6 cells dramatically repressed p27 expression.

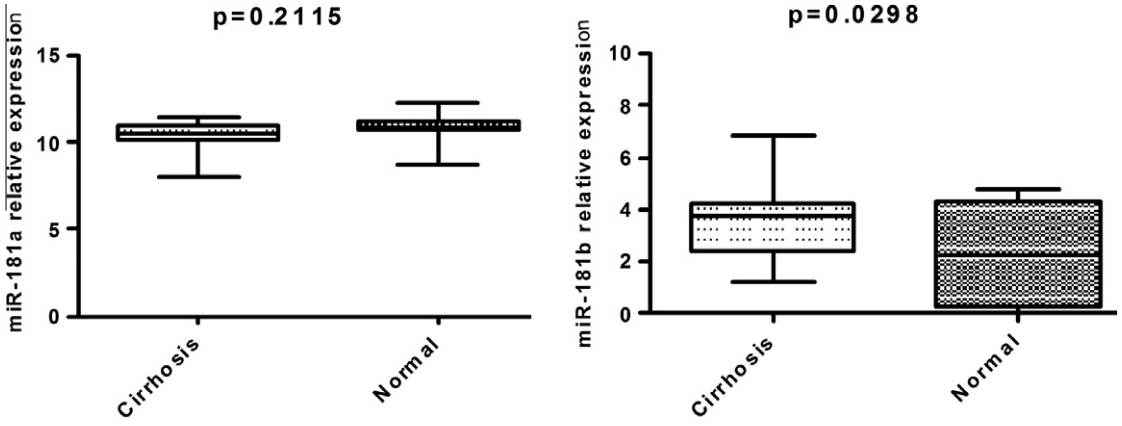


**Fig. 4.** MiR-181b was elevated in the serum of liver cirrhosis patients. The serum levels of miR181b (right panel) were significantly higher in patients with cirrhosis compared with healthy controls ($P < 0.05$). But miR-181a levels in serum had no significant difference between cirrhosis patients and healthy control (left panel).

endogenous p27. Western blot analysis indicated that miR-181b overexpression in HSCT-T6 cells dramatically repressed p27 expression (Fig. 3B). But miR-181a had no effects on luciferase activity and the expression of p27 protein.

### *3.5. miR181b was increased in the serum of patients with liver cirrhosis*

Recent studies have shown that plasma levels of miRNAs are potential biomarkers for various pathological conditions, such as cancer [9–14]. miR-181b was induced by TGF-β1 and regulated the HSC cell proliferation, which indicated its role in liver cirrhosis. Therefore, we studied the expression level of miR-181a and miR-181b in serum of liver cirrhosis patients. We isolated miRNAs from the serum of 22 patients with cirrhosis and compared levels of miR-181 in these patients with 17 healthy controls. Interestingly, the miR-181b serum levels were significantly higher in patients with cirrhosis compared to healthy controls, but miR-181a serum levels had no difference with that of healthy controls (Fig. 4).

## 4. Discussion

Liver fibrosis is characterized by the excessive accumulation of extracellular matrix proteins and develops in most types of chronic liver disease, eventually leading to cirrhosis. Chronic injuries to the liver which can lead to fibrosis include hepatitis, alcohol abuse, and exposure to toxic agents. Activation of HSCs is one of the most pathophysiological aspects of liver fibrosis, which are the main producers of extracellular matrix [15]. TGF-β1 is a key mediator which induce HSCs activation and transformation to fibroblastic cells [16]. So improvement of understanding related to TGF-β1 pathways and HSC activation is important to develop new anti-fibrotic agents.

In this study, we found that TGF-β1 could induce the expression of miR-181a and miR-181b when it stimulated the activation of HSC (α-SMA expression elevation was the character of HSC activation). So we deduced that these two miRNAs may be related to HSC activation. Our results showed that the transient overexpression of miR-181b could promote HSC-T6 cell proliferation and cell cycle progression by increasing the number of S phase cells. Furthermore, as predicted by microRNA.org, p27, a key inhibitor of cell cycle, was the direct target of miR-181a and miR-181b.Our results verified the prediction that p27 expression could inhibited by miR-181b.These results might suggest that TGF-β1 down-regulated P27 depending on miR-181b, sequentially, promoted HSC-T6 proliferation. Excitingly, we observed that miR181b levels were increased in the serum of human patients with cirrhosis, compared to healthy controls. This result provided a clue that miR-181b may be developed as a clinical diagnostic marker. Trials including more patients will be helpful to verify the clinical value of miR-181b.

As for miR-181a, it also could be induced by TGF-β1 in this study, but the change was in much lower folds than miR-181b. We did not

find any relations between miR-181a and cell proliferation, cell cycle, p27 expression in HSC-T6 cell. These results suggested that miR-181a might not be as important as miR-181b in liver fibrosis and cirrhosis. It may be regulated by TGF-β1 in cirrhosis with the unknown mechanism.

Other studies also demonstrated the important role of miRNAs during HSC activation. For example, analysis of changes in miRNA expression in activated rat HSCs identified 12 upregulated miRNAs (miR-874, -29c*, -501, -349, -325-5p, -328, -138, -143, -207, -872, -140, -193) and nine downregulated miRNAs (miR-341, -20b-3p, -15b, -16, -375, -122, -146a, -92b, -126) [17]. Besides, it is reported that the over-expression of miR-16 and miR-15b inhibited HSC proliferation and induced apoptosis through the down-regulation of the mitochondrial associated anti-apoptotic protein Bcl-2 [18,19].

In conclusion, our results indicate that miR-181b could be induced by TGF-β1 and increase the growth of HSCs by directly targeting p27. Moreover, the increased levels of miR-181b in serum of cirrhosis patients suggest that it may be potential diagnostic biomarkers for cirrhosis. Besides, more detail studies could clarify if the expression of miR-181a and miR-181b has the relation to the stages of liver fibrosis.

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