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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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the case of the liver, IFNs also exhibit an antifibrotic action in human chronic hepatitis [21,22] and rodent liver fibrosis models [23]. Our data suggest that miR-29b may be a novel regulator of type I collagen expression in addition to its involvement in the well-known Smad cascade. Moreover, miR-29b upregulation may play a partial role in the antifibrotic action of IFNs.

Materials and methods

Materials. Recombinant human TGF-β1 was purchased from PeproTech (London, UK). Human natural IFNα was obtained from Otsuka Pharmaceutical Co. (Tokushima, Japan). Precursors of miR-29b, -143, and -218, and the negative control were purchased from Ambion (Austin, TX, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit monoclonal antibodies against Smad2 and phospho-Smad2 were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). The mouse monoclonal antibody against SP1 was purchased from Bio Matrix Research Inc. (Chiba, Japan). Rabbit polyclonal antibody against type I collagen was purchased from Rockland Immunochemicals, Inc. (Gilbertsville, PA, USA). Mouse monoclonal antibody against GAPDH was purchased from Chemicon International Inc. (Temecula, CA, USA). Enhanced Chemiluminescence plus detection reagent was purchased from GE Healthcare (Buckinghamshire, UK). Immobilon P membranes were purchased from Millipore Corp. (Bedford, MA, USA). All other reagents were purchased from Sigma Chemical Co. or Wako Pure Chemical Co. (Osaka, Japan).

Preparation of the human hepatic stellate cell line LX-2. The human hepatic stellate cell line (LX-2, donated by Dr. Scott Friedman), which was spontaneously immortalized by growth in low serum, was established as reported previously [24]. Characterizations of the cells are described in detail elsewhere. The cells were maintained on plastic culture plates in DMEM supplemented with 10% FBS. After the culture had continued for the indicated number of days, the medium was replaced with DMEM supplemented with 0.1% FBS plus test agents, and the culture was continued for another 24 h.

Quantitative real-time PCR. Total RNA was extracted from human stellate cells using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNAs were synthesized using 0.5 μg of total RNA, ReverTra Ace (Toyobo, Osaka, Japan), and oligo$(dT)_{12-18}$ primers according to the manufacturer's instructions [25]. Gene expression was measured by real-time PCR using cDNA, real-time PCR Master Mix Reagents (Toyobo), and a set of gene-specific oligonucleotide primers (alpha 1 (I) collagen [Col1A1]: Forward 5′-CCCGGGTTTCAGAGACA ACTTC-3′, Reverse 5′-TCCACATGCTTTATTCCAGCAATC-3′; TGF-β1: Forward 5′-AGCGACTCGCCAGAGTGGTTA-3′, Reverse 5′-GCAGTG TGTTATCCCTGCTGTCA-3′; SP1: Forward 5′-TCGGATGAGCTACA GAGGCACAA-3′, Reverse 5′-GTCACTCCTCATGAAGCGCTTAGG-3′; and GAPDH: Forward 5′-GCACCGTCAAGGCTGAGAAC-3′, Reverse 5′-TGGTGAAGACGCCAGTGGA-3′) with an Applied Biosystems Prism 7500 (Applied Biosystems, Foster City, CA, USA). To detect miRNA expression, the RT reaction was performed using the TaqMan MicroRNA Assay (Applied Biosystems) according to the manufacturer's instructions. The GAPDH level was measured and used to normalize the relative abundance of mRNAs and miRNAs.

Immunoblot. Proteins (20–50 μg) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto Immobilon P membranes. After blocking, the membranes were treated with primary antibodies, followed by peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized by the enhanced chemiluminescence system using the Fujifilm Image Reader LAS-3000 (Fuji Medical Systems, Stamford, CT, USA).

Transient transfection of miRNA precursors. Precursors of miR-29b, -143, and -218, and the negative control were transfected into human stellate cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at a final concentration of 50 nM. Briefly, the cells were plated in DMEM supplemented with 10% FBS at a density of $1–2 \times 10^5$ cells/ml 24 h prior to the transfection. miRNA precursors and Lipofectamine 2000 were mixed at a ratio of 25 (pmol):1 (μl) in Opti-MEM I Reduced Medium (Invitrogen) and incubated for 20–30 min at room temperature. The miRNA precursor–Lipofectamine 2000 complexes were then added to stellate cell culture medium. After 6 h, the culture medium was changed, and TGF-β1 was added at a concentration of 2 ng/ml.

Luciferase reporter assay. 3′UTRs containing putative miRNA target regions of the Col1A1 and SP1 genes were obtained by PCR using human stellate cell cDNA as a template and primer sets as follows: Col1A1–miR-29: Forward 5′-TTCTCGAGGTTCTTGTCTTG ATGTGTCACC-3′, Reverse 5′-TTTCTAGAGAGAGCAGAGGCCTGAGA AG-3′; Col1A1–miR-143: Forward 5′-CTCGAGACTCCCTCCATCCCAA CCT-3′, Reverse 5′-TCTAGAATTGCTGGGCAGACAATAC-3′; Col1A1–miR-218: Forward 5′-CTCGAGGTGGATGGGGACTTGTGAAT-3′, Reverse 5′-TCTAGATTATGTTTGGGTCATTTCCAC-3′; SP1–miR-29: Forward 5′-TTCTCGAGTGGGTGCTACACAGAATGC-3′, Reverse 5′-TTTC TAGAAGACTGTCCTTATTTCCTTGGTA-3′; and SP1–miR-218: Forward 5′-CTCGAGGATGTTTTCCCTTAACTTTTCCT-3′, Reverse 5′-TCT AGACTAAAAGCTTATATCCTCAGCATC-3′. Each of the forward and reverse primers carried the *Xho*I and *Xba*I sites at their 5′-ends. The obtained DNA fragments were inserted into the pmirGLO Vector (Promega, San Luis Obispo, CA, USA). The resulting vectors were dubbed pCol1A1–miR-29/mirGLO, pCol1A1–miR-143/mirGLO, pCol1A1–miR-218/mirGLO, pSP1–miR-29/mirGLO, and pSP1–miR-218/mirGLO. Human stellate cells were seeded on 96-well plates (Microtest 96-well Assay Plate; Becton Dickinson, Franklin Lakes, NJ, USA) in DMEM supplemented with 10% FBS at a density of 2×10^4 cells/well. The following day, they were transfected with 200 ng of reporter plasmid along with miRNA precursors using Lipofectamine 2000 as described above and incubated for an additional 24 h. After incubation, the medium was removed from the wells, and 20 μl of phosphate-buffered saline was added. The Dual-Glo Luciferase Assay System (Promega) was used to analyze luciferase expression according to 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 bar graphs are the means ± SD of at least three independent experiments. Statistical analysis was performed using Student's *t*-test, and $P < 0.05$ was considered significant.

Results and discussion

Regulation of Col1A1 expression by TGF-β1 and IFNα in human stellate cells

Immortalized human stellate cells, LX-2, are classified as an activated phenotype that expresses mRNAs for Col1A1 and other fibrogenetic molecules and are reported to be highly gene-transfectable [24]. At first, we observed that Col1A1 mRNA expression increased dose-dependently by TGF-β1 (Fig. 1A), whereas this upregulation was significantly inhibited by the presence of 100 IU/ml of human IFNα (Fig. 1B).

Extraction of miR-29b, -143, and -218 as candidates interacting with Col1A1 3′UTR

To determine the role of miRNAs in human stellate cell collagen expression, we searched for predictable miRNAs that could interact

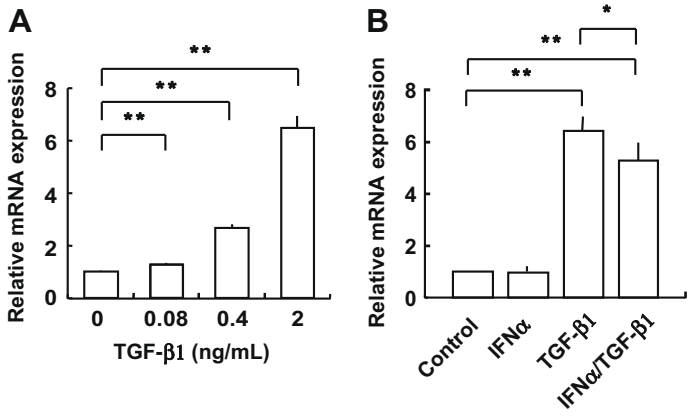

Fig. 1. Regulation of alpha 1 (I) collagen (Col1A1) expression in human stellate cells. (A) Dose-dependent effect of TGF-β1 on Col1A1 mRNA expression. Human stellate cells, LX-2, were treated with TGF-β1 (0, 0.08, 0.4, and 2 ng/ml) for 24 h in DMEM containing 0.1% FBS. (B) Effect of IFNα on Col1A1 mRNA expression in human stellate cells stimulated with TGF-β1. The cells were treated with IFNα (100 IU/ml), TGF-β1 (2 ng/ml), or IFNα (100 IU/ml) + TGF-β1 (2 ng/ml) for 24 h in DMEM containing 0.1% FBS. Control: human stellate cells were cultured for 24 h in DMEM containing 0.1% FBS. mRNA expression was analyzed by real-time PCR. The results are expressed as relative expression against control expression without treatment. $^{*}P < 0.05$; $^{**}P < 0.01$.

with 3′UTR of human Col1A1 mRNA using TargetScan Human Release 5.1 (http://www.targetscan.org/). As a result, miR-29, -98, -129, -133, -143, -196, -218, and let-7 were extracted as candidates. Because further *in silico* analyses among the eight candidates indicated that miR-29b, -143, and -218 were highly homologous to the Col1A1 3′UTR, we checked the expression levels of these miRNAs in human stellate cells by real-time PCR. As a result, miR-143 and -218 expressions were up and downregulated dose-dependently by TGF-β1, respectively, (Fig. 2A and B). Although miR-29b expression was unaffected by TGF-β1, it increased in

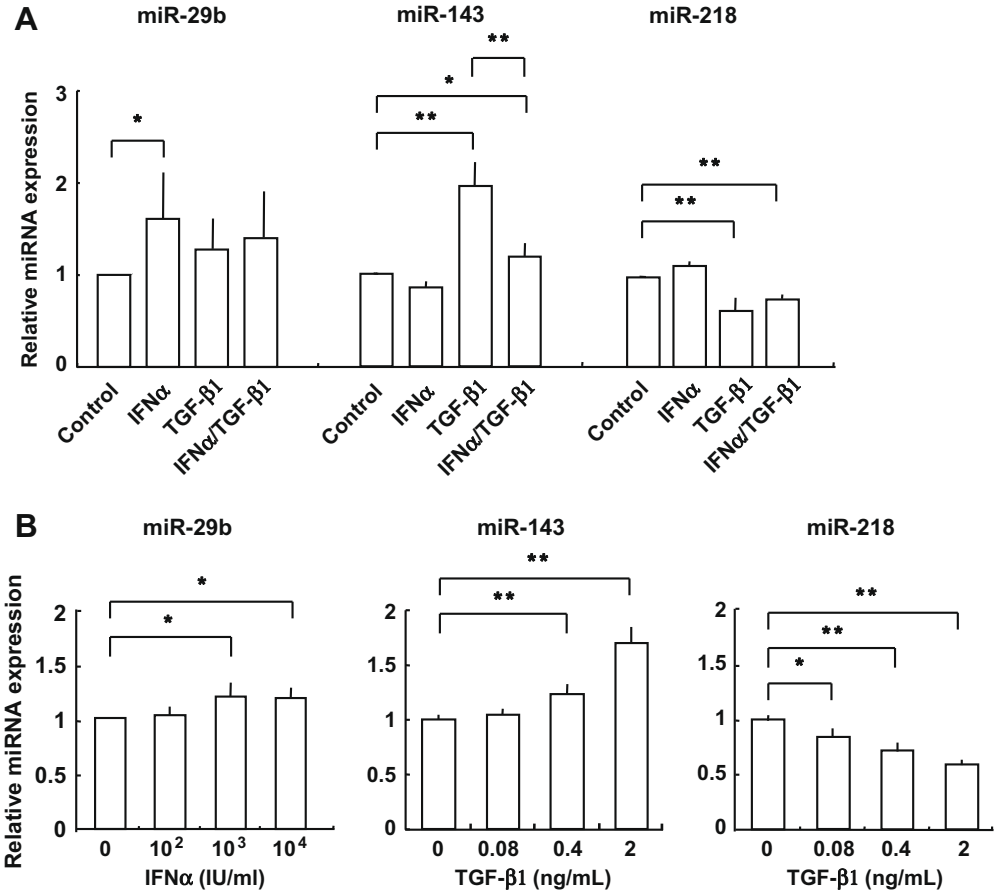

Fig. 2. Expression of miR-29b, -143, and -218 in human stellate cells. (A) Expression of miR-29b, -143, and -218 in human stellate cells, LX-2. The cells were treated with IFNα (100 IU/ml), TGF-β1 (2 ng/ml), or IFNα (100 IU/ml) + TGF-β1 (2 ng/ml) for 24 h in DMEM containing 0.1% FBS. Control: human stellate cells were cultured for 24 h in DMEM containing 0.1% FBS. (B) Dose-dependent effect of IFNα or TGF-β1 on the expression of miR-29b, -143, and -218 in human stellate cells. miRNA expression was analyzed by real-time PCR. The results are expressed as relative expression against control expression without treatment. $^{*}P < 0.05$; $^{**}P < 0.01$.

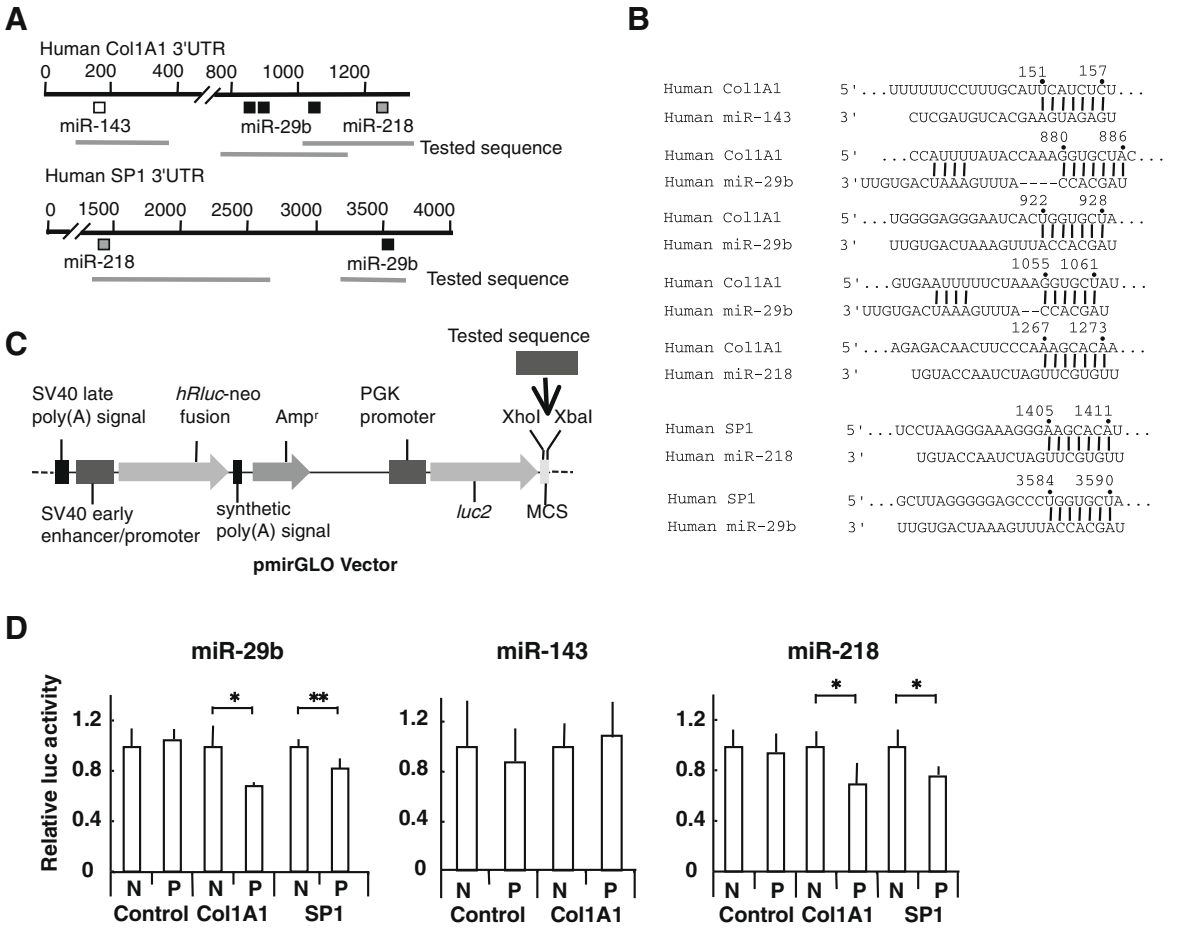

Fig. 3. Interaction of miR-29b, -143, and -218 with the 3′UTRs of alpha 1 (I) collagen (Col1A1) and SP1 mRNAs. (A) Schematic indication of the miRNA binding sites in the 3′UTRs of Col1A1 and SP1 mRNAs based on TargetScan Human Release 5.1 (http://www.targetscan.org/). Each black, white, and gray box indicates miR-29b, -143, and -218, respectively. Tested sequences indicate the regions that were inserted into the luciferase reporter vector. (B) Predicted consequential pairing of the target region and miRNAs. Arabic numerals above indicate the positions relative to the 3′UTR start sites. (C) Luciferase reporter vector structure. The vector contained two expression units; one for the *Renilla* luciferase gene (*hRluc*-neo fusion) expression. This unit was driven by an SV40 early promoter. The other was for the firefly luciferase gene (*luc2*). This unit was driven by a human phosphoglycerate kinase (PGK) promoter and contained multiple cloning sites (MCS) downstream of the *luc2* sequence. Each Col1A1 and SP1 3′UTR containing a putative miRNA target region (tested sequence) was cloned into the MCS. Arrows indicate the gene directions. Amp^R indicates an ampicillin-resistant plasmid gene. (D) Interaction of miR-29b, -143, and -218 with the 3′UTRs of Col1A1 and SP1 mRNAs in human stellate cells. Relative luciferase activity derived from pCol1A1–miR-29/mirGLO and pSP1–miR-29/mirGLO in the presence of miR-29b precursors (left panel), pCol1A1–miR-143/mirGLO in the presence of miR-143 precursors (center panel), and pCol1A1–miR-218/mirGLO and pSP1–miR-218/mirGLO in the presence of miR-218 precursors (right panel). The pmirGLO vector was used as a negative control reporter vector (control). N: cotransfection of reporter vectors along with negative control precursors, which have a scrambled sequence. P: cotransfection of reporter vectors along with miRNA precursors. Firefly and *Renilla* luciferase activities were determined, and firefly luciferase was normalized to *Renilla* luciferase activity. Results are expressed as relative activities against the activity in the presence of negative control precursors. $^{*}P < 0.05$ and $^{**}P < 0.01$.

the presence of IFNα (Fig. 2A and B). Thus, we assumed that these miRNAs might affect type I collagen expression via their interaction with Col1A1 3′UTR in human stellate cells.

Interaction of miR-29b, -143, and -218 with 3′UTRs of Col1A1 and SP1 mRNAs

The prediction of miRNA target regions on Col1A1 3′UTR by TargetScan indicated that Col1A1 3′UTR has three target regions for miR-29b, one for miR-143, and one for miR-218 (Fig. 3A and B). Because collagen gene expression is regulated by miR-192 via an interaction with the transcriptional repressor E-box [26], we additionally considered SP1, which is a transcriptional regulator of Col1A1 expression induced by TGF-β1 [27,28]. The predicted miRNA target regions of SP1 3′UTR contained one target region for miR-29b and one for miR-218 (Fig. 3A and B).

To investigate the direct targeting of Col1A1 by miR-29b, -143, and -218 and that of SP1 by miR-29b and -218, the sequence of each target region was cloned and inserted into the downstream region of the firefly luciferase reporter gene (Fig. 3C). The resulting vectors were dubbed pCol1A1–miR-29/mirGLO, pCol1A1–miR-143/mirGLO, pCol1A1–miR-218/mirGLO, pSP1–miR-29/mirGLO, and pSP1–miR-218/mirGLO. These vectors were cotransfected into human stellate cells with miRNA precursors. As a result, the miR-29b and -218 precursors inhibited luciferase activity derived from the vectors carrying Col1A1 or SP1 3′UTRs (Fig. 3D). In contrast, the miR-143 precursors had no effect on luciferase activity of the vector carrying Col1A1 3′UTR (Fig. 3D). According to these observations, we assumed that the Col1A1 and SP1 3′UTR sequences could be targeted by miR-29b and -218, whereas miR-143, which was induced by TGF-β1 (Fig. 2A and B), had a negligible effect on Col1A1 expression in human stellate cells.

Regulation of type I collagen expression by miR-29b and -218

Next, we examined the effect of miR-29b and -218 overexpression on type I collagen mRNA and protein expression in human stellate cells. Transient transfection of miR-29b precursors signifi-

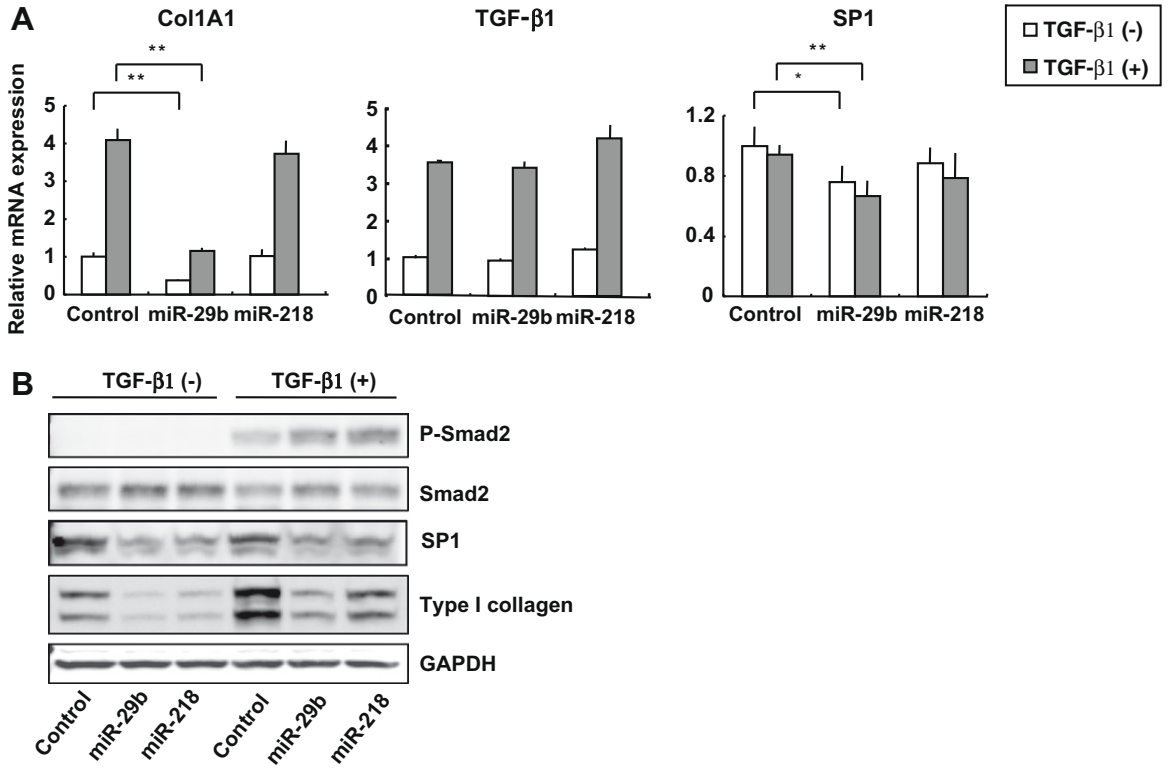

Fig. 4. Effect of miR-29b and -218 on type I collagen expression in human stellate cells. Human stellate cells were cultured in DMEM supplemented with 10% FBS and were transfected with 50 nM miR-29b, -218 precursors, or a negative control, which had a scrambled sequence (control) using Lipofectamine 2000. After 6 h, the medium was changed to DMEM containing 0.1% FBS with or without 2 ng/ml TGF-β1, and the culture was continued for another 24 h. (A) Effect of miR-29b and -218 precursors on the expression of Col1A1, TGF-β1, and SP1 mRNAs in human stellate cells with (gray column) or without (white column) TGF-β1. mRNA expression was analyzed by real-time PCR. The results are expressed as relative expression against control expression. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Effect of miR-29b and -218 precursors on the protein expression of phospho-Smad2 (P-Smad2), Smad2, SP1, type I collagen, and GAPDH in human stellate cells in the presence (+) or absence (−) of TGF-β1.

cantly inhibited type I collagen mRNA and protein expression (Fig. 4A, left panel, and B) in unstimulated human stellate cells. Additionally, transfection of miR-29b precursors completely suppressed the upregulation of type I collagen mRNA and protein under TGF-β1 stimulation. TGF-β1 stimulation induces Col1A1 mRNA expression through a pathway that includes SP1 and phosphorylated Smad2/3 [29]. In our results, upregulation of TGF-β1 mRNA (Fig. 4A, center panel) and phosphorylation of Smad2 (Fig. 4B) under TGF-β1 stimulation were unaffected by the transfection of miR-29b precursors. These results suggested that miR-29b may affect the downstream of phosphorylated Smad2. Moreover, the transfection of miR-29b precursors decreased SP1 mRNA and protein expression (Fig. 4A, right panel, and B). Thus, the miR-29b-induced repression of type I collagen expression could be caused by its direct interaction with Col1A1 3′UTR and additionally by its interaction with SP1 expression in human stellate cells. These observations agree with a report showing the role of miR-29 in collagen expression and cardiac fibrosis after cardiac infarction [30]. In contrast, transfection of miR-218 precursors triggered a negligible change in Col1A1 and SP1 mRNA expression (Fig. 4A, left and right panels) but slightly reduced their protein level (Fig. 4B). Taken together, these results imply that miR-29b is the most potent miRNA with regard to collagen production in human stellate cells.

Conclusions

We found a potent repression of collagen production by miR-29b in human stellate cells. IFNs attenuate and may regress liver fibrosis caused by hepatitis C viral infection [21–23], although the precise molecular mechanism has yet to be demonstrated. The present study using human stellate cells demonstrated that IFNα upregulates miR-29b (Fig. 2B and C), which is a negative regulator of type I collagen production via the interaction with Col1A1 and SP1 3′UTRs. This observation implies the contribution of miR-29b to antifibrotic IFN actions. Targeted delivery of miR-29b to activated stellate cells in the liver could become a new therapeutic strategy for human liver fibrosis in the future.

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