E1 3'UTR contained two target sites for miR-195 (Fig. 5A, B). To investigate the direct interaction between them, the part of the cyclin E1 3'UTR containing the two miR-195 target sites (497 bp) was cloned from LX-2 cells, inserted the downstream of a firefly luciferase reporter gene in a pmirGLO vector (Fig. 5C), and cotransfected into LX-2 cells. As shown in Fig. 5D, luciferase reporter activity decreased significantly in miR-195 precursor-transfected cells compared with cells transfected with a negative control of the precursor. These results suggested a direct interaction between miR-195 and cyclin E1 3'UTR in LX-2 cells. Binding site of miR-195 was not found in p21 3'UTR by TargetScan.

Regulation of cyclin E1 expression by IFN-β and miR-195

To confirm the contribution of miR-195 to the inhibitory effect of IFN-β on cyclin E1 expression, LX-2 cells were first transfected with 50 nM miR-195 inhibitor and then treated with 1,000 IU/ml IFN-β. As shown in Fig. 6A, miR-195 inhibitor blocked the inhibitory effect of IFN-β on cyclin E1 mRNA expression at 16 h and 24 h. Although there was no difference in the cyclin E1 mRNA expression between IFN-β-treated cells and non-treated cells (control) at 48 h, the cyclin E1 mRNA expression level in miR-195 inhibitor plus IFN-β-treated cells was up-regulated compared with non-treated cells (Fig. 6A). Immunoblot analysis revealed that miR-195 inhibitor elevated the cyclin E1 expression level of IFN-β-treated cells at 24 h and 48 h (Fig. 6B).

Discussion

In this study, we showed that IFN-β is more antiproliferative on LX-2 cells than IFN-α, which appears to be contradictory to their known mechanism of action: both IFN-α and

IFN-β exert their activities through the common signaling pathway, beginning with binding to the same type I IFN receptor (IFNAR) consisting of IFNAR1 and IFNAR2, which activate the common components of Janus kinase/Signal Transducer and Activator of Transcription (STAT) pathway (Darnell et al., 1994). However, a similar activity difference between the IFNs has also been demonstrated in colon cancer cell lines (Katayama et al., 2007) and in rat HSCs (Shen et al., 2002). Some studies showed that IFN-β but not IFN-α formed a stable complex with IFNARs, suggesting that IFN-β may interact with IFNAR chains in a manner different from IFN-α (Croze et al., 1996; Russell-Harde et al., 1999).

We showed here that IFN-β down-regulated the expression of cyclin E1 and up-regulated the expression of p21, which caused the cells to be less active proceeding in the transition from G0 to G1 phase and in the progression of S phase. The cell cycle is regulated by various molecules, such as cyclins and CDKs. Cyclin E is essential in activating CDK2. The cyclin E-CDK2 complex phosphorylates pRb at G1 phase, leading to gene transcription activities that are needed in S phase, and also activates the factors involved in DNA replication at early S phase (Golias et al., 2004). It has been reported that cyclin E1 expression increased in non-parenchymal cells of human fibrotic liver and that cyclin E1-deficient mice developed milder liver fibrosis compared with wild-type mice after CCl_4 administration (Nevzorova et al., 2010). These results imply that cyclin E1 regulates the progression of liver fibrosis by accelerating HSC proliferation.

The most frequent miRNAs that targets cyclin E1 are the miR-16 family, which consists of miR-15, -16, -195, -424, and -497 (Liu et al., 2008; Wang et al., 2009). We

here observed the induction of miR-195 by IFN-β. miR-195 was reported to be down-regulated in human HCC tissues and to suppress HCC growth through the targeted interference of cyclin D1, CDK6, and E2F3 in a *xenograft* mouse model (Xu et al., 2009), while it was reported to target cyclin E1 in addition to the above-mentioned factors in A549 cells (Liu et al., 2008). miR-15b and miR-16 are down-regulated concomitantly with HSC activation and their overexpression induces apoptosis and a delay of cell cycle in HSCs by targeting Bcl-2 and cyclin D1 (Guo et al., 2009a; Guo et al., 2009b). However, the role of miR-195 in HSCs remains unknown. We showed here that miR-195 expression was decreased during spontaneous activation of primary-cultured mouse HSCs and that miR-195 interacted with cyclin E1 3'UTR and lowered the expression levels of the cyclin E1 mRNA and protein in LX-2 cells. These results suggest that the down-regulation of miR-195 may associate with the proliferation of HSCs in fibrotic liver similarly to miR-15 and miR-16. In this study, the changes of the protein expression levels of E2F3, CDK6, and cyclin D1, which were reported to be regulated by miR-195 (Xu et al., 2009), were negligible by miR-195, although the exact reason for this phenomenon was not determined. However, because the total context scores obtained by TargetScan were -0.73 for cyclin E1, -0.33 for E2F3, -0.32 for cyclin D1, and -0.09 for CDK6, the result obtained here was thought to be reasonable. In addition, minimal or negligible effect of miR-195 on the expression of E2F3, CDK4, CDK6, and cyclin D1 was compatible with that of IFN-β on these factors. Furthermore, inhibition of miR-195 by miR-195 inhibitor attenuated the effect of IFN-β on cyclin E1 expression, though not so strong. Taken together, it is most likely that the down-regulation of cyclin E1 by IFN-β treatment in HSCs is mediated through miR-195

up-regulation. The mechanism through which IFN-β induces miR-195 in LX-2 cells need to be explored further.

It is well known that IFNs induce the expression of p21 in various cancer cells (Katayama et al., 2007; Sangfelt et al., 1999). We also observed the up-regulation of p21 in IFN-β-treated cells. Therefore, p21, in addition to cyclin E1, may play a role in IFN-induced growth inhibition of HSCs. Until now, it has been reported that IFNs induce p21 expression through the binding of STAT and interferon regulatory factor, which are critical signaling molecules after IFN-IFNAR interaction, to p21 gene promoter (Gartel and Tyner, 1999). Unexpectedly, we found the up-regulation of p21 by miR-195 (Fig. 4). The results obtained here raise a new possibility that the up-regulation of p21 by IFN-β in HSCs may be partially mediated through miR-195.

In conclusion, type I IFN, in particular IFN-β, inhibited the proliferation of human HSCs by delaying the cell cycle in G1 to early S phase through the down-regulation of cyclin E1 and up-regulation of p21. The cyclin E1 down-regulation and p21 up-regulation were partially mediated by miR-195 that was up-regulated by IFN-β. This study raises a new mechanistic aspect of the antifibrotic effect of IFN in liver fibrosis and the possibility of influencing miR-195 as a therapeutic strategy for liver fibrosis.

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Figure legends

Fig. 1. Expression of miR-195 in mouse HSCs during primary culture and growth inhibitory effect of IFN-α and -β on human stellate cells. (A and B) Isolated mouse HSCs were cultured for the indicated periods. The expression levels of miR-195 (A), and α-SMA and cyclin E1 mRNA (B) were measured by real-time PCR. $*P < 0.05$, $**P < 0.01$ compared with 1 day. (C and D) LX-2 cells were incubated with IFN-α or -β (1,000 IU/ml) for 3-7 days (C), or with IFN-α or -β at the concentration of 10-1,000 IU/ml for 7 days (D). Control indicates non-treated cells. The proportion of viable cells was determined using a WST-1 assay. $**P < 0.01$ compared with control.

Fig. 2. Effect of IFN-α and -β on cell cycle distribution in human stellate cells. LX-2 cells synchronized in G0/G1 phase were then incubated with IFN-α or -β (1,000 IU/ml) in DMEM/FBS for the indicated periods. Control indicates non-treated cells. The cell cycle was analyzed by flow cytometry. The white, black, and shaded region indicates the histogram measured by flow cytometry, G0/G1 phase (left) or G2/M phase (right), and S phase, respectively, as analyzed by ModFIT LT software.

Fig. 3. Expression of cell cycle-related genes in stellate cells. LX-2 cells were incubated with IFN-β (1,000 IU/ml) for up to 72 h for determining the expression levels of mRNAs of cyclin D1, cyclin E1, CDK2, CDK4, CDK6, p21, and p27. Control indicates non-treated cells. $*P < 0.05$, $**P < 0.01$ compared with control.

Fig. 4. Regulation of expression of cell cycle regulators by miR-195. (A) LX-2 cells

were incubated with IFN-β (1,000 IU/ml) for up to 72 h for determining the expression levels of miR-195. Control indicates non-treated cells. $*P < 0.05$, $**P < 0.01$ compared with control. (B – D) LX-2 cells were transfected with 50 nM miR-195 precursor or a negative control (control). (B) mRNA expression levels of E2F3, CDK4, CDK6, cyclin D1, cyclin E1, and p21 measured at 24, 48, 72, and 96 h posttransfection. (C) Protein expression of E2F3, CDK4, CDK6, cyclin D1, cyclin E1, and p21 examined at 48, 72, and 96 h posttransfection. (D) Growth of LX-2 cells transfected with miR-195 or untreated control was measured using a WST-1 assay. $*P < 0.05$, $**P < 0.01$ compared with control.

Fig. 5. Interaction of miR-195 with the 3'UTR of cyclin E1 mRNA. (A) Schematic indication of the putative miR-195 target sites in the 3'UTR of the cyclin E1 mRNA. Tested sequences indicate the regions that were inserted into the luciferase reporter vector. (B) Predicted pairing of the target region and miRNAs. (C) Structure of the luciferase reporter vector [14]. The putative miR-195 target region in cyclin E1 3'UTR (tested sequence) was ligated into the MCS. Arrows indicate the gene directions. Amp^R indicates an ampicillin resistance gene. (D) Reporter gene assay of the interaction between the 3'UTR of cyclin E1 mRNA and miR-195 in LX-2 cells. Results are expressed as the relative activities against the activity in the presence of the control. $*P < 0.05$, $**P < 0.01$ compared with control.

Fig. 6. Regulation of cyclin E1 expression by IFN-β and miR-195. LX-2 cells were transfected with 50 nM miR-195 inhibitor or a negative control. After 6 h, the culture

medium was changed and then IFN-β (1,000 IU/ml) was added. Cells were then incubated for the indicated time periods. (A) mRNA expression levels of cyclin E1. (B) Protein expression levels of cyclin E1. GAPDH are for loading adjustment. Control; cells were transfected with a negative control and incubated without IFN-β. $*P < 0.05$, $**P < 0.01$.

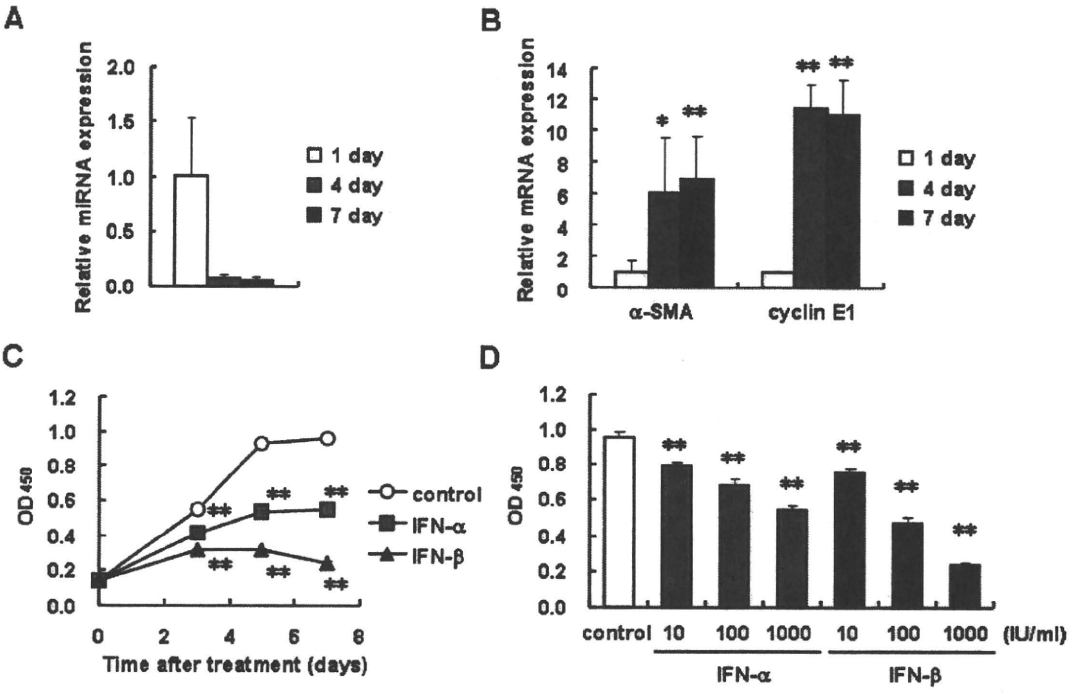


Fig. 1

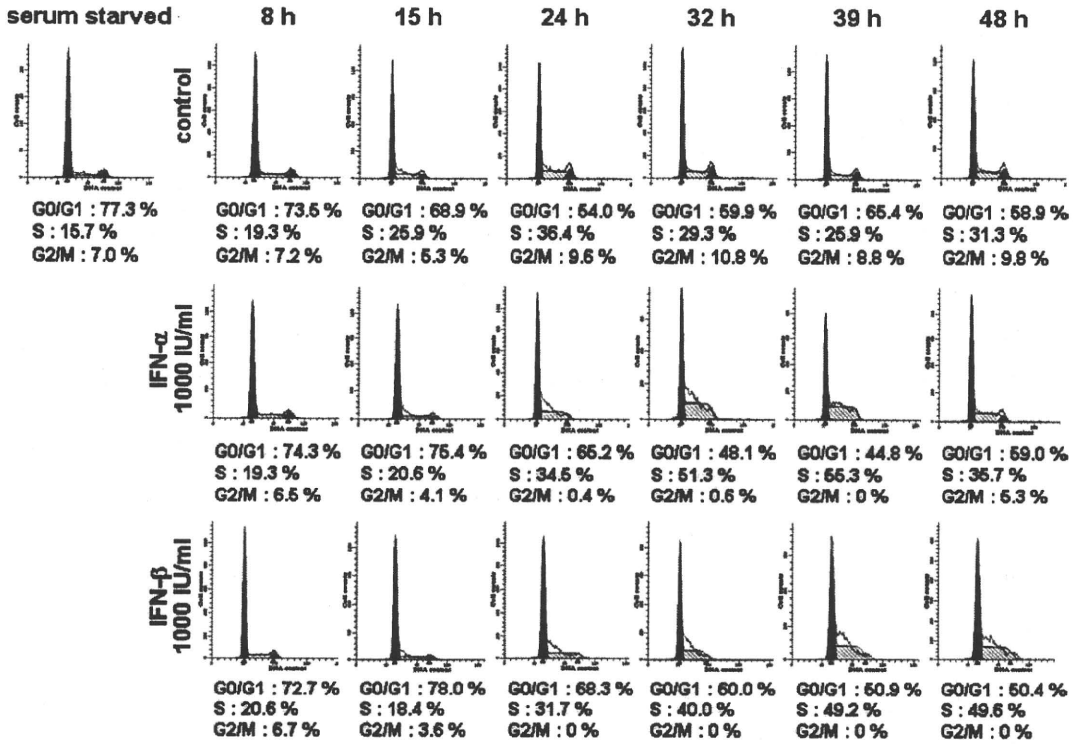
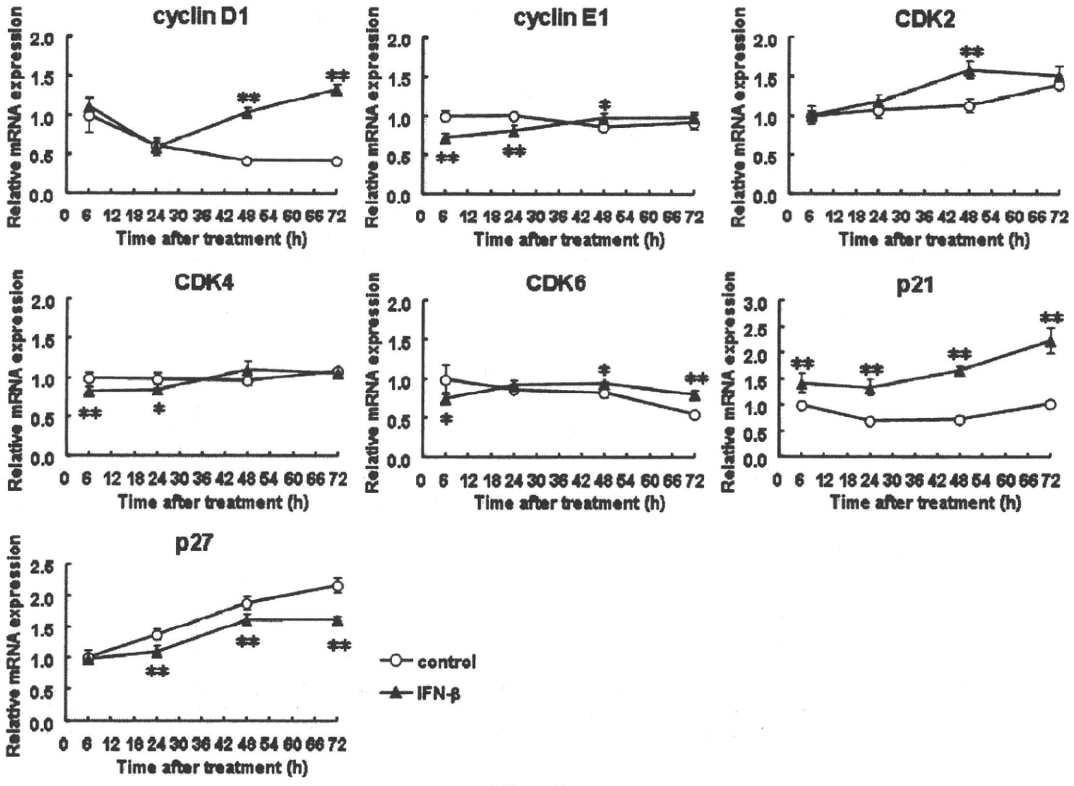


Fig. 2



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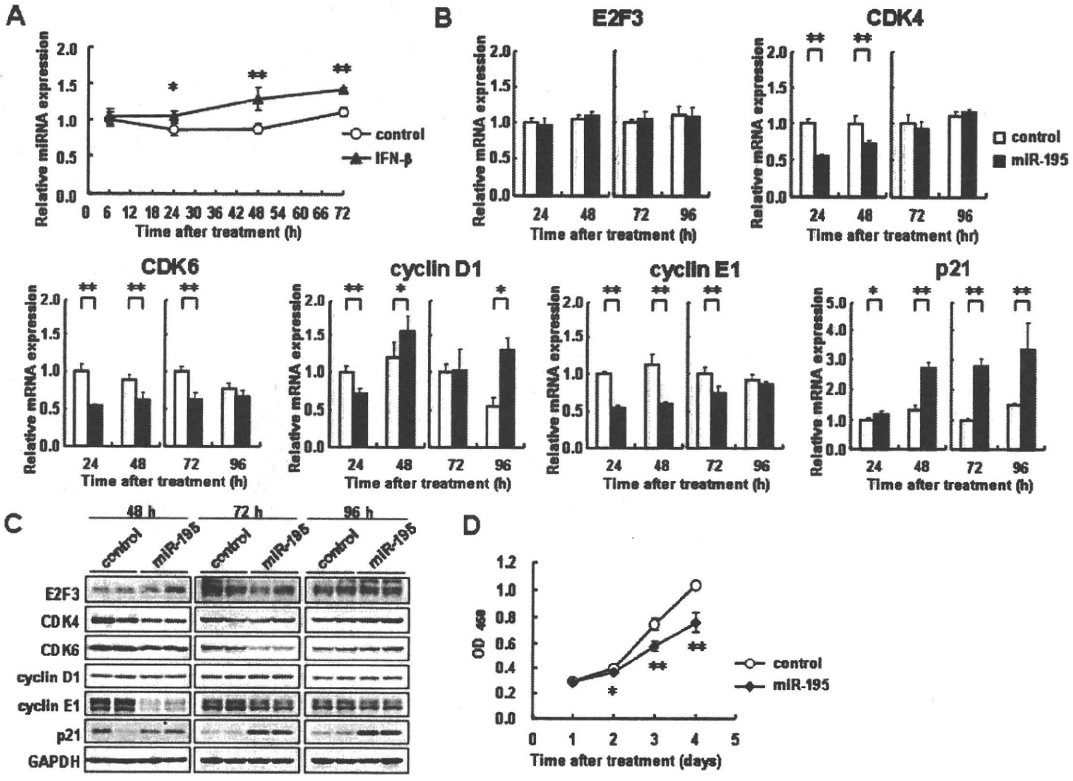

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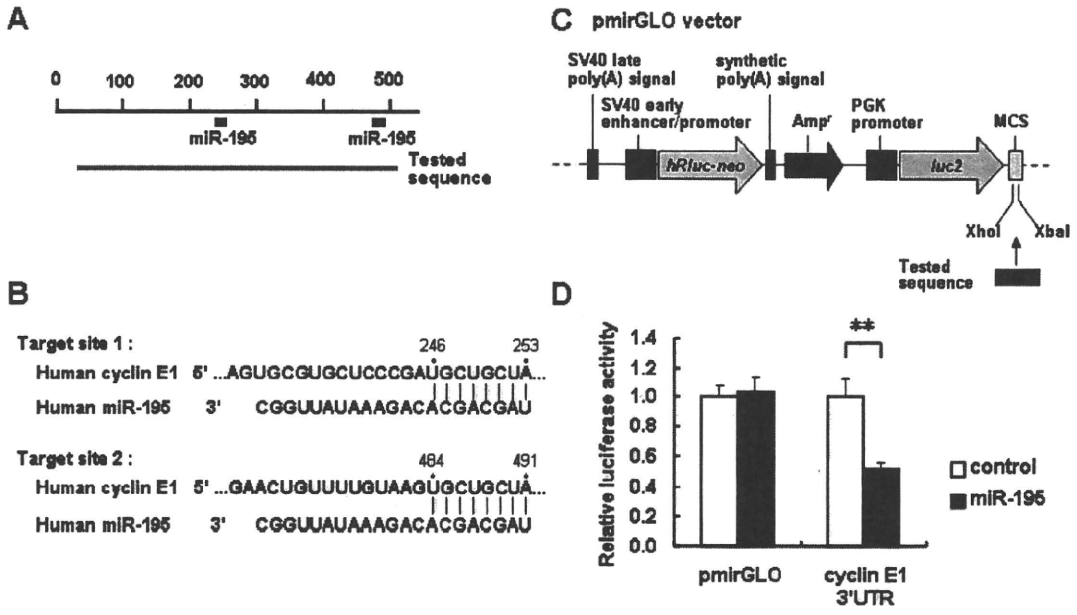

Fig. 5

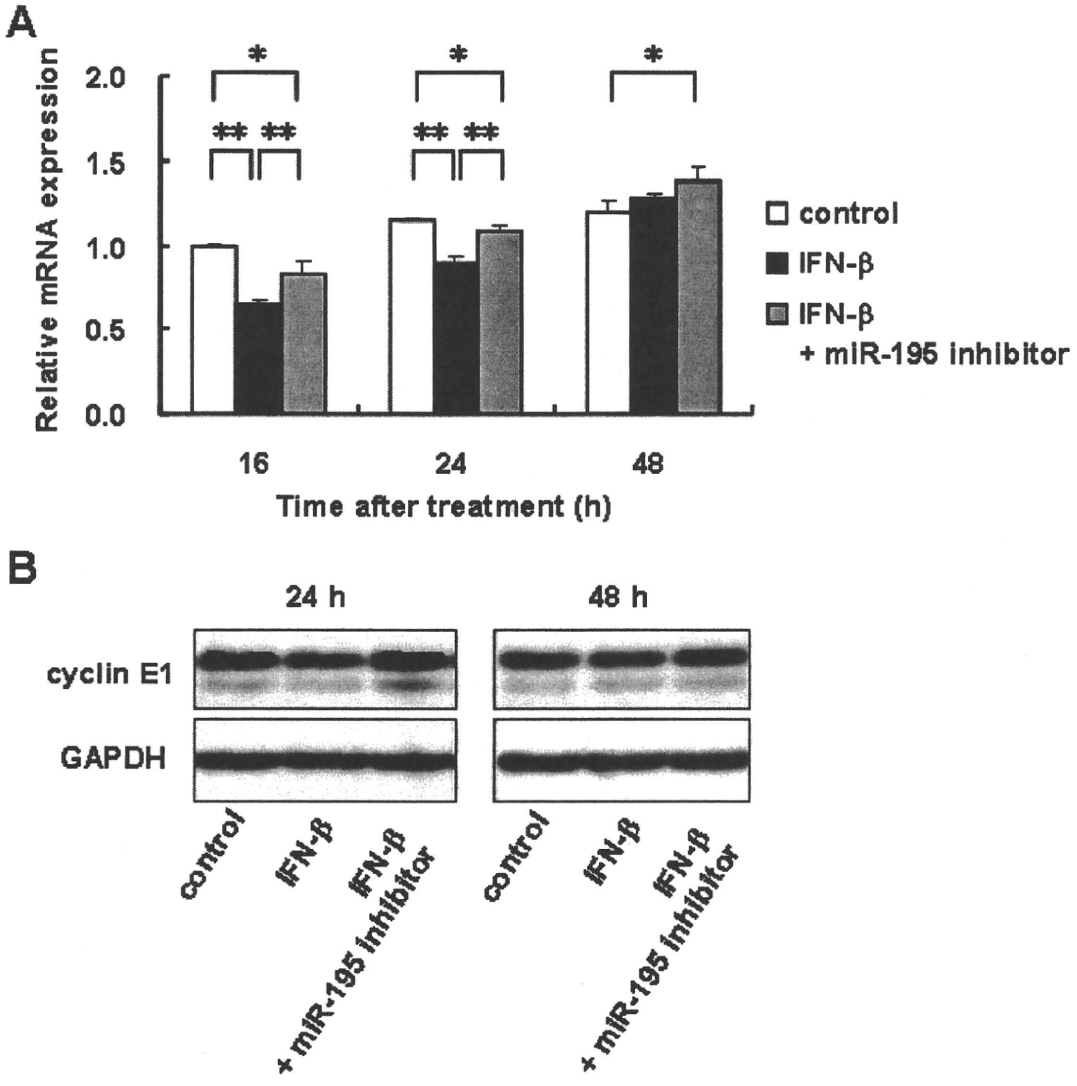

Fig. 6

Table 1. Sequences of primers used in real-time PCR analyses and 3'UTR cloning for luciferase reporter assay

Gene	Accession No.	Sequence
real-time PCR		
CDK6	NM_001259	Forward: 5'-ATATCTGCCTACAGTGCCCTGTCTC-3'
		Reverse: 5'-GTGGGAATCCAGGTTTTCTTTGCAC-3'
Cyclin E1	NM_001238	Forward: 5'-GCAGTATCCCCAGCAAATC-3'
		Reverse: 5'-TCAAGGCAGTCAACATCCA-3'
Cyclin D1	NM_053056	Forward: 5'-GCTGTGCATCTACACCGACAACTC-3'
		Reverse: 5'-AGGTTCCACTTGAGCTTGTTCACC-3'
E2F3	NM_001949	Forward: 5'-CCAACTCAGGACATAGCGATTGCTC-3'
		Reverse: 5'-AGGAATTTGGTCCTCAGTCTGCTGT-3'
GAPDH	NM_002046	Forward: 5'-GCACCGTCAAGGCTGAGAAC-3'
		Reverse: 5'-TGGTGAAGACGCCAGTGGA-3'
p21	NM_000389	Forward: 5'-AGCAGAGGAAGACCATGTGGA-3'
		Reverse: 5'-GGAGTGGTAGAAATCTGTCATGCT-3'
3'UTR cloning		
Cyclin E1	NM_001238	Forward: 5'-TTCTCGAGATCCTTCTCCACCAAAGACAGTT-3'
		Reverse: 5'-TTTCTAGAGAATGGATAGATATAGCAGCACTTACA-3'

The forward and reverse primers for 3'UTR cloning carried the XhoI and XbaI sites at their 5'-ends, respectively.

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Suppression of type I collagen production by microRNA-29b in cultured human stellate cells

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ABSTRACT

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression through imperfect base pairing with the 3′ untranslated region (3′UTR) of target mRNA. We studied the regulation of alpha 1 (I) collagen (Col1A1) expression by miRNAs in human stellate cells, which are involved in liver fibrogenesis. Among miR-29b, -143, and -218, whose expressions were altered in response to transforming growth factor-β1 or interferon-α stimulation, miR-29b was the most effective suppressor of type I collagen at the mRNA and protein level via its direct binding to Col1A1 3′UTR. miR-29b also had an effect on SP1 expression. These results suggested that miR-29b is involved in the regulation of type I collagen expression by interferon-α in hepatic stellate cells. It is anticipated that miR-29b will be used for the regulation of stellate cell activation and lead to antifibrotic therapy.

Introduction

Hepatic stellate cells, which reside in the Disse's space outside the liver sinusoids, maintain a quiescent phenotype and store vitamin A under physiological conditions [1,2]. When liver injury occurs due to alcohol abuse, hepatitis viral infection, or obesity, stellate cells activate in response to inflammatory stimuli and become myofibroblastic cells that express smooth muscle α-actin as a representative marker [2]. Myofibroblastic cells secrete profibrogenic mediators, such as transforming growth factor-β (TGF-β), connective tissue growth factor, and tissue inhibitor of matrix metalloproteinases, and generate extracellular matrix materials including collagens, fibronectin, and laminin; thus, they play a pivotal role in liver fibrogenesis [3]. In particular, collagen production by activated stellate cells is regulated by TGF-β in an autocrine loop, which is accompanied by the induction of TGF-β receptors [4]. Suppression of hepatic stellate cell activation and collagen expression is thus a critical issue to establish therapeutic strategies for human liver fibrosis [1,5].

MicroRNAs (miRNAs) are endogenous small noncoding RNAs that modulate gene expression through imperfect base pairing with the 3′ untranslated region (UTR) of target mRNA, resulting in the inhibition of translation or the promotion of mRNA degradation [6,7]. miRNAs play roles in cell proliferation [8], development [9], and differentiation [10], and their contribution to human diseases such as cancer, cardiomyopathies, and schizophrenia have been reported [11–13]. miR-122 is also involved in the defense system against viral hepatitis C with regard to interferon (IFN)-β therapy [14], and miR-26 expression status is associated with survival and response to adjuvant IFNα therapy in patients with hepatocellular carcinoma [15]. Some miRNAs are involved in liver development and hepatocyte lipid metabolism [16–18].

Recent studies have shown that miRNAs are additionally involved in the alteration of hepatic stellate cell phenotypes; down-regulation of miR-27a and -27b allows culture-activated rat stellate cells to return to a quiescent phenotype with abundant vitamin A storage and decreased cell proliferation [19]; miR-15b and -16, which target the Bcl-2 and caspase signaling pathways, may affect stellate cell activation and liver fibrosis [20]. However, the function of miRNAs in hepatic stellate cell activation and their collagen production is largely unknown.

Here, we show that miR-29b, which is induced in human stellate cells (LX-2) treated with IFNα, is a potential regulator of type I collagen mRNA and protein expression. Although the primary action of IFNs is to eradicate viruses, i.e., hepatitis B and C viruses in

Abbreviations: Col1A1, alpha 1 (I) collagen; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IFN, interferon; miRNAs, microRNAs; TGF-β, transforming growth factor-β; UTR, untranslated region.

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