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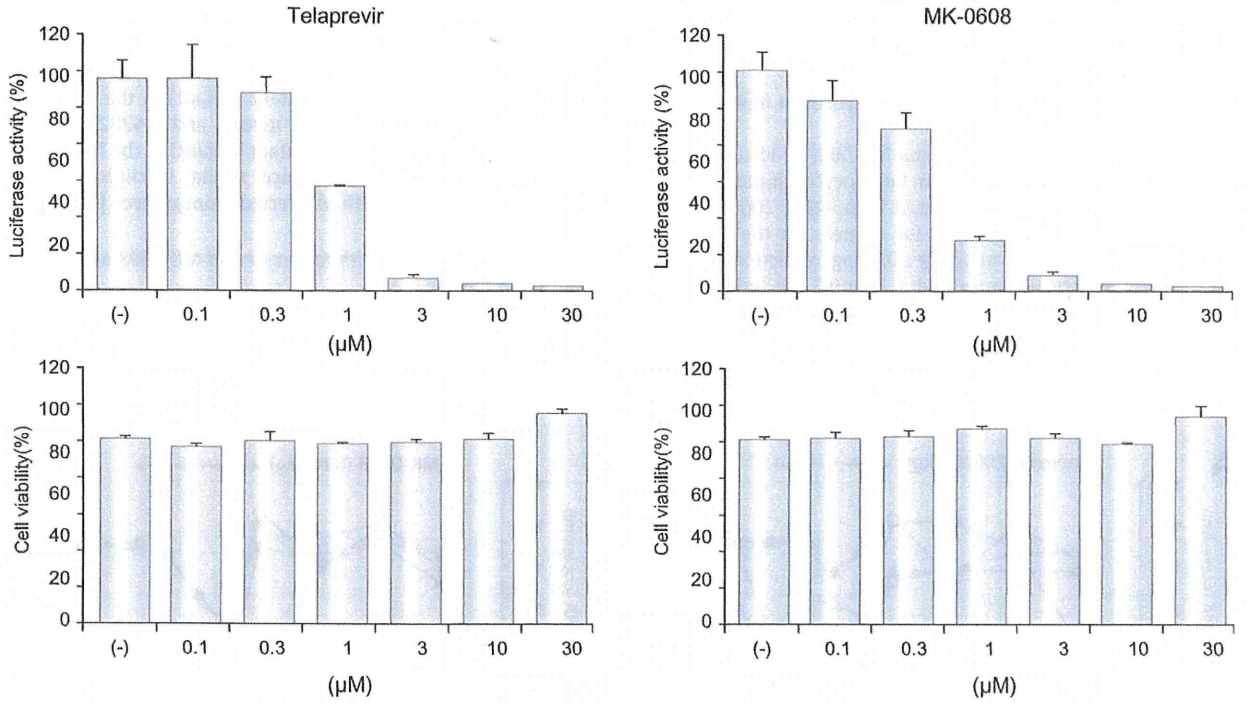

Fig. 1. *In vitro* analysis of susceptibility of HCV replicon cells to anti-HCV drugs. Huh7/Rep-Neo cells were treated for 72 h with the indicated concentrations of either telaprevir or MK-0608. Intracellular HCV RNA replication levels were determined as luciferase activities (upper panel), and expressed relative to cellular viabilities (lower panel). Bars represent means ± SD of three experiments.

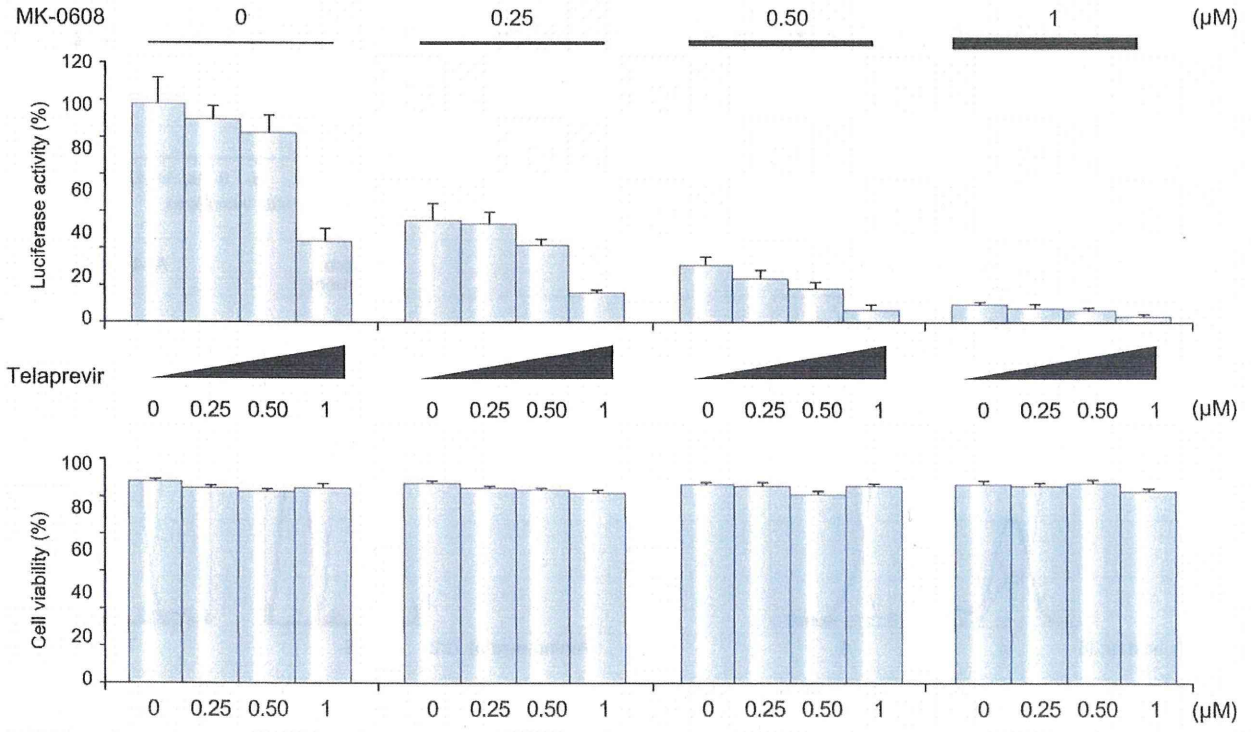

Fig. 2. *In vitro* analysis of susceptibility of HCV replicon cells to combination treatment with anti-HCV drugs. Huh7/Rep-Neo cells were treated for 72 h with the indicated concentration of MK-0608 plus telaprevir. Intracellular HCV RNA replication levels were determined as luciferase activities (upper panel), and expressed relative to cellular viabilities (lower panel). Bars are means ± SD of 3 experiments.

telaprevir and MK-0608 were combined, the anti-HCV effect was increased without cellular damage (Fig. 2).

Effects of telaprevir and MK-0608 on HCV replication in vivo

To analyze the effect of telaprevir and MK-0608 *in vivo*, we used genotype 1b HCV-infected human hepatocyte chimeric mice. Eight HCV-infected mice were treated with either 200 mg/kg of telaprevir or 3 mg/kg of MK-0608 for 4 weeks. At the end of 1 week, treatment resulted in a 1.9 ± 0.7 log reduction of HCV RNA in telaprevir-treated mice and a 2.6 ± 0.2 log reduction in MK-0608-treated mice (Fig. 3A and C). During the treatment, the level of HSA did not decrease. Serum HCV RNA level rebounded in one of the four telaprevir-treated mice and in two of the three MK-0608-treated mice (a MK-0608-treated mouse died after 1 week of treatment). Nucleotide and amino acid sequence analysis showed the emergence of a V36A mutation (NS3-4A protease inhibitor-resistant variant) in the NS3 region (Fig. 3B) in a telaprevir-treated mouse, and a S282T mutation (NS5B polymerase inhibitor-resistant variant) in the NS5B region (Fig. 3D) in MK-0608-treated mice, similar to clinical observations and analysis using HCV-infected chimpanzees [22,23].

Combination treatment with telaprevir and MK-0608 on HCV replication in vivo

Because mono-therapy with either telaprevir or MK0608 resulted in emergence of drug-resistant variants, we analyzed the effect of

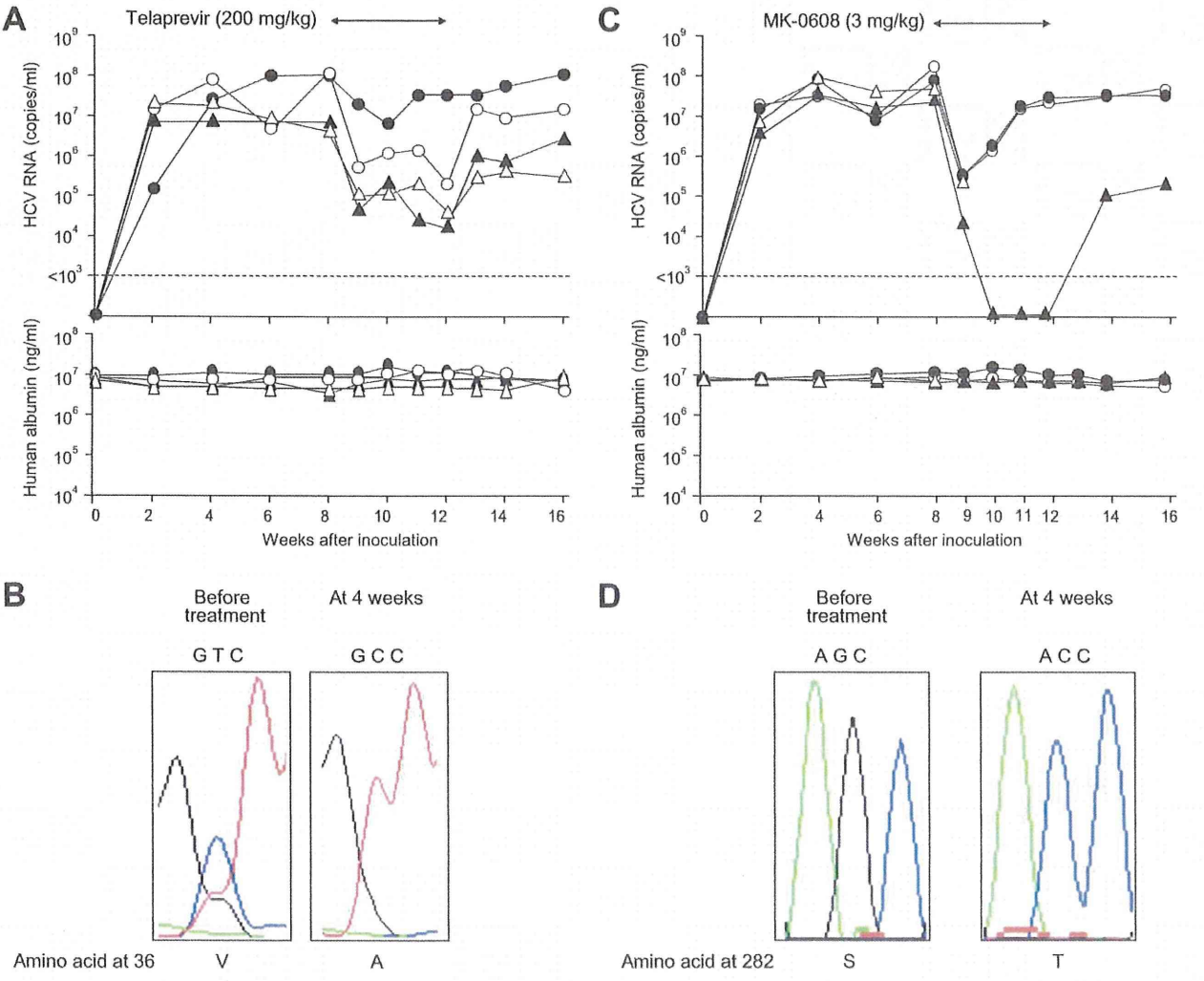

Fig. 3. Antiviral effects of either telaprevir or MK0608 monotherapy on HCV-infected mice. Mice were injected intravenously with 100 μl of HCV-positive human serum samples. Eight weeks after HCV infection, mice were treated with either 200 mg/kg of telaprevir (A) or 3 mg/kg of MK-0608 (C) for 4 weeks. Mice serum samples were obtained at the indicated times, and HCV RNA titer (upper panel) and human serum albumin concentration (lower panel) were analyzed. The horizontal dashed line represents the detection limit (10^3 copies/ml). Note that one telaprevir-treated mouse (A, closed circle) and two MK-0608-treated mice (B, closed circle and open circle) showed a viral breakthrough during the dosing period. Nucleotide and amino acid (aa) sequence analysis of aa 36 in the HCV NS3 (B) or at aa 282 in the NS5B region (D) by direct sequencing in mice serum samples obtained before treatment and at 4 weeks.

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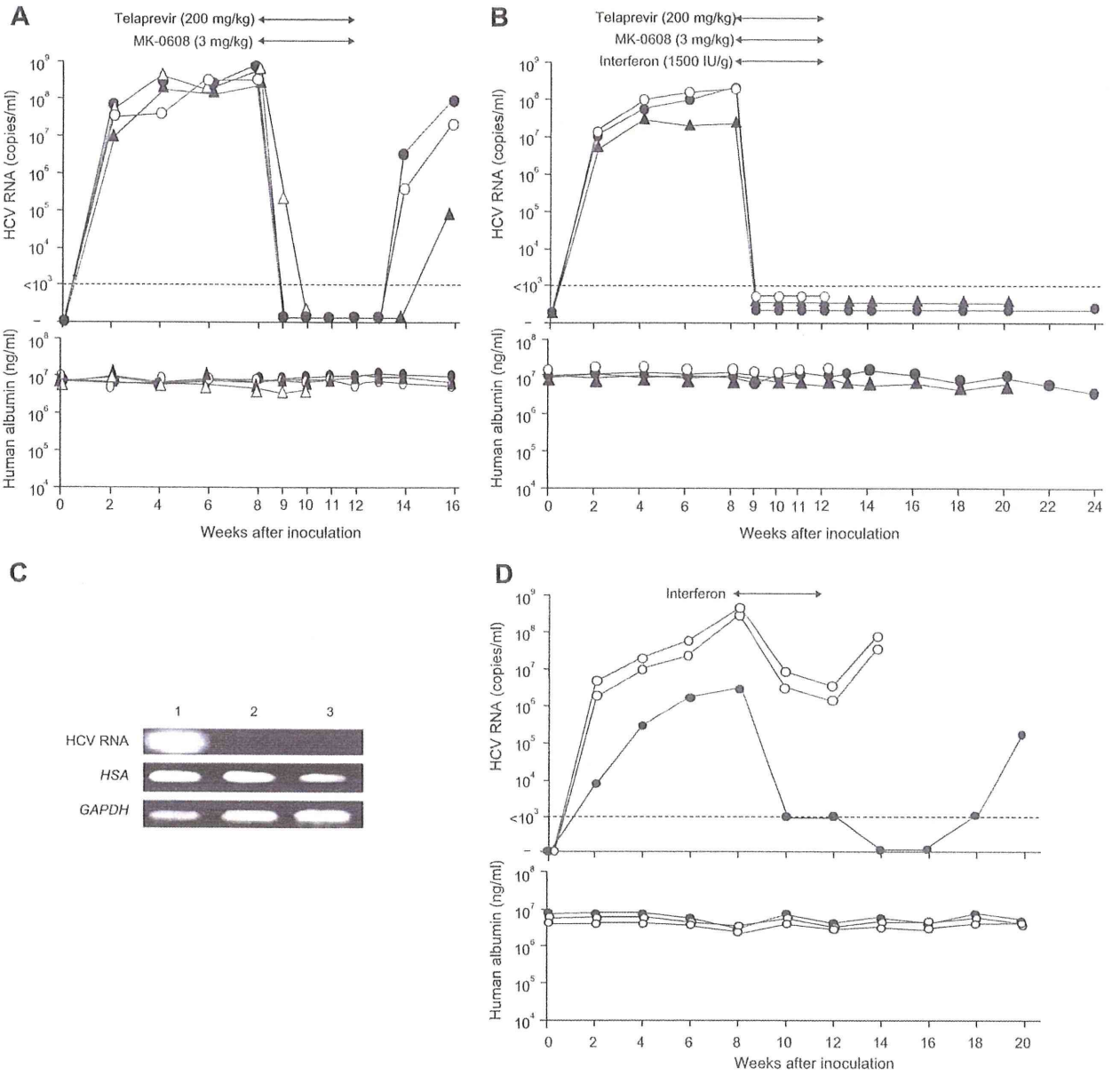

Fig. 4. Antiviral effect of combination treatment on HCV-infected mice. HCV-infected mice were treated with 200 mg/kg of telaprevir plus 3 mg/kg of MK-0608 without (A) or with (B) 1500 IU/g of human interferon-alpha for 4 weeks. Mice serum samples were obtained at the indicated times, and HCV RNA titer (upper panel) and human serum albumin concentration (lower panel) were analyzed. (C) Nested PCR of HCV RNA, human serum albumin (*HSA*) and *GAPDH* in a telaprevir, MK-0608 and interferon-alpha-treated mouse liver at 24 weeks (lane 2). Mice livers with (lane 1) or without (lane 3) HCV-infection were also analyzed. (D) HCV-infected mice were treated with either 1500 (open circles) or 7000 IU/g (closed circles) of interferon-alpha for 4 weeks.

combination treatment of these drugs with or without IFN on HCV replication *in vivo*. Four HCV-infected mice were treated with telaprevir plus MK-0608 for 4 weeks (Fig. 4A). Serum HCV RNA became negative by nested PCR with this combination treatment in all mice. One mouse died after 2 weeks of treatment. During the treatment, no emergence of resistant strains was observed in each of the remaining three mice; however, all mice became positive for HCV RNA again after cessation of the therapy. Another three mice were treated with telaprevir, MK-0608 and IFN-alpha for 4 weeks (Fig. 4B). HCV RNA became undetectable in all three mice 1 week after the beginning of the therapy. After 4 weeks of treatment, one mouse died. In the remaining two mice, HCV RNA did not become positive after cessation of the therapy. One of the remaining two mice died at 20 weeks, and the remaining mouse was sacrificed at 24 weeks (12 weeks after the cessation of therapy). HCV was probably eliminated because no HCV RNA was detected by nested PCR in this mouse liver (Fig. 4C). As a control, HCV-infected mice were treated with 1500 IU/g/day of IFN-alpha alone for 4 weeks, resulting in a two log reduction (Fig. 4D). HCV RNA became undetectable with

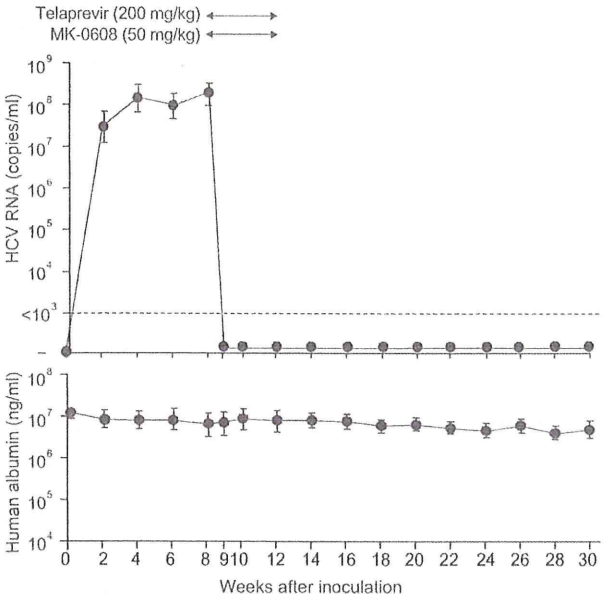

Fig. 5. High doses of MK-0608 and telaprevir combination treatment eliminates virus in HCV-infected mice. HCV-infected mice were treated with 50 mg/kg of MK-0608 and 200 mg/kg of telaprevir for 4 weeks. Mice serum samples were obtained at the indicated times, and HCV RNA titer (upper panel) and human serum albumin concentration (lower panel) were analyzed. Points represent the means ± SD of five mice.

administration of 7000 IU/g/day of IFN-alpha treatment. However, the virus rebounded after cessation of the therapy.

Four-week high dose combination therapy of MK-0608 and telaprevir eliminated HCV from mice

We investigated whether combination treatment with high doses of MK-0608 and telaprevir without IFN eliminates viruses from HCV-infected mice. Five HCV-infected mice were treated with high doses of MK-0608 (50 mg/kg) and telaprevir (200 mg/kg) for 4 weeks. Serum HCV RNA titer became undetectable 1 week after commencement of the therapy and remained undetectable in all mice at 30 weeks (18 weeks after cessation of the therapy) (Fig. 5). No apparent toxicity of the drugs was observed as none of the mice showed a decrease in the level of serum HSA.

Discussion

Since we began performing treatment experiments using human hepatocyte chimeric mice with HCV, we have administered many different drugs to analyze the effects on suppression or eradication of the virus. However, until we performed the experiments described in this study, we have never observed long term absence of the virus following cessation of the therapy [12,24]. Strikingly, after only 4 weeks of triple therapy with IFN, telaprevir and MK0608, was long term absence of the virus in mouse serum after cessation of the therapy visible (Fig. 4B). Furthermore, high dose telaprevir and MK-0608 combination therapy resulted in a similar absence of the virus for 16 weeks after cessation of therapy (Fig. 5). In this study, mice were treated with 200 mg/kg of telaprevir twice a day, and 1 week of the treatment resulted in an approximately 2 log reduction of HCV RNA (Fig. 3A), as has been observed previously in chronic hepatitis C patients treated with 450 mg of telaprevir every 8 hrs [25]. This result suggests that approximately 1/15th of a dose in this mouse model may be equivalent to a dose in humans.

During the observation period, some mice died. We do not think that this is due to the drug regimes because the chimeric mouse is weak, and approximately 50% of mice die spontaneously at week 6 after transplantation [26].

Sustained virological response, the complete elimination of the virus from the human body, is defined as testing negative for HCV RNA in serum for more than 24 weeks after cessation of the therapy. As the chimeric mouse used in this study is a weak animal, we were unable to monitor for absence of the virus beyond 24 weeks following cessation of therapy. However, negative testing for HCV RNA in mouse liver by nested PCR (Fig. 4C) 12 weeks after cessation of the therapy strongly suggests that HCV was completely eliminated from the mouse. Of course the mouse model differs from infection in humans where the virus replicates for years in the livers of infected patients. However, results of this study suggest that we will be able to eliminate the virus in humans by treating patients with regimens similar to those used in this study.

Until recently eradication of the virus with biochemical and histological improvement in chronically infected patients has long been reported only with the use of IFN or PEG-IFN [27,28]. Recently, Suzuki et al. reported for the first time eradication of the virus from chronically infected patients without IFN [29].

Elimination of the virus without IFN is desirable due to the many serious side effects of this drug [3,5–9]. However, emergence of drug resistance is a problem, as demonstrated in this study (Fig. 3) as well as in previous studies using replicon systems and HCV-infected chimpanzees [22,23]. A recent clinical study of NS3-4A and NS5B inhibitor combination therapy has reported that 13 days of this combination treatment achieved robust antiviral suppression in chronic hepatitis C patients [30]. As no study has tested the possibility of development of double drug resistant mutants, we will have to test if long term low dose treatment with any combination of STAT-C compounds might induce emergence of multi-drug resistant strains. Furthermore, as there is no report for emergence of IFN resistant strains, regimens such as combination therapy with multiple STAT-C drugs with a small or standard amount of IFN should be tested to develop the best therapy to eradicate the virus with a minimum of side effects and costs. Our further attempts to test possible combinations in mice to determine the best combination of STAT-C drugs will give us an insight into how to develop more effective therapeutic regimens in humans.

Financial support

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Research Article

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ME3738 enhances the effect of interferon and inhibits hepatitis C virus replication both *in vitro* and *in vivo*

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Background & Aims: ME3738 (22β-methoxyolean-12-ene-3β, 24-diol), a derivative of soyasapogenol B, attenuates liver disease in several animal models of acute and chronic liver injury. ME3738 is thought to inhibit replication of hepatitis C virus (HCV) by enhancing interferon (IFN)-β production, as determined using the HCV full-length binary expression system. We examined the effect of ME3738 combined with IFN-α on HCV replication using the genotype 1b subgenomic replicon system and an *in vivo* mouse HCV model.

Methods: HCV replicon cells (ORN/3-5B/KE cells and Con1 cells) were incubated with ME3738 and/or IFN-α, and then intracellular IFN-stimulated genes (ISGs) and HCV RNA replication were analyzed by reverse-transcription-real time polymerase chain reaction and luciferase reporter assay. HCV-infected human hepatocyte chimeric mice were also treated with ME3738 and/or IFN-α for 4 weeks. Mouse serum HCV RNA titer, HCV core antigen, and ISGs expression in the liver were measured.

Results: ME3738 induced gene expression of oligoadenylate synthetase 1 and inhibited HCV replication in both HCV replicon cells. The drug enhanced the effect of IFN to significantly increase ISG expression levels, inhibit HCV replication in replicon cells, and reduce mouse serum HCV RNA and core antigen levels in mouse livers. The combination treatment was not hepatotoxic as evident histologically and did not reduce human serum albumin in mice.

Conclusions: ME3738 inhibited HCV replication, enhancing the effect of IFN-α to increase ISG expression both *in vitro* and *in vivo*, suggesting that the combination of ME3738 and IFN might be useful therapeutically for patients with chronic hepatitis C.

Keywords: Human hepatocyte chimeric mouse; Interferon-stimulated genes.
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Abbreviations: HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; IL, interleukin; ISG, interferon stimulated gene; MxA, myxovirus resistance protein A; OAS, oligoadenylate synthetase; PKR, double stranded RNA-dependent protein kinase; PCR, polymerase chain reaction; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator; USP18, ubiquitin specific peptidase 18.

Introduction

The hepatitis C virus (HCV) infects an estimated 170 million people worldwide [1] leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [2,3]. To date, the most effective therapy for viral clearance is a 48- or 72-week combination therapy of pegylated interferon (IFN)-α and ribavirin. However, successful eradication of the virus is achieved in only about 50% of treated patients [4–6]. Moreover, therapy induces significant adverse effects, such as fever, fatigue, and anemia [4], resulting in poor tolerability. More effective and less toxic treatment is, therefore, desired.

ME3738 (22β-methoxyolean-12-ene-3β, 24-diol), a derivative of soyasapogenol B [7], attenuates liver disease in several animal models of acute and chronic liver injury induced by concanavalin A, ethanol, lithocholate, and bile duct ligation [8–12]. ME3738 induces interleukin (IL)-6 expression, and serum amyloid A and α1-acid glycoprotein act as downstream targets of the IL-6 signal to protect against concanavalin A-induced liver injury [8–10]. The drug also prevents the progression of hepatic fibrosis in rats with bile duct ligation through suppression of activation and collagen synthesis of hepatic stellate cells [12].

Recently, Hiasa et al. reported that ME3738 inhibited HCV replication by enhancing IFN-β production using the HCV full-length binary expression system that uses full-length genotype 1a HCV complementary DNA plasmid with a T7 promoter sequence and an adenoviral vector expressing T7 polymerase [13]. However, it is not clear if the production of IFN-β and subsequent expression of IFN-stimulated genes (ISGs) was induced by the transcribed HCV genomes through detection by innate

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immune system receptors, including RIG-I. In addition, it is also not clear whether ME3738 has anti-viral effects on genotype 1b HCV, which is the most common and most IFN-resistant genotype in Japan [14].

Recently, HCV-infected mice have been developed by inoculating HCV-infected human serum into urokinase-type plasminogen activator (uPA)-severe combined immunodeficiency (SCID) mice engrafted with human hepatocytes [15,16]. We and other groups had reported that this mouse model is useful for evaluating anti-HCV drugs such as IFN-α and anti-NS3 protease *in vivo* [17–19].

In the present study, we investigated the effects of ME3738 on HCV replication both *in vitro* and *in vivo* using the genotype 1b HCV replicon and HCV-infected human hepatocyte chimeric mice. The results demonstrate that ME3738 itself had an inhibitory effect on HCV replication, and when combined with IFN, ME3738 enhanced the anti-HCV effect of IFN by up-regulation of ISGs, such as oligoadenylate synthetase (*OAS*) *1*, myxovirus resistance protein A (*MxA*), and *ISG15* in HCV replicon cells. We also showed that the combination therapy increased *OAS1*, RNA-dependent protein kinase (*PKR*) and ubiquitin specific peptidase 18 (*USP18*) expression levels, and reduced virus levels effectively without liver cell damage in human hepatocyte chimeric mice.

Material and methods

Cell culture

Cells supporting replication of the genotype 1b-derived subgenomic HCV replicon, ORN/3-5B/KE cells [20] (kindly provided by N. Kato, Okayama University, Japan) and Con-1 cells [21], were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal bovine serum, non-essential amino acids, glutamine, penicillin, and streptomycin (complete DMEM) in the presence of G418 (300 μg/ml; Geneticin, Invitrogen, Carlsbad, CA). ORN/3-5B/KE and Con1 replicon cells (2×10^4) were seeded onto 12-well plates and incubated for 3 days with or without ME3738 (Meiji Seika Kaisha, Tokyo, Japan) [9], human IFN-α (Dainippon Sumitomo Pharma Co., Tokyo), or the combination of both drugs.

Quantitation of HCV RNA and ISG mRNAs

RNA extraction and quantitation of HCV by real-time polymerase chain reaction (PCR) were performed as described previously [19]. Briefly, RNA was extracted from mice serum, livers, or cellular lysate using SepaGene RVR (Sankojunyaku, Tokyo, Japan) and reverse transcribed with a random hexamer and a reverse transcriptase (ReverTraAce; TOYOBO, Osaka, Japan) according to the instructions provided by the manufacturer. Quantitation of HCV RNA was performed using the Real-Time PCR system (Applied Biosystems, Foster City, CA). The primers used for amplification were 5′-GAGTGTCGTGCAGCCTCCA-3′ and 5′-CACTCGCAAGCAC CCTATCA-3′. Quantitation of ISGs (*OAS1*, *MxA*, *PKR*, *USP18* and *ISG15*) was performed using real-time PCR Master Mix (TOYOBO) and TaqMan Gene Expression Assay primer and probe sets (PE Applied Biosystems, Foster City, CA). Thermal cycling conditions were as follows: a precycling period of 1 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Each ISG expression level was expressed relative to the endogenous RNA levels of the housekeeping reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Luciferase reporter assay

After 72 h of IFN and/or ME3738 treatment, ORN/3-5B/KE cells were harvested with Renilla lysis reagent (Promega, Madison, WI) and subjected to the luciferase assay according to the manufacturer's protocol.

Western blotting

The cells were ruptured with 250 μl lysis buffer [10 mM Tris/HCl pH 7.4, 140 mM NaCl and 0.5% (v/v) NP-40] followed by centrifugation for 2 min at 15,000*g*. Cell lysates were subjected to Western blotting using antibodies against NS3 (Novocastra Laboratories, UK) and β-actin (Sigma, Tokyo, Japana) as described previously [22].

WST assay

Cell viability was determined by employing tetrazolium salt, WST-8, using the WST-8 Cell Proliferation Assay Kit (Dojindo Laboratories., Kumamoto, Japan), according to the instructions provided by the manufacturer.

Human serum samples

Human serum samples containing high titers of genotype 1b HCV (2.2×10^6 copies/ml) were obtained from a patient with chronic hepatitis after obtaining written informed consent. Aliquots were stored in liquid nitrogen until use.

Animal treatment

All animal protocols in this study were in accordance with the guidelines of the local committee for animal experiments and under approval of the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University. We transplanted human hepatocytes into $uPA^{+/+}/SCID^{+/+}$ mice as described previously [16]. All mice used in this study were transplanted with frozen human hepatocytes obtained from the same donor. Mice were injected intravenously with 50 μl of HCV-positive human serum samples. Six weeks after HCV infection, mice were fed a normal chow containing 0.15% (w/w) ME3738 for 4 weeks, with or without IFN-α. IFN-α-treatment was provided daily by intramuscular injection of diluted IFN solution. Serum samples were collected every week, and human serum albumin (HSA) concentration and HCV RNA were measured. Mouse serum concentrations of HSA, which correlate with the repopulation rates, were measured as described previously [16]. Serum ME3738 concentrations were measured by liquid chromatography/mass spectrometry/mass spectrometry. After the fourth week of treatment, mice were sacrificed, and livers were either fixed with 4% buffered-paraformaldehyde for histological examination or frozen immediately in liquid nitrogen to measure HCV core antigen. To investigate the expression of ISGs in mouse livers, mice were kept for 1 week with or without 0.45% (w/w) ME3738 and then given a single injection of 1500 IU/g IFN-α. Four hours after injection, mice were sacrificed and liver samples were collected.

Quantitation of HCV core antigen in the mouse liver

Livers were homogenized in phosphate-buffered saline with 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate. The homogenates were centrifuged at 20,000*g* for 30 min. HCV core antigen levels in the supernatant of liver homogenates were measured using enzyme immunoassay as described previously [23].

Statistical analysis

All data are expressed as mean ± SD. Levels of HCV RNA and ISG mRNAs were compared using the Mann–Whitney *U*-test. A p value less than 0.05 was considered statistically significant. All statistical analyses were performed with SPSS 14.0 software (SPSS, Tokyo, Japan).

Results

Antiviral activity of ME3738 on HCV subgenomic replicon

The effect of ME3738 on HCV replication was analyzed *in vitro* using subgenomic HCV replicon cells possessing the luciferase reporter. ORN/3-5B/KE cells were treated with either IFN-α or ME3738 for 72 h. The luciferase reporter assay demonstrated that the HCV RNA replication level decreased depending on the

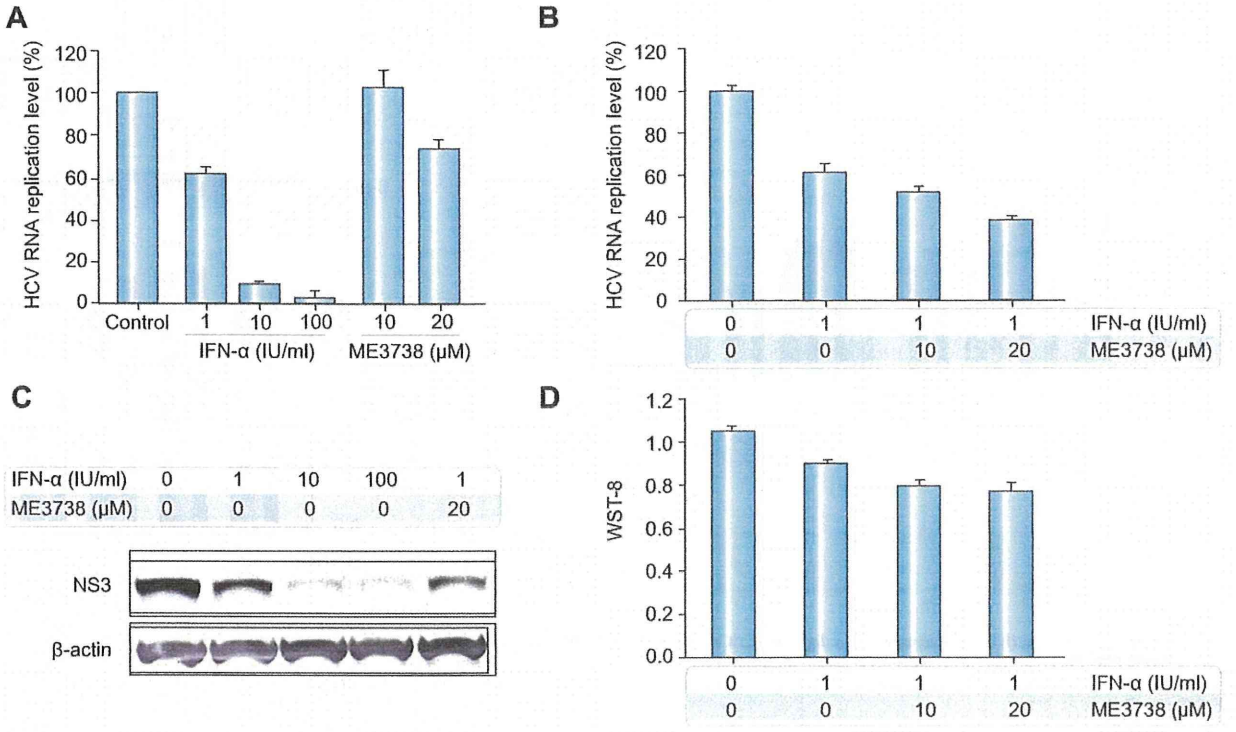

Fig. 1. Effects of ME3738 on HCV replication in the subgenomic HCV replicon, ORN/3-5B/KE cells. ORN/3-5B/KE cells were treated for 72 h with the indicated concentration of interferon (IFN)-α alone, ME3738 alone, or IFN-α plus ME3738. (A and B) Intracellular HCV RNA replication levels were determined as luciferase activity and expressed relative to cellular viability. (C) Cell lysates were analyzed by immunoblotting with antibodies to NS3 and β-actin. (D) Cellular viability was analyzed by WST assay. Data are represented as the mean ± SD of 6 experiments. Control: cells treated with neither ME3738 nor IFN-α.

IFN-treatment dose as reported previously (Fig. 1A) [20]. Treatment with 20 μM of ME3738 also reduced HCV RNA replication. Next, we investigated whether ME3738 enhances the effect of IFN-α. IFN-α (1 IU/ml) plus ME3738 inhibited the HCV RNA replication dose in a dependent manner with ME3738 (Fig. 1B). The level of cellular HCV NS3 protein was reduced depending on IFN-α-treatment and was reduced effectively by IFN-α/ME3738 combination treatment (Fig. 1C). The viability of cells treated with IFN-α/ME3738 combination treatment was lower than that of the control treatment and almost the same as with IFN-α treatment alone (Fig. 1D).

The effect of ME3738 was also tested in a different replicon system, Con-1 cells. ME3738 reduced HCV RNA replication dose dependently in Con-1 cells (Fig. 2A). Similar to ORN/3-5B/KE cells, IFN-α (1 IU/ml) plus ME3738 inhibited HCV RNA replication dose in a dependent manner with ME3738 (Fig. 2A), and the level of cellular HCV NS3 protein was reduced effectively by IFN-α/ME3738 combination treatment (Fig. 2B). The viability of cells treated with IFN-α/ME3738 combination treatment was lower but was not significant with IFN-α treatment alone (Fig. 2C). These results indicate that ME3738 itself has an inhibitory effect on HCV replication and enhances the effect of IFN-α.

Expression of ISGs in ME3738-treated replicon cells

We measured the levels of ISGs in drug-treated ORN/3-5B/KE cells and Con1 cells. IFN-α treatment significantly increased the expression levels of *OAS1*, *MxA*, *PKR*, *USP18* and *ISG15*, which reached maximum levels at 24 h in ORN/3-5B/KE cells (Fig. 3A) and 8 h in Con1 cells (Fig. 3B). ME3738 treatment alone significantly increased the expression of *OAS1* in both cells. IFN-α treatment significantly increased the expression of ISGs; however, IFN-α/ME3738 combination treatment significantly induced the expressions of *OA1S*, *MxA* and *ISG15* to levels higher than IFN-α alone in both cells. These results indicate that ME3738 enhances the effect of IFN-α to increase ISG expression, and this effect may contribute to the inhibition of HCV replication.

Effect of ME3738 on HCV replication in vivo

To further analyze the effects of ME3738, we used genotype 1b HCV-infected human hepatocyte chimeric mice [17,19]. Six weeks after HCV infection, when the mice developed stable viremia (10^6–10^7 copies/ml, data not shown), the animals were treated with ME3738 alone, IFN-α alone, or ME3738/IFN-α for 4 weeks (Fig. 4A). Mouse serum concentrations of ME3738 increased in ME3738- and ME3738 plus IFN-α-treated mice (Table 1). ME3738 alone did not reduce the levels of HCV RNA in mice, while IFN-α-treatment reduced the HCV RNA levels, as reported previously [17]. ME3738 plus IFN-α-treatment significantly reduced HCV to levels lower than that achieved by ME3738 or IFN-α alone. We also measured the HCV core protein level in the livers of treated mice. As shown by replicon experiments, core protein levels were reduced most effectively by the ME3738/IFN-α-combination therapy (Fig. 4B). Since the level of HSA did not decrease in these treatments, it was concluded that

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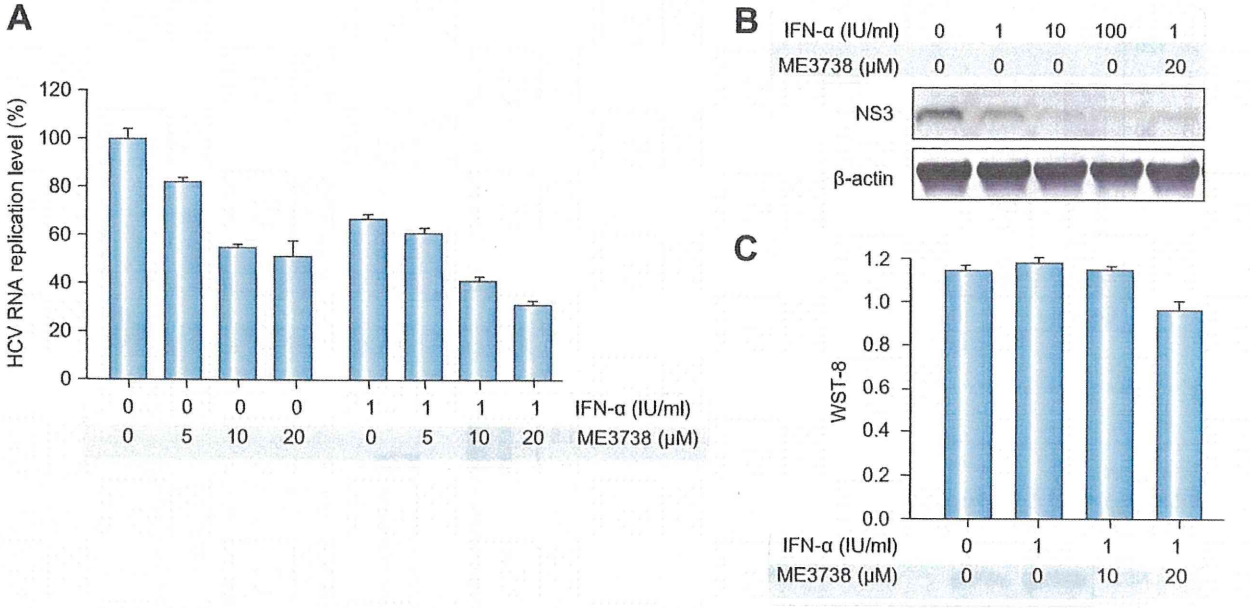

Fig. 2. Effects of ME3738 on HCV replication in the subgenomic HCV replicon, Con1 cells. Con1 cells were treated for 72 h with the indicated concentration of ME3738 alone or IFN-α plus ME3738. (A) Intracellular HCV RNA replication levels were determined via real-time PCR. (B) Cell lysates were analyzed by immunoblotting with antibodies to NS3 and β-actin. (C) Cellular viability was analyzed by WST assay. Data are represented as the mean ± SD of 6 experiments. Control: cells treated with neither ME3738 nor IFN-α.

the reduction of HCV in chimeric mice was not due to toxicity of the drugs (Fig. 4A). This was also supported by histopathological findings, including lack of cytotoxic changes in the livers of all four groups of mice (Fig. 4C). The effect of ME3738 to increase ISG expression was assessed in mouse liver following treatment with a high concentration of ME3738 for 1 week and a single injection of IFN-α. ME3738 alone showed no increase in the expression of ISGs in mouse livers (Fig. 5). IFN-α treatment significantly increased the expression of ISGs; however, IFN-α/ME3738 combination treatment significantly induced the expressions of *OAS1*, *PKR* and *USP18* mRNA levels in mouse livers to levels higher than IFN-α alone. These results indicate that ME3738 inhibits HCV replication, enhancing the effect of IFN-α to increase ISG expression *in vivo*.

Discussion

Although the treatment outcome of chronic HCV infection has improved with the advent of pegylated IFN-α and ribavirin, the eradication rate of HCV is only about 50%. Many patients are unable to receive this therapy because of the harmful side effects or the financial costs. Development of effective, safe and inexpensive therapies should be encouraged.

ME3738 is reported to attenuate various liver pathologies in animals [8–12]. Furthermore, Hiasa et al. reported recently that ME3738 induces IFN-β mRNA expression and inhibits the replication of HCV [13]. We thus attempted in this study to evaluate the effect of ME3738, especially in combination with IFN-α, on HCV.

The results of the present study show that ME3738 induced the gene expression of *OAS* (Fig. 2) and inhibited HCV replication (Fig. 1A). Hiasa et al. reported that ME3738 enhanced the expression of *IFN-β* mRNA and that the enhanced production of IFN-β resulted in the increased expression of ISGs [13]. They showed also that the effect of ME3738 on HCV was abolished following the inhibition of IFN-β expression with siRNA or antibody. Our results are consistent with their findings. The extent of the increase in ISG expression was smaller in Hiasa et al. [13] than in our results. This is probably because they used the T7-genotype 1a-cDNA transient transfection-infection system to produce HCV in HepG2 or Huh7 cells [13,24,25] and assessed the effect of ME3738 by utilizing naturally produced IFN-β. The amount of IFN is likely to be very small in their system compared to that used in our study. We also tried to detect *IFN-β* mRNA in our replicon system but were unable to detect it in our replicon cells (Huh7 based ORN/3-5B/KE cells and Con1 cells). This is probably due to a defect of the innate immune system in producing IFN-β in those cells. This is consistent with their finding that ME3738 had an inferior effect in Huh7 cells than in HepG2 cells to produce ISG products [13].

As we showed in this study, ME3738 enhances the effect of IFN against HCV replication both *in vitro* (Figs. 1B and 2A) and *in vivo* (Figs. 4A and 4B). ME3738 enhanced the effect of IFN-α by increasing the expression levels of ISGs both *in vitro* (Fig. 3) and *in vivo* (Fig. 5). How ME3738 enhances the transcription of ISGs is unknown at this stage. ME3738 was reported initially to protect liver cells against injury through induction of IL-6 [8,9]. IL-6 is reported to provide protection to certain cells [26–28] by preventing apoptosis. In the present study, we tried to detect IL-6 protein in the serum and mRNA in the liver of ME3738-treated mice. However, the levels of both were too low to measure. Further studies should be conducted to elucidate the mechanism by which ME3738 enhances immunity against viral infections.

Our results showed that ME3738 did not reduce cell viability. We also showed that the drug is not hepatotoxic, as inferred by HSA level and liver histology. Since ME3738 is reported to

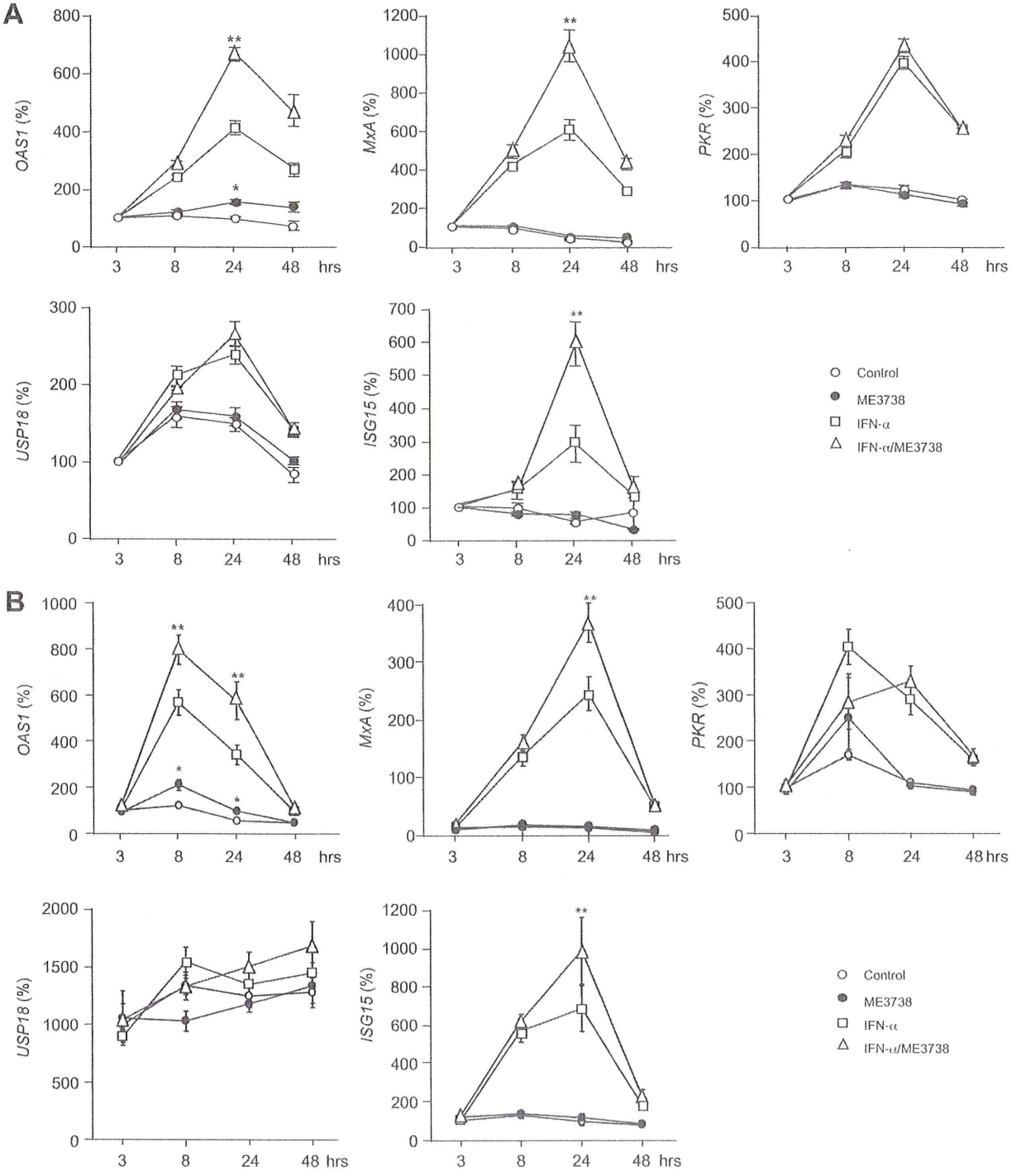

Fig. 3. Effects of ME3738 on the expression of interferon-stimulated genes. ORN/C-5B/KE cells (A) and Con1 cells (B) were treated with 20 μM of ME3738 and/or 1 IU/ml of interferon (IFN)-α for 48 h. Intracellular gene expression levels of oligoadenylate synthetase (*OAS*), myxovirus resistance protein A (*MxA*), double stranded RNA-activated protein kinase (*PKR*), *USP-18* and interferon-stimulated gene (*ISG*) *15* were measured at the indicated times. RNA levels were expressed relative to *GAPDH* mRNA. Data are shown as the mean ± SD of 6 experiments. Control: cells treated with neither ME3738 nor IFN-α. (*p <0.05 compared with Control, **p <0.05 compared with IFN-α treatment).

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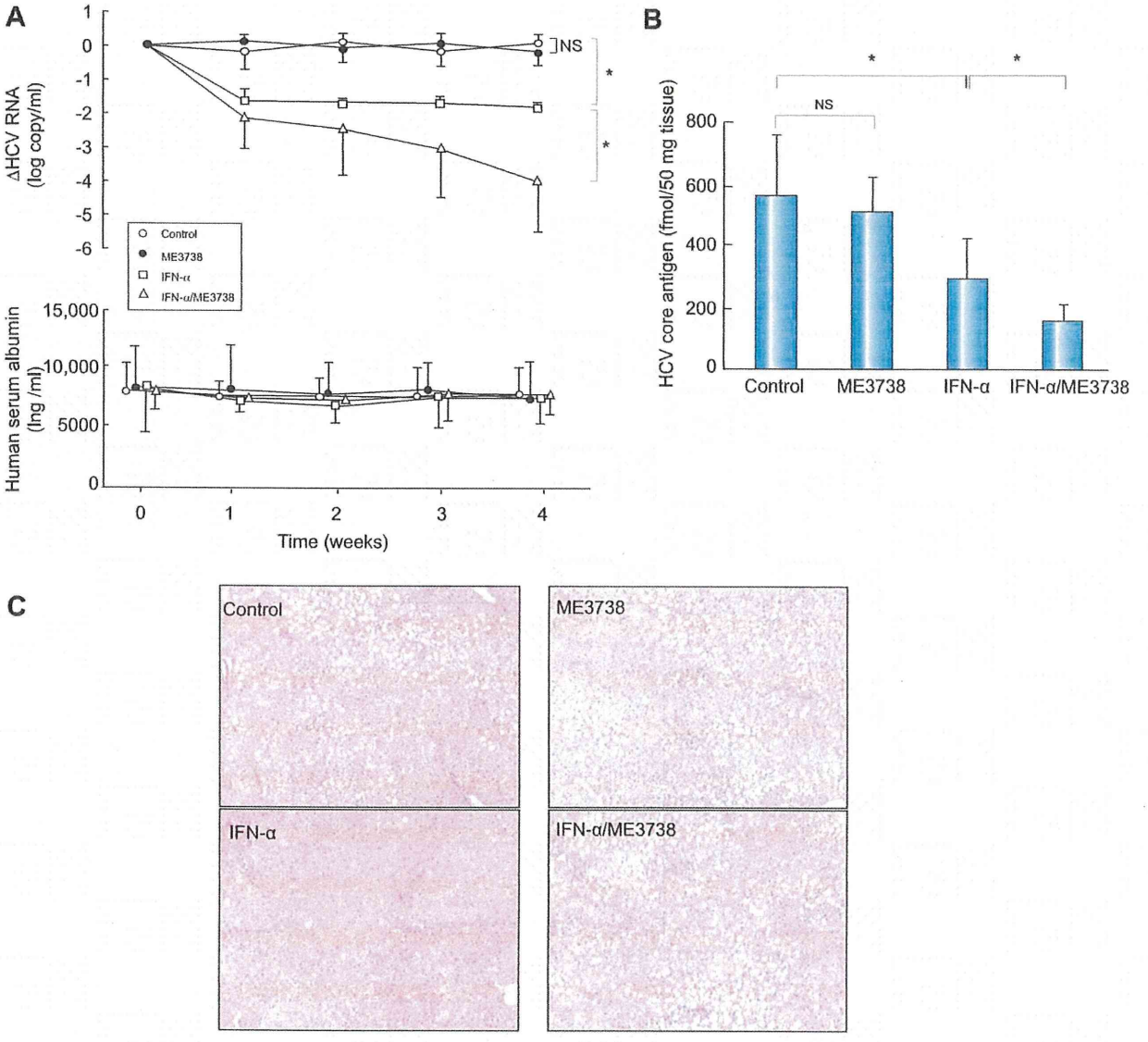

Fig. 4. ME3738 enhances the effect of IFN in mice with HCV infection. Mice were injected intravenously with 50 μl of HCV-positive human serum samples. Six weeks after HCV infection, mice were treated with ME3738 and/or interferon (IFN)-α for 4 weeks. (A) Mouse serum samples were obtained every week, and HCV RNA titer (upper panel) and human serum albumin concentration (lower panel) were analyzed. (B) HCV core antigen was measured in the mouse livers after 4 weeks of treatment. Data are mean ± SD of 6 mice. (*p <0.05; **p <0.01; NS, not significant). (C) Liver samples obtained from mice were stained with hematoxylin-eosin (Original magnification, 100×). Note the lack of specific changes in the mice of each group. Control: HCV-infected mice treated with neither ME3738 nor IFN-α.

Table 1. Concentrations of ME3738 in mouse serum samples.

	Control	ME3738	IFN-α	ME3738/ IFN-α
ME3738 (μM)	<0.01	4.02 ± 0.90	<0.01	2.44 ± 0.21

Concentrations of ME3738 in serum samples obtained from mice after 4 weeks of treatment were measured by liquid chromatography/mass spectrometry/mass spectrometry. Data are shown as mean ± SD of three mice. Control: HCV-infected mice treated with neither ME3738 nor IFN-α.

attenuate liver disease in several animal models of acute and chronic liver injury [8–12], the drug could be suitable for treatment of patients with chronic hepatitis C. In the current regimen of PEG-IFN and ribavirin combination therapy, IFN reduces the replication rate of the virus by inducing expression of ISGs in liver cells. Ribavirin enhances the effect of IFN synergistically through an unknown mechanism. ME3738 also enhances the effect of IFN similarly to ribavirin and may protect liver cells from apoptosis. Combination therapy using these three drugs might yield excellent anti-viral and anti-inflammatory effects. Alternatively, ME3738 could be used instead of ribavirin if the drug shows a superior effect in combination with IFN. Further animal and human studies should be conducted to develop an effective regimen for the treatment of patients with chronic hepatitis C.

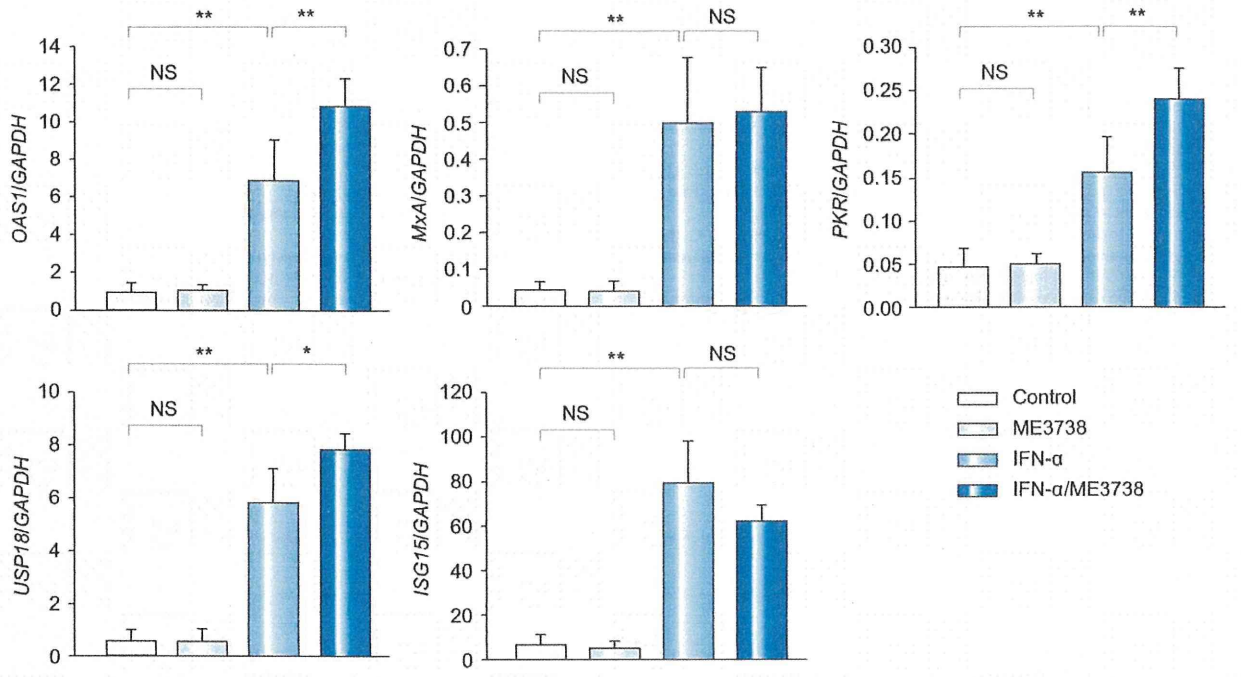

Fig. 5. Interferon-stimulated gene expression in mouse liver samples. Mice were treated with or without 0.45% (w/w) ME3738 for 1 week and then given a single injection of 1500 IU/g IFN-α. Four hours after IFN-α injection, interferon stimulated gene expression in mouse livers was measured. RNA levels are expressed relative to *GAPDH* mRNA. Data are presented as mean ± SD of six mice. Control: Mice treated with neither ME3738 nor IFN-α. (*p <0.05; **p <0.01; NS, not significant).

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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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

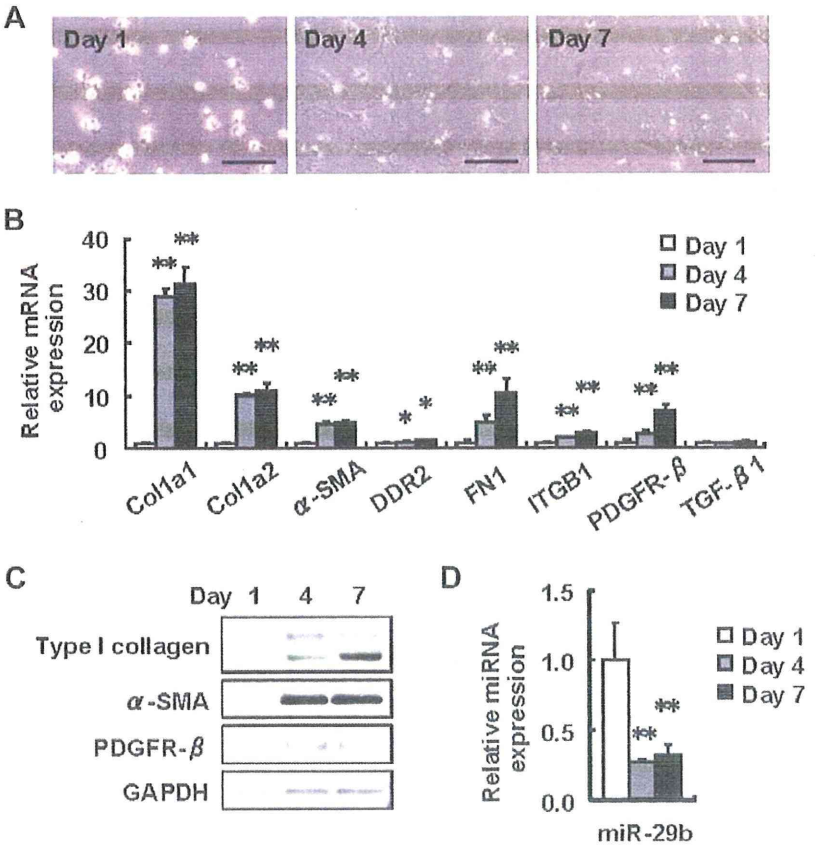

Fig. 1. Expression of miR-29b in mouse primary HSCs during culture. HSCs were isolated from mouse liver (Day 0) and cultured for the indicated periods. (A) Phase-contrast microscopy. Scale bar, 200 μm. (B) mRNA expression levels of Col1a1, Col1a2, α-SMA, DDR2, FN1, ITGB1, PDGFR-β and TGF-β1 were analyzed 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.

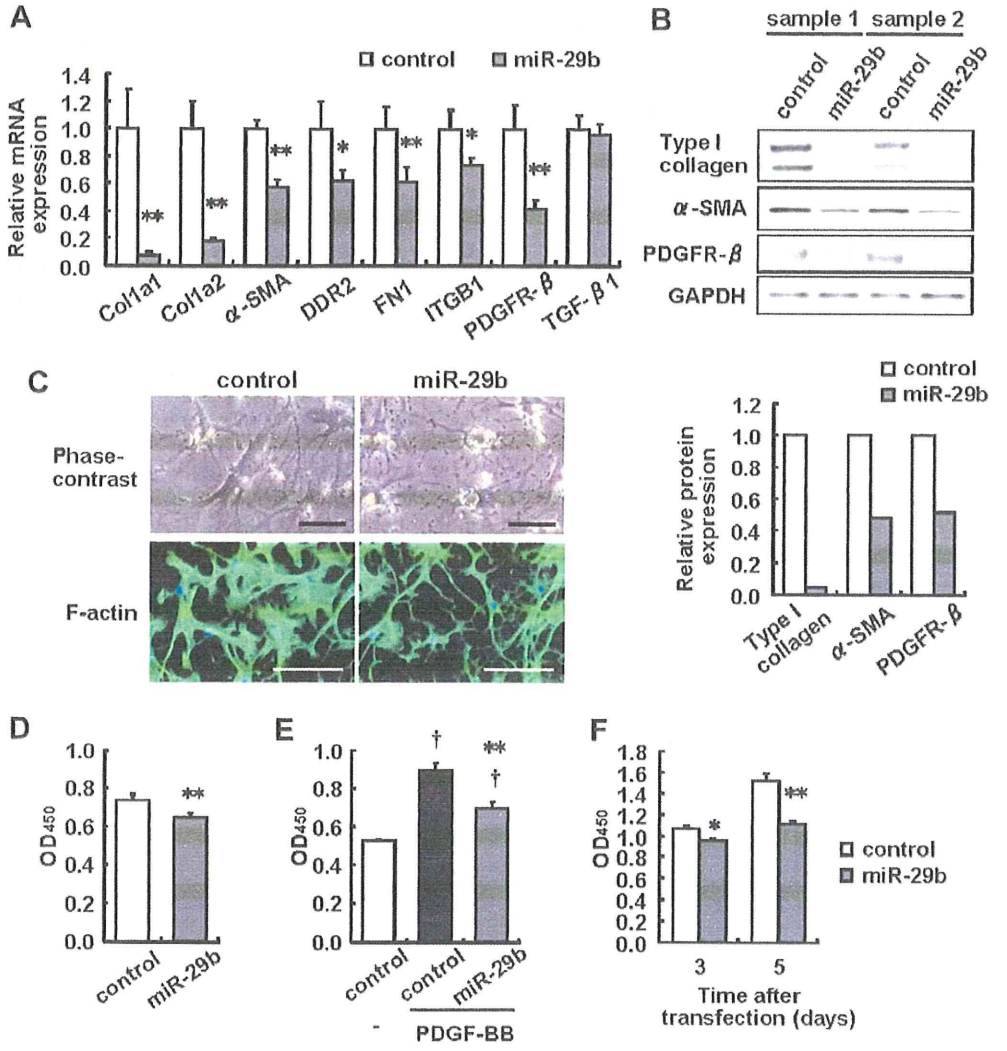

Fig. 2. Effects of miR-29b overexpression on the activation of HSCs. (A–D) Mouse HSCs were transfected with 10 nM miR-29b precursor or a negative control (control) on Day 1 and incubated for 3 days. (A) mRNA expression levels of Col1a1, Col1a2, α-SMA, DDR2, FN1, ITGB1, PDGFR-β and TGF-β1 were analyzed 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

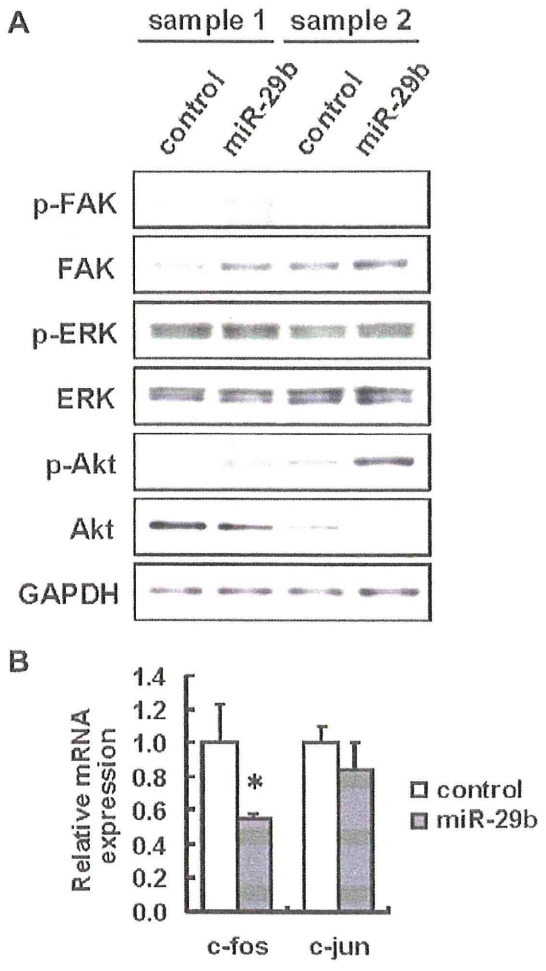

Fig. 3. Effects of miR-29b overexpression on the ECM- and growth factor-related signaling in mouse primary HSCs. Mouse HSCs were transfected with 10 nM miR-29b precursor or a negative control (control) on Day 1 and were incubated for 3 days. (A) Phosphorylation of FAK (Y397), ERK (T202/Y204) and Akt (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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Journal of Cellular Physiology

Down-Regulation of Cyclin E1 Expression by MicroRNA-195 Accounts for Interferon-β-Induced Inhibition of Hepatic Stellate Cell Proliferation

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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.

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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 (Friedman, 2000; Bataller and Brenner, 2001). 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 (Mallat et al., 1995; Fort et al., 1998; Shen et al., 2002; Inagaki et al., 2003; Chang et al., 2005; Tanabe et al., 2007; Ogawa et al., 2009), 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; microRNA-122 (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,b). 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

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