Abstract

Recent studies have suggested that interferons (IFNs) have an antifibrotic effect in the liver independent of their antiviral effect although its detailed mechanism remains largely unknown. Some microRNAs have been reported to regulate pathophysiological activities of hepatic stellate cells (HSCs). We performed analyses of the antiproliferative effects of IFNs in HSCs with special regard to microRNA-195 (miR-195). We found that miR-195 was prominently down-regulated in the proliferative phase of primary-cultured mouse HSCs. Supporting this fact, IFN-β induced miR-195 expression and inhibited the cell proliferation by delaying their G1 to S phase cell cycle progression in human HSC line LX-2. IFN-β down-regulated cyclin E1 and up-regulated p21 mRNA levels in LX-2 cells. Luciferase reporter assay revealed the direct interaction of miR-195 with the cyclin E1 3'UTR. Overexpression of miR-195 lowered cyclin E1 mRNA and protein expression levels, increased p21 mRNA and protein expression levels, and inhibited cell proliferation in LX-2 cells. Moreover miR-195 inhibition restored cyclin E1 levels that were down-regulated by IFN-β. In conclusion, IFN-β inhibited the proliferation of LX-2 cells by delaying cell cycle progression in G1 to S phase, partially through the down-regulation of cyclin E1 and up-regulation of p21. IFN-induced miR-195 was involved in these processes. These observations reveal a new mechanistic aspect of the antifibrotic effect of IFNs in the liver.

Introduction

Hepatic fibrosis is characterized by excessive accumulation of extracellular matrices (ECM) and is a common feature of chronic liver diseases. Hepatic stellate cells (HSCs) are considered to play multiple roles in the fibrotic process. HSCs maintain a quiescent phenotype and store vitamin A under physiological conditions. When liver injury occurs, they become activated and trans-differentiate into myofibroblastic cells, whose characteristics include the proliferation, loss of vitamin A droplets, expression of α-smooth muscle actin (α-SMA), secretion of profibrogenic mediators and ECM (Bataller and Brenner, 2001; Friedman, 2000). Therefore, controlling the population and activation of HSCs should be a potential therapeutic target against liver fibrosis.

Interferons (IFNs) are cytokines with antiviral, immunomodulatory, and cell growth inhibitory effects. IFN-α and -β are classified as type I IFNs (Pestka et al., 1987; Uze et al., 2007), which are generally applied for the therapy of eradication of hepatitis B and C viruses. Studies using rodent models and cultured HSCs have also suggested that IFNs have a direct antifibrotic potential independently of their antivirus activity (Chang et al., 2005; Fort et al., 1998; Inagaki et al., 2003; Mallat et al., 1995; Ogawa et al., 2009; Shen et al., 2002; Tanabe et al., 2007), although the detailed molecular mechanisms of these effects of IFNs remain to be clarified.

Recently, microRNAs (miRNAs), which are endogenous small non-coding RNA, have become a focus of interest as post-transcriptional regulators of gene expression through interaction with the 3' untranslated region (3'UTR) of target mRNAs (Bartel, 2004). miRNAs are known to participate in cell proliferation, development, differentiation, and metabolism (Bartel, 2004). Moreover, it has been reported that expression of miRNAs

could alter hepatic pathophysiology; miR-122 is involved in the IFN-β-related defense system against viral hepatitis C (Pedersen et al., 2007), and miR-26 is associated with survival and response to adjuvant IFN-α therapy in patients with hepatocellular carcinoma (HCC) (Ji et al., 2009a). Regarding HSCs, miR-15b and miR-16 are down-regulated upon HSC's activation, and their overexpression induces apoptosis and a delay in the cell cycle (Guo et al., 2009a; Guo et al., 2009b). Knockdown of miR-27a and miR-27b in activated HSCs allowed a switch to a more quiescent phenotype and decreased cell proliferation (Ji et al., 2009b). miR-150 and miR-194 suppress proliferation, activation, and ECM production of HSCs (Venugopal et al., 2010). Recently, we showed that miR-29b was induced by IFN and suppressed type I collagen production in LX-2 cells (Ogawa et al., 2010).

In the present study, we measured the levels of miR-195 in primary-cultured mouse HSCs and found that its expression was markedly reduced in their activation phase, suggesting the regulatory role of miR-195 in the activation/deactivation process of HSCs. Because miR-195 is categorized into the same family as miR-15b and miR-16 and has been reported to regulate cell cycle by targeting E2F3, CDK6, and cyclin D1 (Xu et al., 2009), we suspect the involvement of miR-195 in the proliferation of HSC and in type I IFN, in particular IFN-β, -induced inhibition of their growth.

Materials and Methods

Materials. Human hepatic stellate cell line LX-2 was donated by Dr. Scott L. Friedman (Mount Sinai School of Medicine, New York, NY, USA) (Xu et al., 2005). Necessary reagents and materials were obtained from the following sources: Dulbecco's

modified Eagle's medium (DMEM) from Sigma Chemical Co. (St. Louis, MO, USA); fetal bovine serum (FBS) from Invitrogen (Carlsbad, CA, USA); human natural IFN-α and IFN-β from Otsuka Pharmaceutical Co. (Tokushima, Japan) and Toray Industries Inc. (Tokyo, Japan), respectively; precursor and inhibitor of miR-195, and the corresponding negative controls from Ambion (Austin, TX, USA); mouse monoclonal antibody against cyclin E1, cyclin D1 and p21, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from MBL (Nagoya, Japan), Cell Signaling Technology Inc. (Beverly, MA, USA), and Chemicon International Inc. (Temecula, CA, USA), respectively; rabbit polyclonal antibodies against cyclin-dependent kinase (CDK) 6 and E2F3 from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); goat polyclonal antibody against CDK4 from Santa Cruz Biotechnology Inc.; enhanced Chemiluminescence plus detection reagent from GE Healthcare (Buckinghamshire, UK); Immobilon P membranes from Millipore Corp. (Bedford, MA, USA); reagents for cDNA synthesis and real-time PCR from Toyobo (Osaka, Japan); a cell counting kit from Dojindo Laboratories (Kumamoto, Japan); and all other reagents from Sigma Chemical Co. or Wako Pure Chemical Co. (Osaka, Japan).

Cells. LX-2 cells were maintained in DMEM supplemented with 10% FBS (DMEM/FBS) and were plated at a density of 0.7–1.5 × 10^4 cells/cm^2 24 h prior to biological assay. Biological assays were done in DMEM/FBS unless stated otherwise. Mouse primary HSCs were isolated from male C57BL/6 mice by the pronase-collagenase digestion method as described previously (Uyama et al., 2006) and were cultured in DMEM/FBS.

Transient transfection of miRNA precursors and inhibitors. Precursor of miR-195,

which was a double-strand RNA mimicking endogenous miR-195 precursor, and the negative control with a scrambled sequence were transfected into LX-2 cells using Lipofectamine 2000 (Invitrogen) at a final concentration of 50 nM in accordance with the manufacturer's instructions. Briefly, miRNA precursor and Lipofectamine 2000 were mixed at a ratio of 25 (pmol):1 (μl) in Opti-MEM I Reduced Medium (Invitrogen), incubated for 20 min at room temperature, and were then added to the cultures. After 24 h, the culture medium was replaced with fresh medium. Inhibitor of miR-195, which was designed to bind to endogenous miR-195 and inhibit its activity, and the negative control with a scrambled sequence were transfected similarly. After 6 h, the culture medium was changed and IFN-β was added successively.

Cell proliferation assay. LX-2 cells were plated at a density of 2×10^3 cells/well in 96-well plates 24 h prior to experiments. The culture medium was replaced by fresh medium containing different concentrations of IFNs at day 0 and 3. After 3, 5, and 7 days of treatment, cell proliferation was measured by WST-1 assay. In another experiment, the cells were plated at a density of 3×10^3 cells/well in 96-well plate for 24 h prior and were then transfected with the miR-195 precursor as described above. After 24 h, the medium was changed and the culture was continued for an additional 1–3 days before the measurement of cell proliferation.

Cell cycle analysis. Cells were serum starved for 24 h and then the medium was replaced with IFN-containing DMEM/FBS. At the indicated time points after treatment, the cells were harvested by trypsinization, washed in phosphate buffered saline (PBS), and fixed in ice-cold 70% ethanol. The cells were washed in PBS and resuspended in PBS containing 500 μg/ml RNase A and incubated for 20 min. Cellular DNA was

stained with propidium iodide at a final concentration of 25 μg/ml for 20 min. The cells were analyzed using a FACSCalibur HG flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). A total of 20,000 events were counted for each sample. Data were analyzed using ModFIT LT software (Verity Software House, Topsham, ME, USA).

Quantitative real-time PCR. Quantitative real-time PCR was performed according to the method described elsewhere with use of a set of gene-specific oligonucleotide primers (Table 1) using an Applied Biosystems Prism 7500 (Applied Biosystems, Foster City, CA, USA) (Ogawa et al., 2010). To detect miR-195 expression, the reverse transcription reaction was performed using a TaqMan microRNA Assay (Applied Biosystems) in accordance with the manufacturer's instructions. The expression level of GAPDH was used to normalize the relative abundance of mRNAs and miR-195.

Immunoblotting. Cells were lysed in RIPA buffer [50 mM Tris/HCl, pH7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] containing Protease Inhibitor Cocktail, Phosphatase Inhibitors Cocktail 1, and Phosphatase Inhibitor Cocktail 2 (Sigma). Proteins (20 μg) were electrophoresed in a 10% SDS-polyacrylamide gel and then transferred onto Immobilon P membranes (Ogawa et al., 2010). Immunoreactive bands were visualized by the enhanced chemiluminescence system using a Fujifilm Image Reader LAS-3000 (Fuji Medical Systems, Stamford, CT, USA).

Luciferase reporter assay. Interaction of miR-195 to the 3'UTR of the cyclin E1 gene was tested according to the reported method (Ogawa et al., 2010). The 3'UTR of the cyclin E1 gene containing putative miR-195 target regions was obtained by PCR using cDNA derived from LX-2 and a primer set listed in Table 1. The obtained DNA

fragments (497 bp) were inserted into a pmirGLO Vector (Promega, San Luis Obispo, CA, USA). LX-2 cells, plated in 96-well plates at a density of 2×10^4 cells/well 24 h prior to experiment, were transfected with 200 ng of reporter plasmid and miRNA precursor using Lipofectamine 2000. After 24 h, the medium was changed to 20 μl of PBS. The Dual-Glo Luciferase Assay System (Promega) was used to analyze luciferase expression in accordance with the manufacturer's protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity to adjust for variations in transfection efficiency among experiments.

Statistical analysis. Data presented as graphs are the means ± S.D. of at least three independent experiments. Statistical analysis was performed using Student's t-test. $P < 0.05$ was considered significant.

Results

Reduction of miR-195 expression during activation of primary-cultured HSCs

It has been known that, when maintained in a plastic culture plate, freshly isolated primary-cultured HSCs undergo spontaneous activation and transformation into myofibroblastic cells that express α-SMA and produce fibrogenic mediators, such as type I collagen and transforming growth factor-β. In our preliminary experiments using primary-cultured mouse HSCs, we noticed that the cells drastically decreased the expression of miR-195 when they underwent spontaneous activation (unpublished observation). The present study confirmed this notion as shown in Fig. 1A. miR-195 expression level certainly decreased in activation process of primary-cultured mouse HSCs. In contrast, the expression levels of α-SMA and cyclin E1 mRNA increased (Fig.

1B). Accordingly, we considered that miR-195 plays a role as an antiproliferative and antiactivating miRNA in HSCs. As a matter of fact, there was a study showing that miR-16 family including miR-195 inhibits proliferation of lung cancer cells by silencing cyclins D1 and E1, and CDK6 (Liu et al., 2008). The result indicated by Fig. 1 and the cited study together drove us to explore the IFN's antiproliferative action on HSCs (Mallat et al., 1995; Shen et al., 2002), focusing on miR-195 and cell cycle-related genes.

Effects of IFN-α and -β on proliferation of HSCs

First, we investigated the effects of type I IFNs on the proliferation of LX-2 cells using a WST-1 assay. LX-2 cells in control culture continued to grow during the experimental period of 7 days (Fig. 1C). IFN-α and -β both, but the latter more actively, decreased cell proliferation time-dependently at a concentration of 1,000 IU/ml, supporting the previous studies (Mallat et al., 1995; Shen et al., 2002). Dose-dependency of the growth inhibition is shown in IFN concentrations from 10 to 1,000 IU/ml (Fig. 1D).

Effects of IFN-α and -β on cell cycle distribution

To elucidate the mechanism of the growth inhibitory effect of IFN, we next examined the change in cell cycle distribution in response to IFN-α and -β treatment by flow cytometry. LX-2 cells were synchronized in G0/G1 phase by serum starvation for 24 h. In non-treated cells (control), population in G0/G1 phase was reduced after serum exposure, which was accompanied by the increase of population in S phase. This cell cycle transition peaked at 24 h (Fig. 2, upper panel). In cells treated with IFN-α or -β, the G0/G1 phase population was larger and the S phase population was smaller than in the control cells at 15 h and 24 h. In addition, the accumulation of cells in early S phase

was observed at 32–48 h (Fig. 2, middle and lower panels). These delays in cell cycle shift were more potent in IFN-β-treated cells than in IFN-α-treated cells. It was concluded that type I IFN hampered HSC proliferation through a delay in the cell cycle at the transition from G1 to S phase and in the progression of S phase.

Regulation of cyclin E1 and p21 expression by IFN-β

IFN-β was chosen in the following experiments because of its more potent inhibition of cell cycle progression than IFN-α, as described above. The transition from G1 to S phase and the progression of S phase are known to be influenced by various regulators (Golias et al., 2004). Among them, we found that IFN-β significantly decreased cyclin E1 mRNA expression levels by 0.6 – 0.7-fold at 6 h and 24 h and increased p21 mRNA expression levels by 1.4 – 2.3-fold at 6h, 24h, 48 h, and 72 h in LX-2 cells (Fig. 3). The expression levels of CDK4 and CDK6 was also reduced by IFN-β at early phase with less extent. The others showed negligible change within 24 h although variable dynamics were seen thereafter; changes of cyclin D1, CDK2, and p27 expression at late phase were toward cell cycle promotion with currently unknown reason.

Regulation of miR-195 expression by IFN-β

The result indicated from Fig. 1 strongly suggested the possibility that IFN-β increase the expression of miR-195 in LX-2 cells. To test this possibility, we examined the expression levels of miR-195 in IFN-β-treated LX-2 cells. As a result, the miR-195 expression level was significantly increased by IFN-β treatment at 24 h, 48 h and 72 h (Fig. 4A).

Regulation of cyclin E1 and p21 expression by miR-195

The results obtained from experiments shown in Fig. 3 and Fig. 4A led us to

hypothesize that IFN-β up-regulates the expression of miR-195, which then down-regulates the expression of cyclin E1 and up-regulates the expression of p21. In addition, there had been a study reporting that miR-195 targets E2F3, CDK6, and cyclin D1 in addition to cyclin E1 (Xu et al., 2009). Under these considerations, we examined the changes in the expression levels of the above-mentioned cell cycle-related molecules and CDK4 by introducing miR-195 precursor into LX-2 cells. Transfection of miR-195 precursor increased the miR-195 expression levels in LX-2 cells by up to 10,000–30,000 times compared with those in cells transfected with negative control (data not shown). Cyclin E1 mRNA and protein expression levels showed a remarkable reduction up to 72 h as result of miR-195 overexpression (Fig. 4B, C). On the other hand, p21 mRNA and protein expression levels showed a marked increase. CDK4, CDK6, and cyclin D1 expression levels were significantly changed at the mRNA level, but negligibly at the protein level. E2F3 mRNA and protein expression levels were unchanged (Fig. 4B, C). These results suggested that miR-195 mainly regulated cyclin E1 and p21 expression in LX-2 cells. Moreover, transfection of miR-195 precursor (50 nM) decreased the proliferation of LX-2 cells in the WST-1 assay (Fig. 4D). These results showed that miR-195 down-regulates endogenous cyclin E1 expression and up-regulates p21 expression, resulting in the attenuation of cell cycle progression and cell proliferation.

Interaction of miR-195 with cyclin E1 3'UTR in LX-2 cells

Next, we examined whether miR-195 interacted directly with cyclin E1 3'UTR in LX-2 cells. The predicted miRNA target sites for miR-195 in the cyclin E1 3'UTR were analyzed using TargetScan Human Release 5.1 (http://www.targetscan.org/). The cyclin

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