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Suppression of hepatic stellate cell activation by microRNA-29b

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ABSTRACT

MicroRNAs (miRNAs) participate in the regulation of cellular functions including proliferation, apoptosis, and migration. It has been previously shown that the miR-29 family is involved in regulating type I collagen expression by interacting with the 3′UTR of its mRNA. Here, we investigated the roles of miR-29b in the activation of mouse primary-cultured hepatic stellate cells (HSCs), a principal collagen-producing cell in the liver. Expression of miR-29b was found to be down-regulated during HSC activation in primary culture. Transfection of a miR-29b precursor markedly attenuated the expression of Col1a1 and Col1a2 mRNAs and additionally blunted the increased expression of α-SMA, DDR2, FN1, ITGB1, and PDGFR-β, which are key genes involved in the activation of HSCs. Further, overexpression of miR-29b led HSCs to remain in a quiescent state, as evidenced by their quiescent star-like cell morphology. Although phosphorylation of FAK, ERK, and Akt, and the mRNA expression of c-jun was unaffected, miR-29b overexpression suppressed the expression of c-fos mRNA. These results suggested that miR-29b is involved in the activation of HSCs and could be a candidate molecule for suppressing their activation and consequent liver fibrosis.

1. Introduction

Liver fibrosis is characterized by excessive accumulation of extracellular matrices (ECMs) and is a common feature of chronic liver injury. Hepatic stellate cells (HSCs) are considered to be the primary population that contributes to fibrogenic reactions by producing ECM in response to liver trauma. HSCs, which reside in the space of Disse outside the liver sinusoids, maintain a quiescent phenotype and store vitamin A under physiological conditions. When liver injury occurs, they become activated and trans-differentiate into myofibroblast-like cells, which are proliferative cells that lose their vitamin A droplets, express α-smooth muscle actin (α-SMA), and secrete profibrogenic mediators and ECM proteins [1,2]. Therefore, controlling the activation of the HSC population is considered a potential therapeutic target for liver fibrosis.

MicroRNAs (miRNAs) are endogenous, small, non-coding RNAs that work as post-transcriptional regulators of gene expression through their interaction with the 3′ untranslated region (3′UTR) of target mRNAs [3]. They participate in various biological phenomena, such as cell proliferation, development, differentiation, and metabolism [3]. Regarding HSCs, it was reported that miR-15b and miR-16 are down-regulated upon HSC activation and that their overexpression induces apoptosis and a delay in the cell cycle progression of HSCs [4,5]. Knockdown of miR-27a and miR-27b in activated HSCs reportedly allowed their reversion to a quiescent phenotype and decreased their rate of cell proliferation [6]. MiR-150 and miR-194 were reported to suppress proliferation, activation, and ECM production by HSCs [7]. We also reported the involvement of miR-195 in the proliferation of HSCs when treated with interferon [8].

Previously, we showed that miR-29b was induced by interferon treatment and that it suppressed type I collagen production in the human HSC line LX-2 [9]. Moreover, Roderburg et al. reported that miRNAs in the miR-29 family were significantly decreased in the

Abbreviations: BSA, bovine serum albumin; Col1a1, alpha 1 (I) collagen; Col1a2, alpha 2 (I) collagen; DDR, discoidin domain receptor; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FBS, fetal bovine serum; FN, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSC, hepatic stellate cell; ITGB1, integrin β1; miRNA, microRNA; PBS, phosphate buffered saline; PDGFR-β, platelet-derived growth factor receptor-β; PI3K, phosphatidylinositol-3 kinase; SDS, sodium dodecyl sulfate; α-SMA, α-smooth muscle actin; TGF-β, transforming growth factor-β; 3′UTR, 3′ untranslated region.

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fibrotic liver tissue of humans and mice [10]. Thus, it has been speculated that the change in the expression of miR-29 is closely related to the development of liver fibrosis. Although analyses of miR-29 functions were performed on ECM metabolism in these reports, the cells used in these experiments were immortalized cell lines that had already been activated and had become myofibroblastic, which does not always reflect miR-29 function in quiescent HSCs *in vivo*. Therefore, it is important to evaluate the effect of miR-29 on the activation of primary-cultured HSCs. These cells are known to undergo spontaneous activation and transdifferentiation into myofibroblastic cells in culture, similarly to those *in vivo*. Activated HSCs express α-SMA and produce fibrogenic mediators, such as type I collagen and transforming growth factor-β (TGF-β).

Here, we show the effects of miR-29b on the activation of HSCs using freshly isolated primary-cultured mouse HSCs. Overexpression of miR-29b suppressed cell viability and the expression of α-SMA. These effects seemed to be independent of the activation of focal adhesion kinase (FAK), extracellular signal-regulated kinase (ERK), and phosphatidylinositol-3 kinase (PI3K)–Akt, but were partially dependent on the reduction of c-fos mRNA.

2. Materials and methods

2.1. Cells

Primary HSCs were isolated from 12- to 16-week-old male C57BL/6N mice (Japan SLC Inc., Shizuoka, Japan) by pronase–collagenase digestion and subsequent purification by a single-step Nycodenz gradient, as previously described [11]. All animals received humane care, and the experimental protocol was approved by the Committee of Laboratory Animals according to institutional guidelines. Isolated HSCs were cultured on plastic dishes or glass chamber slides in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin. The purity of cultures was evaluated by observation of the characteristic stellate cell shape using phase-contrast microscopy.

The human HSC line LX-2 was donated by Dr. Scott L. Friedman (Mount Sinai School of Medicine, New York, NY, USA) [12]. LX-2 cells were maintained in DMEM as described above.

2.2. Transient transfection of a miR-29b precursor

The miR-29b precursor (Ambion, Austin, TX, USA), which was a double-strand RNA mimicking the endogenous miR-29b precursor, and a negative control (Ambion) were transfected into mouse HSCs and LX-2 cells using Lipofectamine RNAiMAX (Invitrogen) at a final concentration of 10 nM in accordance with the manufacturer's instructions. Briefly, the miRNA precursor and Lipofectamine RNAiMAX were mixed at a ratio of 5 (pmol):1 (μl) in Opti-MEM I Reduced Medium (Invitrogen), incubated for 20 min at room temperature, and then added to the cultures.

2.3. Quantitative real-time PCR

Total RNA was extracted from cells using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). Fifty nanograms of total RNA was reverse-transcribed to cDNA using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) in accordance with the manufacturer's instructions. Gene expression was measured by real-time PCR using cDNA, SYBR Green real-time PCR Master Mix (Toyobo), and a set of gene-specific oligonucleotide primers [alpha 1 (I) collagen (Col1a1): forward 5′-CCTGGCAAAGACGGACTCAAC-3′, reverse 5′-GCTGAAGT CATAACCGCCACTG-3′; alpha 2 (I) collagen (Col1a2): forward 5′-AA GGGTCCCTCTGGAGAACC-3′, reverse 5′-TCTAGAGCCAGGGAG AC-CCA-3′; α-SMA: forward 5′-TCCCTGGAGAAGAGCTACGAACT-3′, reverse 5′-AAGCGTTCGTTTCCAATGGT-3′; discoidin domain receptor (DDR) 2: forward 5′-CGAAAGCTTCCAGAGTTTGC-3′, reverse 5′-GCT TCACAACACCACTGCAC-3′; fibronectin (FN) 1: forward 5′-GATGCC GATCAGAAGTTTGG-3′, reverse 5′-GGTTGTGCAGATCTCCTCGT-3′; β1 integrin (ITGB1): forward 5′-CAACCACAACAGCTGCTTCTAA-3′, reverse 5′-TCAGCCCTCTTGAATTTTAATGT-3′; platelet-derived growth factor receptor-β (PDGFR-β): forward 5′-GCGTATCTATATCT TTGTGCCAGA-3′, reverse 5′-ACAGGTCCTCGGAG TCCAT-3′; c-fos: forward 5′-AGAAGGGGCAAAGTAGAGCA-3′, reverse 5′-CAGCTCC CTCCTCCGATT-3′; c-jun: forward 5′-CCAGAAGATGGTGTGGTGTTT-3′, reverse 5′-CTGACCCTCTCCCCTTGC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5′-TGCACCACCAACTGCT-TAG-3′, reverse 5′-GGATGCAGGGATGATGTTC-3′] using an Applied Biosystems Prism 7500 (Applied Biosystems, Foster City, CA, USA). To detect miR-29b 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-29b.

2.4. Immunoblots

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 Inhibitor Cocktail 1, and Phosphatase Inhibitor Cocktail 2 (Sigma). Proteins (2.5–10 μg) were electrophoresed in a 5–20% gradient SDS–polyacrylamide gel (ATTO Co., Tokyo, Japan) and were then transferred onto Immobilon P membranes (Millipore, Bedford, MA, USA). After blocking, the membranes were incubated with primary antibodies [mouse monoclonal antibody against α-SMA (Dako, Ely, UK); rabbit polyclonal antibody against type I collagen (Rockland Immunochemicals, Inc., Gilbertsville, PA, USA); rabbit polyclonal antibodies against PDGFR-β and GAPDH (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); rabbit polyclonal antibodies against FAK and phospho-FAK (Y397) (Cell Signaling Technology Inc., Beverly, MA, USA); and mouse monoclonal antibodies against ERK, phospho-ERK (T202/Y204), Akt, and phospho-Akt (S473) (Cell Signaling Technology Inc.)] followed by peroxidase-conjugated secondary antibodies (Dako). Immunoreactive bands were visualized by the enhanced chemiluminescence system (Amersham, Roosdaal, Netherlands) using a Fujifilm Image Reader LAS-3000 (Fuji Medical Systems, Stamford, CT, USA).

2.5. F-actin staining

HSCs on glass chamber slides were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 min and were permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. The nonspecific background signal was blocked with 1% bovine serum albumin (BSA) in PBS for 20 min. F-actin was stained with MFP488-phalloidin (Mobitec, Goettingen, Germany) in PBS with 1% BSA for 20 min. 4′,6-diamidino-2-phenylindole (DAPI) (Dojindo Laboratories, Kumamoto, Japan) was used for counterstaining.

2.6. Cell viability assay

The cell viability was evaluated by the WST-1 assay based changes in absorbance at 450 nm. Freshly isolated mouse HSCs or LX-2 cells were plated in 96-well plates at a density of 1.5×10^4 or 3×10^3 cells/well, respectively. The following day, cells were transfected with the miR-29b precursor or a negative

control as described above and were incubated for an additional 3 or 5 days before the assessment of cell viability. In another experiment, mouse HSCs that were transfected with the miR-29b precursor the day before were serum-starved overnight and then stimulated with PDGF-BB (10 ng/ml) (R&D Systems, Minneapolis, MO, USA). After incubation for 3 days, cell viability was assessed by the WST-1 assay.

2.7. Statistical analysis

Data presented as bar graphs are the means ± SD of at least three independent experiments. Statistical analysis was performed using the Student's *t*-test, and $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Expression of miR-29b in mouse HSCs during spontaneous activation

At 1 day of culture after isolation, mouse HSCs adhered to plastic plates and exhibited round cell bodies with numerous lipid droplets similar to those observed in lipocytes (Fig. 1A). Cell bodies then began to gradually spread and flatten, increasing in size, and losing lipid droplets, resulting in the activated myofibroblastic phenotype (Fig. 1A). In addition to the changes in cell appearance, mRNA expression levels of α-SMA, Col1a1, Col1a2, FN1, DDR2, ITGB1, and PDGFR-β significantly increased at Days 4 and 7 of culture as compared to Day 1 (Fig. 1B). Immunoblot analyses confirmed the increases of type I collagen, α-SMA, and PDGFR-β protein levels at Days 4 and 7 (Fig. 1C). These molecules have already been reported to be up-regulated in activated HSCs and involved in fibrosis [2]. Thus, the primary mouse HSCs used in this study were in an activated state. Although TGF-β1 is known as a key regulator of collagen production and fibrosis [13], its mRNA expression level in mouse HSCs remained unchanged due to an unknown reason in this study (Fig. 1B). In contrast, miR-29b expression in mouse HSCs was significantly decreased to 28% and 32% at Days 4 and 7, respectively, as compared to Day 1 (Fig. 1D). These findings raised the possibility that a reduction in miR-29b contributed to the up-regulation of the fibrosis-related genes listed above.

3.2. Effects of miR-29b overexpression on the activation of HSCs

To investigate this possibility, we next examined the effects of miR-29b overexpression on the activation of HSCs. Overexpression of miR-29b was achieved by the transient transfection of a synthesized miR-29b precursor, which was a double-strand RNA mimicking the endogenous miR-29b precursor. As shown in Fig. 2A, transfection of the miR-29b precursor markedly suppressed mRNA expression of Col1a1 and Col1a2 to 8% and 18%, respectively. Transfection significantly reduced mRNA expression of FN1 to 61% and also affected the expression of HSC activation-related molecules, such as α-SMA, DDR2, ITGB1, and PDGFR-β to 57%, 62%, 73%, and

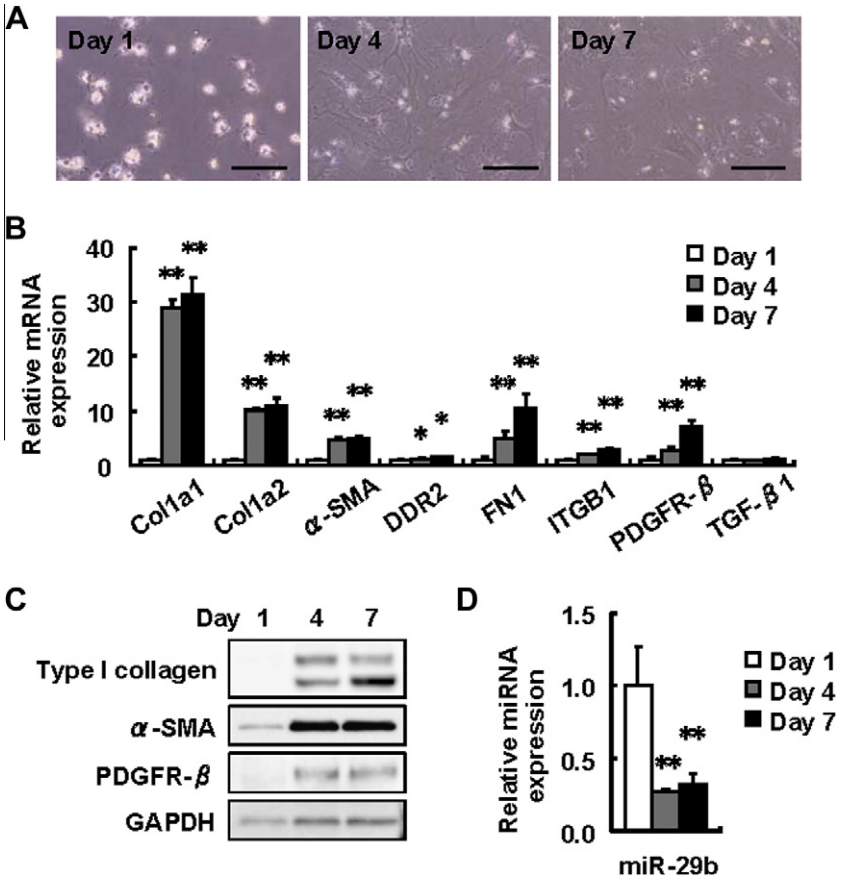

Fig. 1. Expression of miR-29b in mouse primary HSCs during culture. HSCs were isolated from mouse liver (Day 0) and cultured for the indicated periods. (A) Phase-contrast microscopy. Scale bar, 200 μm. (B) mRNA expression levels of Col1a1, Col1a2, α-SMA, DDR2, FN1, ITGB1, PDGFR-β and TGF-β1 were analyzed by real-time PCR. Results are expressed as relative expression against the expression on Day 1 of corresponding genes. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with Day 1. (C) Protein expression levels of type I collagen, α-SMA and PDGFR-β were analyzed by Western blot. GAPDH served as an internal control. (D) miR-29b expression level was analyzed by real-time PCR. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with Day 1.

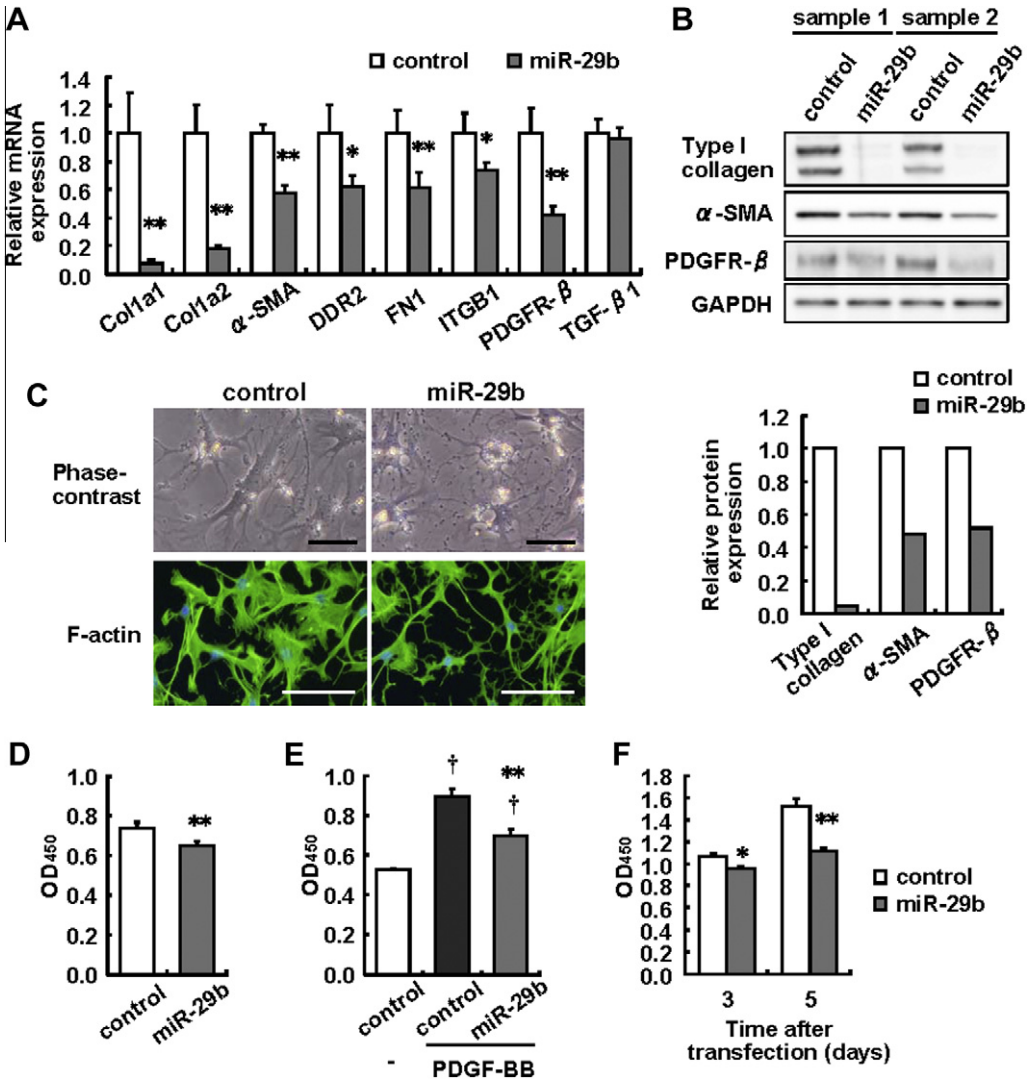

Fig. 2. Effects of miR-29b overexpression on the activation of HSCs. (A–D) Mouse HSCs were transfected with 10 nM miR-29b precursor or a negative control (control) on Day 1 and incubated for 3 days. (A) mRNA expression levels of Col1a1, Col1a2, α-SMA, DDR2, FN1, ITGB1, PDGFR-β and TGF-β1 were analyzed by real-time PCR. The results are expressed as relative expression against the expression of untreated control. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control. (B) Protein expression levels of type I collagen, α-SMA and PDGFR-β were analyzed by Western blot. GAPDH served as an internal control. The lower graph indicates the densitometric results of $n = 2$. (C) Phase-contrast microscopy (upper) and MFP488-phalloidin staining for F-actin (lower). Scale bar, 100 μm. (D) Cell viability was evaluated by WST-1 assay. $^{**}P < 0.01$ compared with control. (E) Mouse HSCs were transfected with miR-29b precursor or a negative control (control) on Day 1. Twenty-four hours later, cells were serum-starved overnight, stimulated with or without PDGF-BB (10 ng/ml) and incubated for an additional 3 days. In Day 6, cell viability was evaluated by WST-1 assay. $^{**}P < 0.01$ compared with control plus PDGF-BB. $^{\dagger}P < 0.05$ compared with control plus non-treat. (F) LX-2 cells were transfected with miR-29b precursor or a negative control (control) and incubated for the indicated periods. Cell viability was evaluated by WST-1 assay. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control.

42%, respectively. The TGF-β1 mRNA level was unaffected. At the protein level, expression of type I collagen, α-SMA, and PDGFR-β was suppressed by the overexpression of the miR-29b precursor (Fig. 2B). Col1a1, Col1a2, ITGB1, and PDGFR-β are predicted targets of miR-29b according to the miRNA target prediction databases TargetScan (http://www.targetscan.org/), miRBase (http://www.mirbase.org/), and mircrorna.org (http://www.microrna.org). Therefore, the suppression of these proteins might be due to the direct interaction of miR-29b with the 3′UTR of their corresponding mRNAs. Although α-SMA, DDR2, and FN1 are not predicted targets of miR-29b, their mRNA levels were suppressed. Thus, this effect was thought to be a secondary action of miR-29b over-expression. That is, it is suggested that miR-29b can not only target Col1a1, Col1a2, ITGB1, and PDGFR-β, but can also suppress the activation of HSCs by regulating other unidentified mechanisms, resulting in the suppression of α-SMA, DDR2, and FN1. In support of these results, morphological transformation from the quiescent to the myofibroblastic cell shape, as shown in Fig. 1A, was impeded in miR-29b precursor-transfected cells (Fig. 2C); miR-29b precursor-transfected cells exhibited star-like morphology with small cell bodies and slender dendritic processes as compared to negative control-transfected cells at Day 4. Staining with MFP-phalloidin, which labels F-actin, also confirmed cytoskeletal changes in miR-29b precursor-transfected HSCs. Taken together; these results suggest that miR-29b is able to suppress HSC activation as well as ECM expression.

3.3. Effect of miR-29b overexpression on number of HSCs

Activated HSCs are known to acquire proliferation abilities [1,2]. We considered the possibility that miR-29b was able to regulate the number of HSCs. As shown by the WST-1 assay, when the miR-29b precursor was transfected into HSCs at Day 1, the cell number observed at Day 4 was significantly reduced to 88% of the negative control-transfected cells (Fig. 2D). Treatment of HSCs with 10 ng/mL PDGF-BB, a key mitogen for HSCs [14], significantly

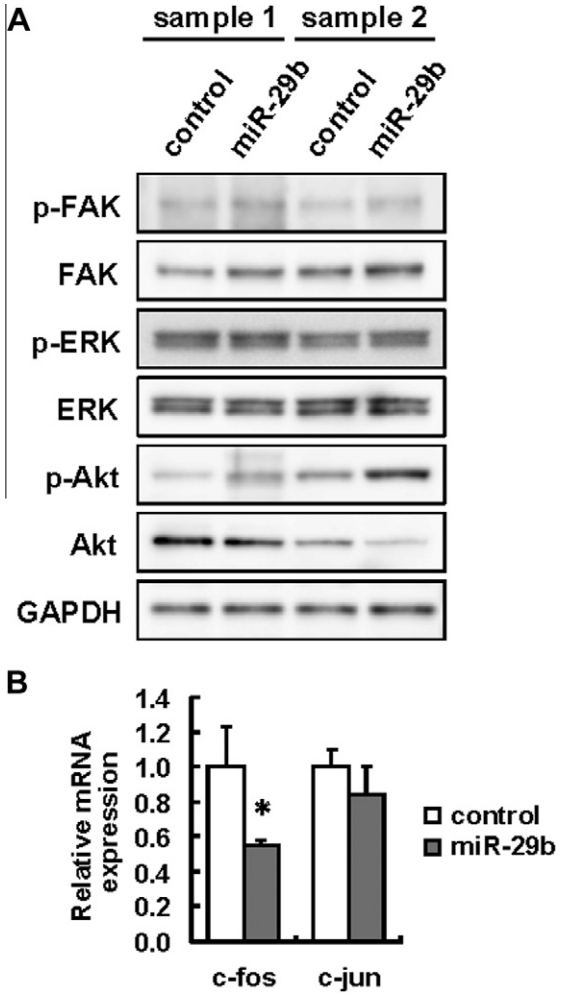

Fig. 3. Effects of miR-29b overexpression on the ECM- and growth factor-related signaling in mouse primary HSCs. Mouse HSCs were transfected with 10 nM miR-29b precursor or a negative control (control) on Day 1 and were incubated for 3 days. (A) Phosphorylation of FAK (Y397), ERK (T202/Y204) and Akt (S473) was analyzed by Western blot. (B) mRNA expression levels of c-fos and c-jun were analyzed by real-time PCR. The results are expressed as relative expression against the expression of control. $^{*}P < 0.05$ compared with control.

increased the cell number up to 1.7 times that of the non-treated cells (Fig. 2E), whereas overexpression of miR-29b inhibited this increase. Furthermore, in LX-2 cells, transfection of the miR-29b precursor decreased cell viability to 89% and 81% at 3 and 5 days following transfection, respectively (Fig. 2F). These results suggested that miR-29b is able to suppress the proliferation of HSCs and that down-regulation of miR-29b during HSC activation may contribute to their active proliferation.

3.4. Effects of miR-29b overexpression on the ECM- and growth factor-related signaling in primary mouse HSCs

The question of how miR-29b functions in blocking HSC activation was also examined. We showed that overexpression of miR-29b suppressed Col1a1, Col1a2, FN1, DDR2, ITGB1, and PDGFR-β expression (Fig. 2A and B). DDR2 is a receptor tyrosine kinase that is activated by the binding of collagen and was reported to be involved in the proliferation of HSCs and in the expression of matrix metalloproteinase-2 [15,16]. ITGB1 is a member of the integrin family and works as a FN or collagen receptor by forming a heterodimer with the integrin α subunit. ITGB1 is reported to be involved in the production of type I collagen and monocyte chemotactic protein-1 in HSCs [17,18]. PDGFR-β is a receptor of PDGF and is involved in the proliferation of activated HSCs [19,20]. Because it is known that intracellular signaling molecules such as FAK, ERK, and PI3K/Akt are key mediators for DDR2, ITGB1, and PDGFR-β [14,21–24], their down-regulation by miR-29b may affect downstream signaling, resulting in the inhibition of both activation and proliferation of HSCs. To verify this hypothesis, we investigated the effect of miR-29b overexpression on the activation of FAK, Akt, and ERK. Activation of these kinases was evaluated by immunoblot analyses to detect the phosphorylation of each protein. Unexpectedly, phosphorylation of FAK, ERK, and Akt was unaffected by miR-29b overexpression (Fig. 3A). Next, we also examined the mRNA expression of c-fos and c-jun, which form the transcription factor AP-1 complex and are located downstream of these signal kinases. Although transfection of the miR-29b precursor failed to alter c-jun expression, it significantly reduced c-fos mRNA expression to 55% (Fig. 3B). Because AP-1 is known to be one of the key transcription factors for the initiation of HSC activation [25,26], this fact indicates that effects of miR-29b may be partially mediated by c-fos down-regulation.

4. Conclusion

We confirmed that miR-29b expression decreased during HSC activation and found that overexpression of miR-29b is able to attenuate the activation and trans-differentiation of HSCs, although the precise molecular mechanism for this effect remains unknown. Changes in miR-29b expression seem to profoundly affect the activation of HSCs.

Conflict of interest

The authors have no conflict of interest to declare.

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