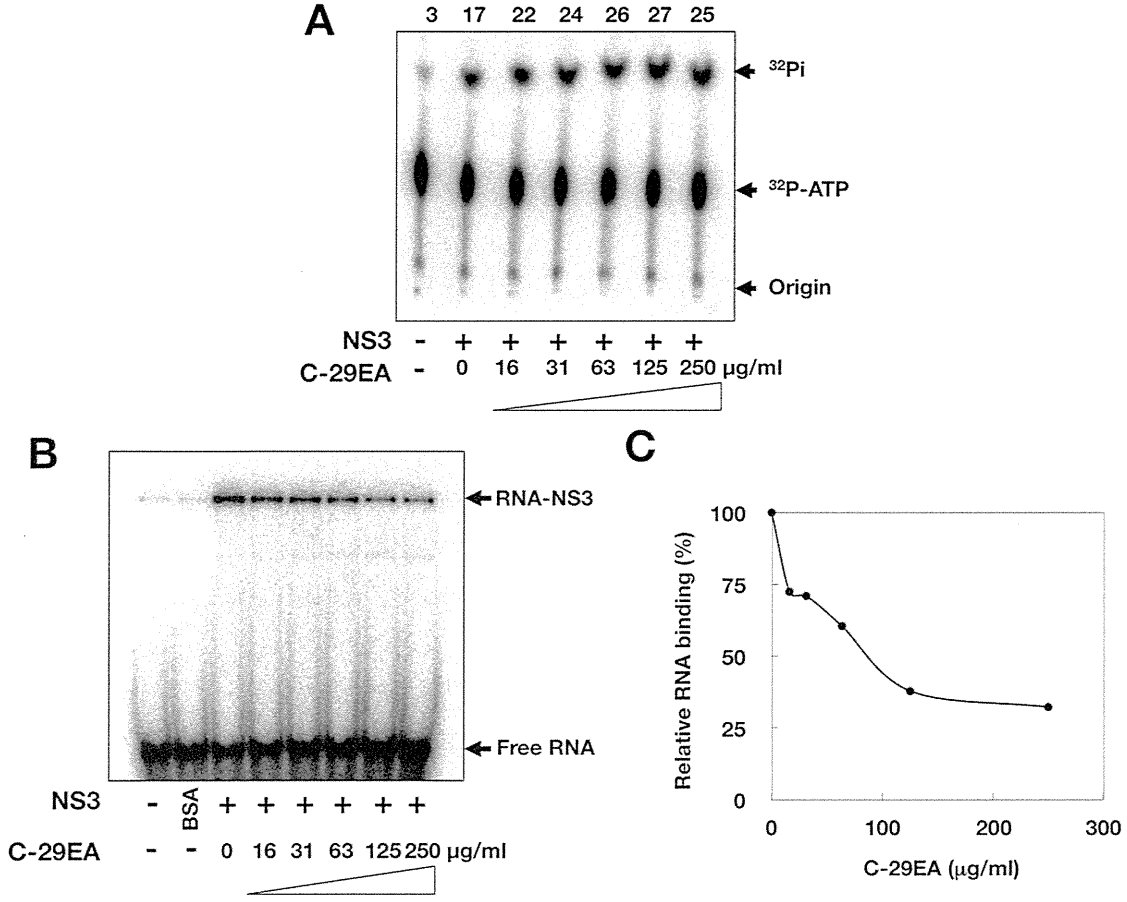

Figure 6. Effect of C-29EA on ATPase and RNA-binding activities of NS3 helicase. (A) The reaction mixtures were incubated with [γ-^{32}P] ATP as described in Materials and Methods. The reaction mixtures were subjected to thin-layer chromatography. The start positions and migrated positions of ATP and free phosphoric acid are indicated as 'Origin', '^{32}P-ATP', and '^{32}P-Pi', respectively, on the right side of the figure. The data represent three independent experiments. Treatment with DMSO corresponds to '0'. (B) Gel mobility shift assay for RNA-binding activity of NS3 helicase. The reaction was carried out with 0.5 nM labeled ssRNA at the indicated concentrations of C-29EA or DMSO. The reaction mixture was subjected to gel mobility shift assay. (C) The relative RNA-binding ability was calculated with band densities in each lane and presented as a percentage of RNA-NS3 in the total density. The data represent three independent experiments. Treatment with DMSO corresponds to '0'.
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HCV NS3 is well known to play a crucial role in viral replication through helicase and protease activities [5,39]. The N-terminal third of NS3 is responsible for serine protease activity in order to process the C-terminal portion of polyprotein containing viral nonstructural proteins [32]. The remaining portion of NS3 exhibits ATPase and RNA-binding activities responsible for helicase activity, which is involved in unwinding double-stranded RNA during replication of genomic viral RNA [40–42]. A negative-strand RNA is synthesized based on a viral genome (positive strand) after viral particles in the infected cells are uncoated, and is then used itself as a template to synthesize a positive-stranded RNA, which is translated or packaged into viral particles. Thus, both helicase and protease activities of NS3 are critical for HCV replication and could be targeted for the development of antiviral agents against HCV.

NS3 helicase activity was inhibited by treatment with C-29EA in a dose-dependent manner with an IC_{50} of 18.9 μg/ml (Fig. 5A). RNA-binding activity, but not ATPase activity, was inhibited by treatment with C-29EA (Fig. 6). Treatment with C-29EA did not significantly affect the HCV-IRES activity and did not induce interferon-stimulated gene 2′,5′-OAS (Figs. 3 and 4). Furthermore, the serine protease activity of NS3 was inhibited by using C-29EA with an IC_{50} of 10.9 μg/ml (Fig. 7). These results suggest that *Amphimedon* sp. includes the unknown compound(s) that could suppress NS3 enzymatic activity to inhibit HCV replication. Although the mechanism by which treatment with C-29EA could inhibit HCV replication has not yet been revealed, the unknown compound(s) may be associated with the inhibition of NS3 protease and helicase, leading to the suppression of HCV replication. However, other effects of extract C-29EA on HCV replication could not be excluded in this study.

The compound 1-N, 4-N-bis [4-(1H-benzimidazol-2-yl)phenyl] benzene-1,4-dicarboxamide, which is designated as $(BIP)_2B$, was reported to be a potent and selective inhibitor of HCV NS3 helicase [43]. This compound competitively decreases the binding ability of HCV NS3 helicase to nucleic acids. The compound $(BIP)_2B$ inhibited RNA-induced stimulation of ATPase, although it did not directly affect the ATP hydrolysis activity of NS3 helicase. Thus, $(BIP)_2B$ could not affect ATPase activity without RNA or with a high concentration of RNA. Treatment with C-29EA inhibited helicase activity and viral replication but not ATPase activity (Figs. 1B, 2, 5, and 6). This extract suppressed the binding of RNA to helicase but exhibited no suppression of ATPase by NS3 helicase. Thus, the inhibitory action of extract C-29EA seems

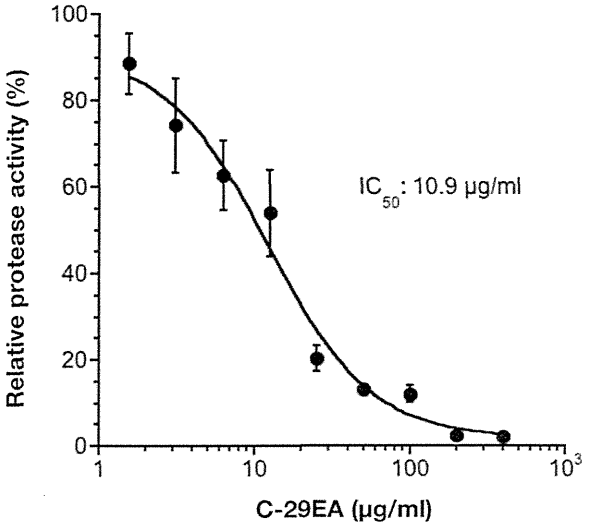

Figure 7. Effect of C-29EA on the activity of NS3 serine protease. NS3/4A serine protease was mixed with various concentrations of C-29EA or DMSO (0) in the reaction mixture and then incubated at 37°C for 120 min. The initial velocity at each concentration of C-29EA was calculated during 120 min reaction. The initial velocity in the absence of C-29EA was defined as 100% of relative protease activity. The data are presented as the mean ± standard deviation for three replicates.
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different from that of $(BIP)_2B$. The quinolone derivative QU663 was reported to inhibit the unwinding activity of NS3 helicase by binding to an RNA-binding groove irrespective of its own ATPase activity [44]. The compound QU663 may competitively bind the RNA-binding site of NS3 but not affect ATPase activity, resulting in the inhibition of unwinding activity. In this study, treatment with C-29EA inhibited the RNA-binding activities of NS3 helicase but did not affect ATPase activity (Fig. 6). Furthermore, treatment with C-29EA suppressed the viral replication of HCV in an HCV cell culture system derived from several virus strains (Figs. 1 and 2, Table 2). The mechanism of C-29EA on the inhibition of NS3 helicase may be similar to that of compound QU663.

It is unknown whether one or several molecules included in C-29EA are critical for the inhibition of protease and helicase activities. The serine protease NS3/4A is one of the viral factors targeted for development into antiviral agents. Improvements in HCV therapy over the past several years have resulted in FDA approval of telaprevir (VX-950) [15,45] and boceprevir (SCH503034) [46,47]. Several studies suggest that the activities of NS3/4A protease and helicase in the full-length molecule enhance each other [48,49]. The NS3/4A protease has formed a complex with macrocyclic acylsulfonamide inhibitors [50,51]. Schiering et al. recently reported the structure of full-length NS3/4A in complex with a macrocyclic acylsulfonamide protease inhibitor [52], although the structure of full-length HCV NS3/4A in complex with a protease inhibitor has not been reported. The inhibitor binds to the active site of the protease, while the P4-capping and P2 moieties of the inhibitor are exposed toward the helicase interface and interact with both protease and helicase residues [52]. An unknown compound included in C-29EA might interact with both protease and helicase domains of NS3 to inhibit their activities. However, our data in this study have not excluded the possibility that several compounds included in C-29EA are related to the inhibition of protease and helicase of NS3/4A.

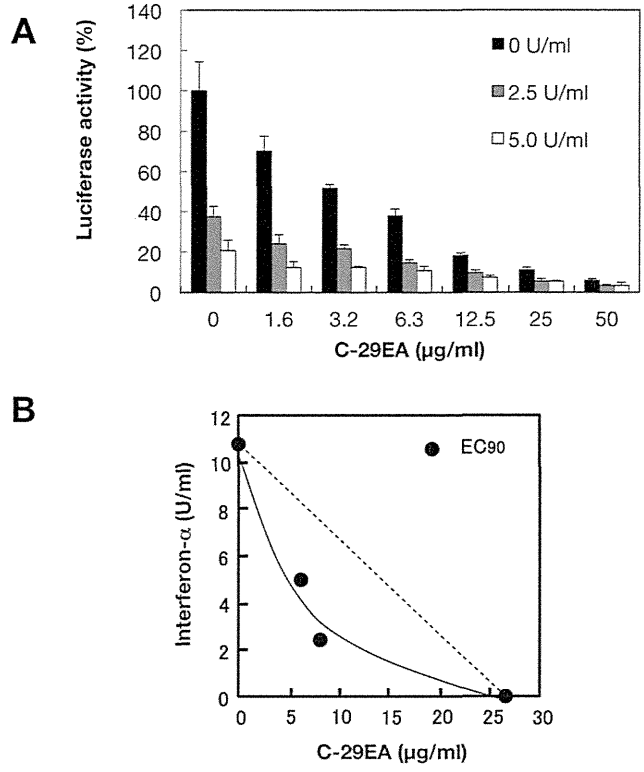

Figure 8. Effect of C-29EA on the antiviral activity of interferon-alpha. (A) The Huh7 cell line, including the subgenomic replicon RNA of genotype 1b strain Con1, was incubated in medium containing various concentrations of C-29EA or DMSO (0) in the presence or the absence of interferon-alpha. Luciferase assay were carried out as described in Materials and Methods. Error bars indicate standard deviation. The data represent three independent experiments. (B) Isobole plots of 90% inhibition of HCV replication. The broken line indicates the additive effect in the isobologram.
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In conclusion, we showed that the EtOAc extract from *Amphimedon* sp. significantly inhibits HCV replication by suppressing viral helicase and protease activities. The purification of an inhibitory compound from the extract of *Amphimedon* sp. will be necessary in order to improve its efficacy by chemical modification.

Materials and Methods

Preparation of Extracts from Marine Organisms

All marine organisms used in this study were hand-collected by scuba diving off islands in Okinawa Prefecture, Japan. No specific permits were required for the described field studies. We do not have to obtain a local government permit to collect invertebrates except for stony corals and marine organisms for fisheries, which we did not collect in this study. The areas where we collected are not privately-owned or protected in any way. We did not collect any invertebrates listed in the red data book issued by Ministry of Environment, Japan. The sponges, tunicates, and soft corals used in this study are not listed at all. Hence, no specific permits are required for this collection in the same way as the previous report of Aratake et al. [53].

The sponge from which C-29EA was extracted was identified as *Amphimedon* sp. and deposited at Naturalis under the code RMNH POR 6100. Each specimen was soaked in acetone. The acetone-extract fraction prepared from each specimen was concentrated.

The resulting material was fractionated as an EtOAc- and water-soluble fraction. The water-soluble fraction was dried up and solubilized in MeOH. The EtOAc- and the MeOH-soluble fractions were used for screening. All samples were dried and then solubilized in dimethyl sulfoxide (DMSO) before testing.

Cell Lines and Virus

The following Huh-7-derived cell lines used in this study were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 0.5 mg/ml G418. The Lunet/Con1 LUN Sb #26 cell line, which harbors the subgenomic replicon RNA of the Con1 strain (genotype 1b), was kindly provided by Ralf Bartenschlager [26]. Huh7/ORN3-5B #24 cell line, which harbors the subgenomic replicon RNA of the O strain (genotype 1b) was reported previously [54] and used for screening in this study (Table 1). HCV replicon cell line derived from genotype 2a strain JFH1 was described previously [55]. The surviving cells were infected with the JFH-1 virus at a multiplicity of infection (moi) of 0.05. The viral RNA derived from the plasmid pJFH1 was transcribed and introduced into Huh7OK1 cells according to the method of Wakita et al. [56]. The infectivity of the JFH1 strain was determined by a focus-forming assay [56].

Quantitative Reverse-transcription PCR (qRT-PCR) and Estimation of Core Protein

The estimation of viral RNA genome was carried out by the method described previously [57] with slight modification. Total RNAs were prepared from cells and culture supernatants by using an RNeasy mini kit (QIAGEN, Tokyo, Japan) and QIAamp Viral RNA mini kit (QIAGEN), respectively. First-strand cDNA was synthesized by using a high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA) with random primers. Each cDNA was estimated by using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Fluorescent signals of SYBR Green were analyzed by using an ABI PRISM 7000 (Applied Biosystems). The HCV internal ribosomal entry site (IRES) region was amplified using the primer pair 5′-GAGTGTCGTGCAGCCTCCA -3′ and 5′- CACTCGCAAG-CACCCTATCA -3′. Expression of HCV core protein was determined by an enzyme-linked immunosorbent assay (ELISA) as described previously [57].

Determination of Luciferase Activity and Cytotoxicity in HCV Replicon Cells

HCV replicon cells were seeded at 2×10^4 cells per well in a 48-well plate 24 h before treatment. C-29EA was added to the culture medium at various concentrations. The treated cells were harvested 72 h post-treatment and lysed in cell culture lysis reagent (Promega, Madison, WI, USA) or *Renilla* luciferase assay lysis buffer (Promega). Luciferase activity in the harvested cells was estimated with a luciferase assay system (Promega) or a *Renilla* luciferase assay system (Promega). The resulting luminescence was detected by the Luminescencer-JNR AB-2100 (ATTO, Tokyo, Japan) and corresponded to the expression level of the HCV replicon. Cell viability was measured by a dimethylthiazol carboxymethoxy-phenylsulfophenyl tetrazolium (MTS) assay using a CellTiter 96 aqueous one-solution cell proliferation assay kit (Promega).

Effects on Activities of Internal Ribosome Entry Site (IRES)

Huh7 cells were transfected with pEF.Rluc.HCV.IRES.Feo or pEF.Rluc.EMCV.IRES.Feo and then were established in medium containing 0.25 mg/ml G418, as described previously [58]. These cell lines were seeded at 2×10^4 cells per well in a 48-well plate 24 h before treatment, treated with 15 μg/ml extract C-29EA, and then harvested at 72 h post-treatment. The firefly luciferase activities were measured with a luciferase assay system (Promega). The total protein concentration was measured using the BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL, USA) to normalize luciferase activity.

Western Blotting and Reverse-transcription Polymerase Chain Reaction (RT-PCR)

Western blotting was carried out by a method described previously [30]. The antibodies to NS3 (clone 8G-2, mouse monoclonal, Abcam, Cambridge, UK), NS5A (clone 256-A, mouse monoclonal, ViroGen, Watertown, MA, USA), and beta-actin were purchased from Cell Signaling Technology (rabbit polyclonal, Danvers, MA, USA) and were used as the primary antibodies in this study. RT-PCR was carried out by a method described previously [30,58].

Assays for RNA Helicase, ATPase, and RNA-binding Activities

A continuous fluorescence assay based on photoinduced electron transfer (PET) was described previously [29] and was slightly modified with regard to the reaction mixture [30]. The NS3 RNA unwinding assay was carried out by the method of Gallinari et al. [59] with slight modifications [30]. NS3 ATPase activity was determined by the method of Gallinari et al. [59] with slight modifications [30]. RNA binding to NS3 helicase was analyzed by a gel mobility shift assay [30,31]. The gene encoding NS3 helicase was amplified from the viral genome of genotype 1b and was introduced into a plasmid for the expression of a recombinant protein [38,60]. The radioactive band was visualized with the Image Reader FLA-9000 and quantified by Multi Gauge V 3.11 software.

NS3 Protease Assay

The fluorescence NS3 serine protease assay based on fluorescence resonance energy transfer (FRET) was carried out by the modified method using the SensoLyte^{TM} 520 HCV protease assay kit (AnaSpec, Fremont, CA, USA). In brief, NS3 protein with a two-fold excess of NS4A cofactor peptide (Pep4AK) was prepared in 1× assay buffer provided with the kit. HCV NS3/4A protease was mixed with increasing concentrations of C-29EA and incubated at 37°C for 15 min. The reaction was started by adding the 5-FAM/QXL 520 substrate to the reaction mixture containing 180 nM HCV NS3/4A protease and various concentrations (0–400 μg/ml) of C-29EA. The resulting mixture (20 μl) was incubated at 37°C for 120 min using a LightCycler 1.5 (Roche Diagnostics, Basel, Switzerland). The fluorescence intensity was recorded every minute for 120 min. The NS3 serine protease activity was calculated as the initial reaction velocity and presented as a percentage of relative activity to that of the control examined with DMSO solvent but not C-29EA, in the same way as described in the fluorescence helicase assay [29].

Analysis of Drug-drug Interaction

The effects of drug combinations were evaluated using the isobologram method [33]. Various doses of C-29EA and interferon-alpha on 90% inhibition of HCV replication were combined to generate an isoeffect curve (isobole) to determine drug–drug interaction. Concave, linear, and convex curves exhibit synergy, additivity, and antagonism, respectively.

Statistical Analysis

The results are expressed as the mean ± standard deviation. The significance of differences in the means was determined by Student's *t*-test.

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Author Contributions

Conceived and designed the experiments: MN MT YS ST NA NN AY JT KM. Performed the experiments: YF KAS AF YM OF HT AY. Analyzed the data: MI NK NS SM NE. Wrote the paper: YF AY JT KM. Collected marine organisms: JT. Identified the sponge: NJdV.

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Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Let-7b is a novel regulator of hepatitis C virus replication

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Abstract The non-coding microRNA (miRNA) is involved in the regulation of hepatitis C virus (HCV) infection and offers an alternative target for developing anti-HCV agent. In this study, we aim to identify novel cellular miRNAs that directly target the HCV genome with anti-HCV therapeutic potential. Bioinformatic analyses were performed to unveil liver-abundant miRNAs with predicted target sequences on HCV genome. Various cell-based systems confirmed that let-7b plays a negative role in HCV expression. In particular, let-7b suppressed HCV replicon activity and down-regulated HCV accumulation leading to reduced infectivity of HCVcc. Mutational analysis identified let-7b binding sites at the coding sequences of NS5B and 5′-UTR of HCV genome that were conserved among various HCV genotypes. We further demonstrated that the underlying mechanism for let-7b-mediated suppression of HCV RNA accumulation was not dependent on inhibition of HCV translation. Let-7b and IFNα-2a also elicited a synergistic inhibitory effect on HCV infection. Together, let-7b represents a novel cellular miRNA that targets the HCV genome and elicits anti-HCV activity. This study thereby sheds new insight into understanding the role of host miRNAs in HCV pathogenesis and to developing a potential anti-HCV therapeutic strategy.

Keywords microRNA · Let-7b · HCV

Ju-Chien Cheng and Yung-Ju Yeh contributed equally to this work.

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Abbreviations

miRNA	microRNA
HCV	Hepatitis C virus
MRE	MicroRNA responsive element
IFNα-2a	Peginterferon alpha-2a
IFN	Interferon
LF2000	Lipofectamine 2000
DMEM	Dulbecco's modified Eagle's medium
FITC	Fluorescein isothiocyanate
DAPI	4′,6-diamidino-2-phenylindole

Introduction

Hepatitis C virus (HCV) frequently causes chronic infection, leading to hepatic fibrosis and hepatocellular carcinoma [1]. Due to the lack of viral vaccine, the population affected by HCV infection is increased substantially [2]. With the strong side-effects and the moderate successful rate associated with the first-line interferon (IFN)-based

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treatment [3], development of effective therapeutic regimens is still an emerging focus in the control of HCV infection.

Small molecules such as telaprevir have been developed to alleviate disease progression. However, the infidelity of HCV RNA polymerase constantly causes mutation and genome instability that result in the generation of drug-resistant viral strain [4, 5]. Targeting the host factors with important roles in viral infection offers an alternative strategy for development of anti-HCV regimen [6]. Apart from host proteins, a new class of small non-coding endogenous RNA molecule microRNA (miRNA) has been recently unveiled [7]. Although it is not yet fully clarified, miRNA is involved in various biological functions, including the response to HCV infection [7–10]. For example, miR-122 enhances whereas mir-199a* suppresses HCV replication and viral production [11–13]; interferon β (IFN-β)-mediated attenuation of viral replication is associated with an increase in miRNAs that have predicted target sequences within the HCV genome [14]. In addition, miRNA effectors including Argonaute 2 (Ago2) and DDX6 were found to positively regulate HCV replication [15, 16]. These findings suggest that cellular miRNAs regulate HCV gene expression and play roles in the host response against HCV infection.

In this study, bioinformatic analyses were performed to identify liver miRNAs targeting the HCV genome. Various cellular and viral systems were used to confirm bioinformatic prediction and to investigate the functional effects of the selected miRNAs. Our data reveal for the first time that let-7b is a negative regulator of HCV replication with the effective target sequences located on the 5′-untranslation region (UTR) and NS5B coding region of the HCV genome. The suppressive effect of let-7b on HCV RNA is not through translation inhibition. Besides, let-7b and Peginterferon alpha-2a (IFNα-2a) elicit a synergistic inhibitory effect on HCV infection. The roles of let-7b in the regulation of HCV pathogenesis and in the development of novel anti-HCV therapeutic strategy are discussed.

Materials and methods

Materials

The plasmid pRep-Feo and the replicon cells Huh7/Rep-Feo were obtained from Dr. Naoya Sakamoto (Tokyo Medical and Dental University). The plasmids pFL-J6/JFH, pJ6/JFH(p7-Rlu2A), and pJ6/JFH(p7-Rlu2A)GNN, the ConI replicon cells and Huh7.5 [17, 18], were kindly provided by Professor Charles Rice (The Rockefeller University, NY). The plasmid JC1-Luc2A, which replaced the Rluc gene of pJ6/JFH(p7-Rlu2A) to firefly luciferase (Luc) gene was kindly provided by Professor Robert T. Schooley (University of California San Diego, CA). Pre-miRNA, miRNA inhibitors and negative control for miRNA and miRNA inhibitors were purchased from Ambion (Austin, TX). The pLKO.1-shGFP control plasmid (clone ID: TRCN0000072197) and the two pLKO.1-shHMGA2 plasmids (clone ID: TRCN0000021965 and TRCN0000021968) were purchased from National RNAi Core Facility (Academia Sinica, Taiwan). The Lipofectamine 2000 (LF2000) and RNAiMAX transfection reagents were purchased from Invitrogen (Carlsbad, CA). The anti-HCV NS5A antibody was purchased from Bio-Design (Carmel, NY). The anti-HCV Core antibody was purchased from Affinity BioReagents (Golden, CO). The anti-β-actin antibody was purchased from Sigma (St. Louis, MO). The IFNα-2a was purchased from Roche (Mannheim, Germany). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium (MTS) reduction assay and the luciferase assay reagents were purchased from Promega (Madison, WI).

Identification of liver miRNAs targeting the HCV genome

Bioinformatic strategy for the identification of liver miRNAs targeting the HCV genome is presented in Fig. 1. Briefly, two published miRNAs expression profiles [19, 20] were used to select for liver-abundant miRNAs. The miRNA is defined as liver abundance when the expression level of a specific liver miRNA divided by the average expression level of the total liver miRNAs is greater than one. The 23 miRNAs (Supplementary Table 1) that were identified as liver abundant in the two profiling databases were subject to bioinformatic analyses using miRanda, RNAhybrid, TargetScan, and PITA [21–24] to predict their target sequences on all six HCV genotypes (Supplementary Table 2). According to the calculation of miRanda, a filter was set to select for miRNAs of which the prediction scores for at least four genotypes were higher than 155. These miRNAs were considered as the candidate liver miRNAs targeting the HCV genome.

Cell culture and viability assay

Human hepatoma Huh7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The Huh7.5 cells that represent a Huh7 subline and are highly permissive for HCV replication were maintained in DMEM with 1% non-essential amino acid (NEAA). The ConI cells were cultured in DMEM supplemented with 10% FBS and 750 μg/ml G418. The Huh7/Rep-Feo subgenome replicon cells were maintained in the same medium except that 1%

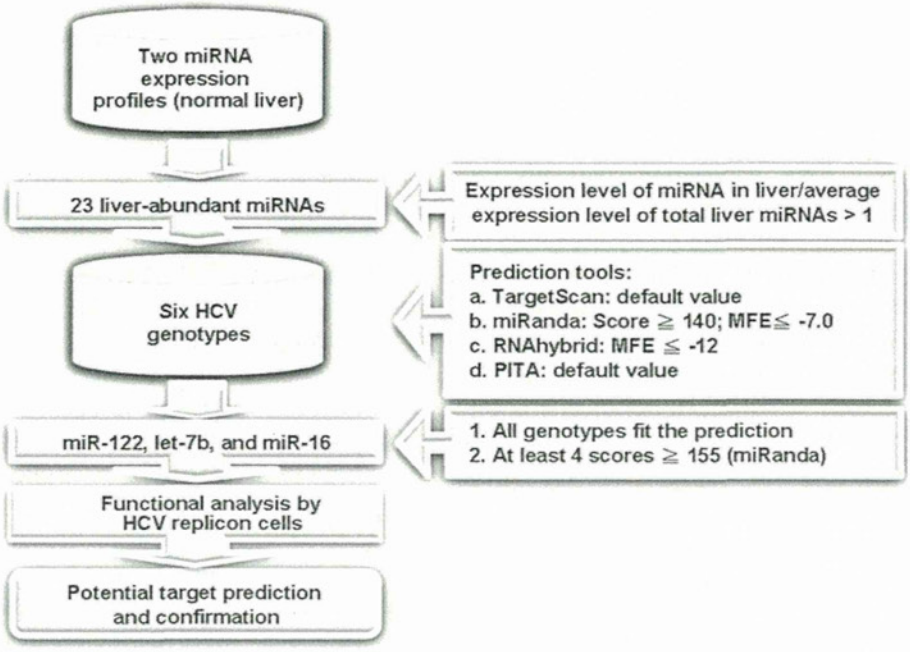

Fig. 1 Bioinformatic strategy for identifying liver miRNAs with target sites on HCV genome. Two published normal liver tissue miRNA expression profiles were used to select liver-abundant miRNAs for bioinformatics analyses as described in "Materials and methods"

NEAA was added and only 250 μg/ml G418 was used [25]. Viable cells were determined by the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit.

Plasmid construction

For luciferase (luc) reporter plasmids, the predicted miRNA responsive element (MRE) for let-7b, as listed in Table 1, was inserted into the *Eco*RI/*Xba*I site downstream of the luciferase gene in phDab2-luc [26] to generate pluc-c-let-7b, pluc-MRE1, pluc-MRE2, pluc-MRE3, and pluc-MRE4. For generation of HCV subgenome mutants with nucleotide mismatch at the ''seed region'' of let-7b or "S1 binding site" of miR-122, site-directed mutagenesis was performed by QuickChange (Stratagene, CA) using pRep-Feo as the template [25] and the primer sets for mMRE1, mMRE2, mMRE3, and miR-122-mut as listed in Table 1. To generate a capped RNA transcript encoding firefly luciferase (FLuc) for use as an internal control in the transient translation assay, the FLuc gene from the plasmid pGL3-Promoter (Madison, WI) was first digested by the restriction enzyme *Nco*I. The nucleotides at the sticky end were filled up as blunt end by Klenow DNA polymerase and the FLuc gene was then excised by the restriction enzyme *Xba*I. On the other hand, pRL-TK plasmid was digested by *Nhe*I and the sticky end was filled up by Klenow DNA polymerase followed by *Xba*I restriction enzyme digestion to remove the Renilla luciferase gene (RLuc). The Fluc gene fragment was then cloned into pRL-TK to replace RLuc to generate pLUC-TK. This plasmid contains a T7 promoter and can transcribe mRNA after linearization by *Bam*HI.

Transient transfection and luciferase activity assay

For transient transfection, Huh7/Rep-Feo cells were seeded at a density of 1×10^4 cells/well for 24 h and the miRNA precursor or inhibitor was transfected into cells by LF2000. At 72 h after transfection, the luciferase activity was quantified using the Bright-Glo luciferase assay reagent. On the other hand, 293T cells were seeded at a density of 2×10^4 cells/well and the reporter plasmid, pRL-TK and miRNA precursor (100 nM) were cotransfected into cells by LF2000. At 24 h after transfection, the firefly and Renilla luciferase activities were quantified using the Dual-Glo luciferase assay reagent.

For RNA transfection, the *Xba*I-digested wild-type pRep-Feo or the mutant subgenome plasmid was subject to in vitro transcription for RNA synthesis. The Huh7.5 cells were transfected with 10 μg HCV RNA, 100 pmol miRNA, and 10 μg pRL-TK by electroporation using Gene Pulser II (Bio-Rad) at 260 V and 950 μF.

For permissive assay, Huh7 cells were seeded at a density of 2×10^5 cells/well and were transfected with the miRNA precursor (100 pmol) by RNAiMAX (Invitrogen) for 24 h. The transfected cells were subsequently infected with HCVcc (6×10^6 copies/ml) for 4 h. After washing away the virus, the cells were cultured for 72 h and the HCV RNA was detected from the infected cells by real-time reverse transcription-PCR (RT-PCR).

Table 1 The sequences for the primers used in this study

Primer name	Primer sequences	Size (mer)
For luciferase reporter constructs[a]		
c-let-7b	S:5′-CTAGA**AACCACACAACCTACTACCTCA**G-3′	22
	AS:5′-AATTC**TGAGGTAGTAGGTTGTGTGGTTT**-3′	
MRE1	S:5′-CTAGA**CACCATGAGCACGAATCCTAAACCTCA**G-3′	27
	AS: 5′-AATTC**TGAGGTTTAGGATTCGTGCTCATGGTG**T-3′	
MRE2	S: 5′-CTAGA**GGCAAAAGGGTGTACTACCTCA**G-3′	22
	AS: 5′-AATTC**TGAGGTAGTACACCCTTTTGCC**T-3′	
MRE3	S: 5′-CTAGA**AGCCACTTGACCTACCTCA**G-3′	19
	AS: 5′-AATTC**TGAGGTAGGTCAAGTGGCT**T-3′	
MRE4	S:5′-CTA**GAGCCGCATGACTGCAGAGAGTGCTGATACTGGCCTCT**G-3′	38
	AS: 5′-AATTC**AGAGGCCAGTATCAGCACTCTCTGCAGTCATGCGGCT-**3′	
For in vitro mutagenesis[b]		
mMRE1	F: 5′-GAGCACGAATCCTAA**TGGAGT**AAGAAAAACCAAAGG-3′	36
	R: 5′-CCTTTGGTTTTTCTT**ACTCCA**TTAGGATTCGTGCTC-3′	
mMRE2	F: 5′-CTGGCAAAAGGGTGTA**TTA**TCTCACTCGCGATCCCAC-3′	47
	R: 5′-GTGGGATCGCGAGUGAG**ATAA**TACACCCTTTTGCCAG-3′	
mMRE3	F: 5′-CATTGAGCCACTTGACCT**TCCG**CAGATCATTGAACGACTC-3′	40
	R: 5′-GAGTCGTTCAATGATCTG**CGGA**AGGTCAAGTGGCTCAATG-3′	
miR-122 mut	F: 5′-CCCGATTGGGGGCGACAC**AG**CACCATAGATCACTCCCC-3′	38
	R: 5′-GGGGAGTGATCTATGGTG**CT**GTGTCGCCCCCAATCGGG-3′	
For synthesis of mature miRNA[c]		
7b	S: 5′UGAGGUAGUAGGUUGUGUGGUU 3′ AS:	22
	5′UUCCACACAACCUACUACCUCA 3′	
m7b	S: 5′UGA**ACUAA**UAGGUUGUGUGGUU 3′ AS:	22
	5′UUCCACACAACCUA**UUAGU**UCA 3′	

S sense strand, *AS* antisense strand *F* forward primer, *R* reverse primer

[a] The *bold letters* indicate the predicted sequence while the other sequence was generated for cloning into *EcoRI*/*Xba*I restriction enzyme site

[b,c] The *bold letters* indicate the mutated nucleotides

Ribonucleoprotein immunoprecipitation (RIP) assay

The RIP assay was performed using the miRNA isolation kit (Wako Laboratory Chemicals, Osaka, Japan) according to the manufacturer's instruction. Briefly, 10 μg of Rep-Feo subgenomic RNA was obtained by in vitro transcription and was co-transfected with 100 pmol of the indicated miRNA into Huh7.5 cells by electroporation. At 6 h after transfection, the cells were lysed in 1 ml of cell lysis solution (20 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$, 200 mM NaCl, and 0.05% NP40). After centrifugation, the supernatant was collected and mixed with anti-human Ago2 monoclonal antibody-conjugated agarose beads for 2 h at 4°C. After several washes with cell lysis solution, the HCV RNAs associated with Ago2-containing miRNA ribonucleoprotein (miRNP) complexes were eluted and were quantified by real-time RT-PCR. For knockdown of endogenous let-7b, the let-7b inhibitor (100 pmol) was transfected into Huh7.5 cells for 24 h followed by electroporation of the cells with the Rep-Feo HCV subgenomic RNA mutated at the miR-122 binding site (10 μg) and 100 pmol of let-7b inhibitor.

Western-blot analysis

The cell lysates were harvested and separated by 10% SDS-PAGE. The expression of HCV viral protein was detected using ECL kit (Perkin-Elmer) as described previously [27].

Production of HCVcc infectious particles and infectivity inhibition assay

The HCVcc infectious particle was produced as described previously [28]. Briefly, in vitro transcribed J6/JFH-based HCV genomic RNA was electroporated into Huh7.5 cells. The virus-containing supernatant was clarified by low-speed centrifugation, passed through a 0.45-μm filter, and concentrated by ultracentrifugation.

For infectivity inhibition assay, Huh7.5 cells were seeded in a six-swell plate at a density of 2×10^5 cells/well. At 24 h after plating, 100 nM miRNA was transfected into the cells using LF2000. HCVcc (0.1 MOI) was then added to each well for 4 h and the transfection complex was replaced with 2% FBS-containing medium for 72 h. The cells were fixed and stained by anti-Core antibody following by FITC-conjugated second antibody, counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and the infectious foci were counted using fluorescence microscopy.

For JC1-Luc2A HCV reporter virus, Huh7.5 cells were seeded in a 96-well plate at a density of 1×10^4 cells/well. At 24 h after plating, HCV reporter virus (0.01 MOI) was added to each well for 4 h. Then 100 nM of the indicated miRNA was transfected into the infected cells using RNAiMax and the transfection complex was replaced with 2% FBS-containing medium for 72 h. The cell lysates were collected for luciferase activity and MTS assay.

RNA isolation and real-time quantitative RT-PCR

Total RNAs were extracted using ReZol method and were quantified using a NanoDrop spectrophotometer. For quantification of HCV RNA expression, total cellular RNA (100 ng) was subject to one-step RT-PCR (25 μl) containing 2× TaqMan master mix and the primer/probe set for HCV (HCV-F: 5′-TGCGGAACCGGTGAGTACA-3′, HCV-R: 5′-CTTAAGGTTTAGGATTCGTGCTCAT-3′, and probe: 5′-CACCCTATCAGGCAGTACCACAAGG CC-3′). The reaction condition was one cycle of 48°C for 30 min, one cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 s followed by 60°C for 1 min using the ABI Prism 7000 Sequence Detection System. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalization control. HCV RNA expression was quantified by the $\Delta\Delta C_t$ method, where C_t represented the threshold cycle.

The TaqMan® microRNA Assay System was used for miRNA detection and quantification. Briefly, the RT reaction was performed in a final volume of 15 μl containing 1.5 μl of 10× RT buffer, 2.5 μl of total RNA (25 ng), 3 μl of 5× miRNA-specific RT primer, 0.15 μl of 100 mM dNTP, 0.2 μl of 40 U/μl RNase inhibitor, and 1 μl of MultiScribe reverse transcriptase (50 U/μl). The reaction condition was 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. Real-time PCR was then performed in a 20-μl PCR containing 1.33 μl of RT product, 10 μl of 10× TaqMan Universal PCR master mix, and 1 μl of the primer and probe mix from the TaqMan® MicroRNA Assay Kit. The reaction condition was 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The expression of RNU6B gene was used as the internal control.

HCV translation assay

Huh7.5 cells were seeded into a six-well plate at a density of 4×10^5 cells/well. At 24 h after transfection of let-7b miRNA (100 nM), the replication-deficient J6/JFH (p7-Rlu2A) GNN mutant RNA (1.25 μg/well) was transfected together with the capped and polyadenylated FLuc mRNA (125 ng/well) by LF2000. After 4 h, cells were harvested and dual luciferase activity assays were performed.

Statistical analysis

Statistical analysis was performed by Student's t test. $p < 0.05$ was considered as statistically significant.

Results

Identification and functional characterization of liver miRNAs with potential recognition sequences on HCV genome

A bioinformatic strategy as described in the "Materials and methods" section was developed to search for novel miRNAs with potential recognition sequences on HCV genome (Fig. 1). Three miRNAs including miR-122, let-7b, and miR-16 were uncovered. To elucidate whether these miRNAs have any functional effect on HCV infection, Huh7/Rep-Feo replicon cells (genotype 1b) were transfected with the indicated miRNAs and the luciferase activity was determined (Fig. 2a, b). In accord with a previous report [11], miR-122 enhanced HCV expression ($p < 0.05$). Notably, let-7b significantly suppressed HCV expression ($p < 0.01$) while miR-16 had only a moderate effect ($p = 0.343$).

To further confirm that let-7b can regulate HCV RNA accumulation, mutated let-7b (m7b) was designed to change three nucleotides on the wild-type let-7b sequences (Fig. 2b; Table 1). As calculated and predicted by miRanda, no m7b target sequence was found on HCV genome (data not shown). After transfection into Huh7/Rep-Feo replicon cells, m7b abrogated the inhibitory effect of let-7b on the luciferase activity (Fig. 2b) thereby demonstrating that let-7b is a negative regulator of HCV expression.

The effects of the three selected miRNAs on HCV expression were also evaluated using ConI replicon cells (Fig. 2c). These miRNAs were transfected into the replicon cells and the expression of viral proteins was determined at 72 h after transfection. Western-blot analysis revealed that miR-122 increased NS5A expression, while let-7b but not miR-16 caused a decrease in NS5A (Fig. 2c, left panel). Furthermore, the let-7b mutant form m7b lost its inhibitory effect on HCV and did not alter NS5A expression (Fig. 2c,

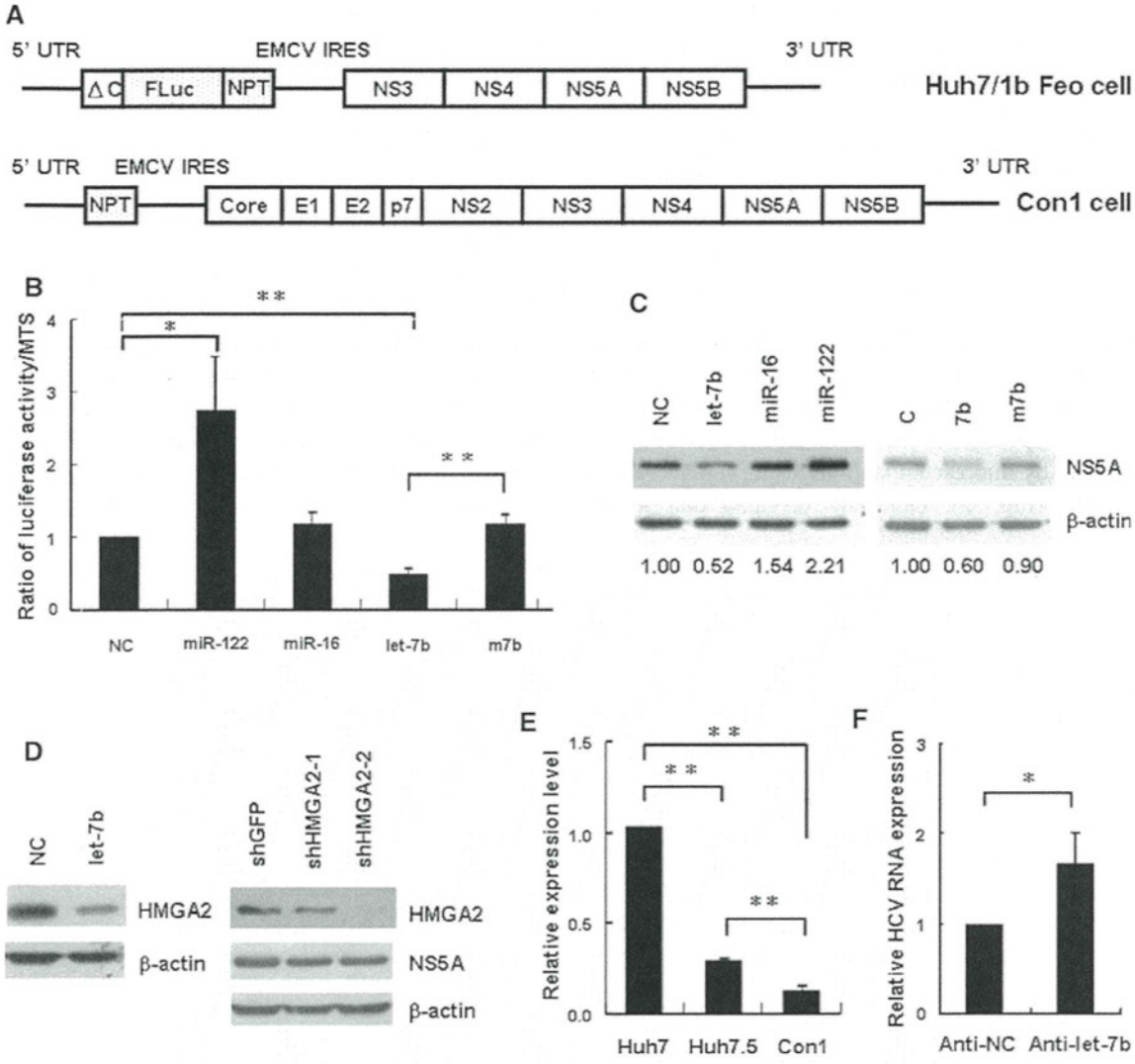

Fig. 2 Characterization of miRNAs with putative target sites on HCV genome. **a** Genomic structures of HCV maintaining in Huh7/Rep-Feo and ConI cells. **b** The miRNA precursors or mutant let-7b (m7b) were transfected into Huh7/Rep-Feo cells. MTS and luciferase activity assays were then performed at 72 h post-transfection. **c** The miRNA precursors (*left panel*) or the mutant form of let-7b (*right panel*) were transfected into the ConI cells. Western-blot analysis was then performed using the anti-NS5A and anti-β-actin antibody at 72 h post-transfection. The ratios for the relative band intensities of NS5A after normalization with β-actin were shown. *NC* negative control miRNA. **d** The precursors of let-7b (*left panel*) or shHMGA2 (*right panel*) were transfected into ConI replicon cells. Western-blot analysis was then performed using the anti-NS5A and anti-β-actin antibody at 72 h post-transfection. *NC* negative control miRNA. **e** Real-time RT-PCR of let-7b was performed using the total RNAs from the indicated cells. RNU6B was used as an internal control for normalization. The data represented the mean ± SD ($n = 3$; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). **f** The let-7b inhibitor (Anti-let-7b) or control inhibitor (Anti-NC) was transfected into Huh7 cells, respectively. HCV RNA expression was quantified by real-time RT-PCR using the total RNAs from the indicated transfected cells. The expression of GAPDH was used as a control for normalization. The data represented the mean ± SD ($n = 3$; $^*p < 0.05$)

right panel). These data thereby implicate that let-7b elicits suppressive activity in HCV protein expression.

HMGA2 is one of the major let-7b target genes and is down-regulated in let-7b-transfected ConI cells as previous reported (Fig. 2d, left panel) [29]. To rule out down-regulation of host transcripts accounts for the inhibitory effect of let-7b on HCV expression, HMGA2 was knockdown by two independent shHMGA2 plasmids. Although HMGA2 was significantly down-regulated in the shHMGA2 expressing cells, no effect was observed for the expression of the viral protein NS5A (Fig. 2d, right panel). These data indicate that down-regulation of HMGA2 does not contribute to the effect of let-7b on HCV expression.

To further delineate the association between let-7b and HCV infectivity, let-7b expression in various HCV-associated cell lines were determined. As shown in Fig. 2e, let-7b expression in the HCV permissive Huh7.5 cells was less than its expression in the parental Huh7 cells ($p < 0.01$).

Consistent with these observations, ConI cells bearing replicated HCV genome also had much lower let-7b (Fig. 2e, $p < 0.01$). Furthermore, Huh7 cells were more permissive for HCVcc infection when let-7b was inactivated by the let-7b inhibitor (Fig. 2f, $p < 0.05$). These data indicate that the cells capable of persistent HCV replication are usually associated with a low level of let-7b expression.

Let-7b reduces HCVcc infectivity

The HCVcc system was used to elucidate the role of let-7b in HCV infectivity. Let-7b was transfected into Huh7.5 cells followed by infection with HCVcc derived from J6/JFH-1 (genotype 2a). Hepatitis C virus expression was then monitored by fluorescent staining using the anti-HCV Core antibody (Fig. 3a). Our data revealed that let-7b reduced HCV infectivity for 42% ($p < 0.05$) while miR-122 enhanced the infectivity for 63% ($p < 0.05$) when compared to the cells expressing negative control miRNA (Fig. 3b). The HCV RNA was also decreased in let-7b-transfected cells (Fig. 3c, $p < 0.05$) indicating that let-7b suppresses HCV RNA level leading to a decrease in viral production.

To further confirm the negative regulatory effect of let-7b on HCVcc production, Huh7.5 cells were infected with the JC1-Luc2A HCV reporter virus and the luciferase activity was used to evaluate HCV viral production. Our data revealed that let-7b reduced 75% of the HCV reporter virus luciferase activity (Fig. 3d, $p < 0.001$) when compared to the cells expressing negative control miRNA. As a control, miR-122 increased 87% of the HCV reporter virus luciferase activity ($p < 0.05$). In contrast, mutation of

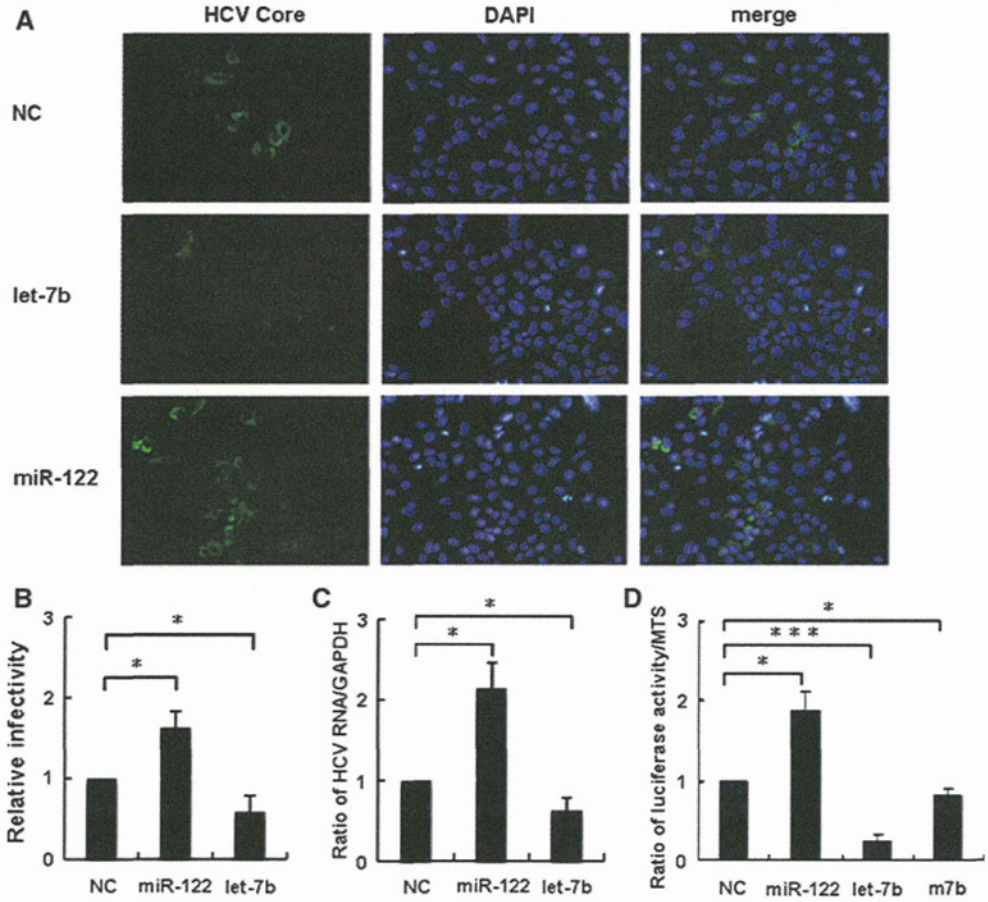

Fig. 3 Let-7b reduces HCVcc infectivity. **a** The indicated miRNAs were transfected into Huh7.5 cells for 24 h followed by infection with J6/JFH-based HCVcc. After 72 h, the cells were stained by anti-Core antibody. Nuclei were visualized by DAPI staining. *NC* negative control. **b** The infectious foci were counted by fluorescence microscopy. The infectivity for the cells transfected with negative control miRNA (NC) was set as one. The data represented the mean ± SD ($n = 3$; $^*p < 0.05$). **c** HCV RNA expression was quantified by real-time RT-PCR using the total RNAs from the indicated transfected cells. The expression of GAPDH was used as a control for normalization. The data represented the mean ± SD ($n = 3$; $^*p < 0.05$). **d** The miRNA precursors or the mutant let-7b (m7b) were transfected into Huh7.5 cells for 24 h followed by infection with JC1-luc2A HCV reporter virus. After 72 h, cell viability was determined by MTS assay and the cell extracts were collected for luciferase activity assay. The relative firefly luciferase versus MTS activity was shown and the negative control miRNA was arbitrarily denoted as one. The data represented the mean ± SD ($n = 3$; $^*p < 0.05$; $^{***}p < 0.001$)

let-7b (m7b) diminished its inhibitory effect on HCV expression and resulted in a slight inhibition of HCV reporter virus luciferase activity ($p < 0.05$). Together, these data implicate that inhibition of HCV RNA expression accounts for the suppressive effect of let-7b on HCV infection.

Let-7b physically interacts with the HCV genome

Argonaute 2 is the core component of miRNA-induced silencing complex (miRISC), which binds the guide miRNA to silence target mRNAs [30]. To determine whether Ago2 together with let-7b and HCV RNA form a miRISC complex, HCV RNA was co-transfected with let-7b into Huh7.5 cells followed by immunoprecipitation using the anti-Ago2 antibody (Ago2-IP). Because the "site 1" sequence (Table 1) is the most important HCV genome sequence for miR-122 binding and for regulation of HCV by miR-122, "site 1" mutation S1-p34 m (miR-122-mut) [11, 12] was introduced into the HCV subgenome to minimize the interference from the endogenous miR-122. The amount of Ago2-IP-associated HCV RNA was quantified by real-time RT-PCR. As shown in Fig. 4a, both HCV RNA subgenomes with wild-type or mutant miR-122 binding site were found to associate with the miRNP complex, while let-7b promoted HCV-miRNP interactions when miR-122 binding site was mutated. The total amount of let-7b associating with the Ago2-IP fraction was unchanged (Fig. 4b). Furthermore, knockdown of endogenous let-7b in Huh7.5 cells followed by Ago2-IP revealed that both the amounts of HCV RNA (Fig. 4c) and let-7b (Fig. 4d) in the Ago2-IP fraction were dramatically decreased. These data thereby indicate that let-7b physically interacts with HCV RNA in the Ago2-containing miRNP complex.

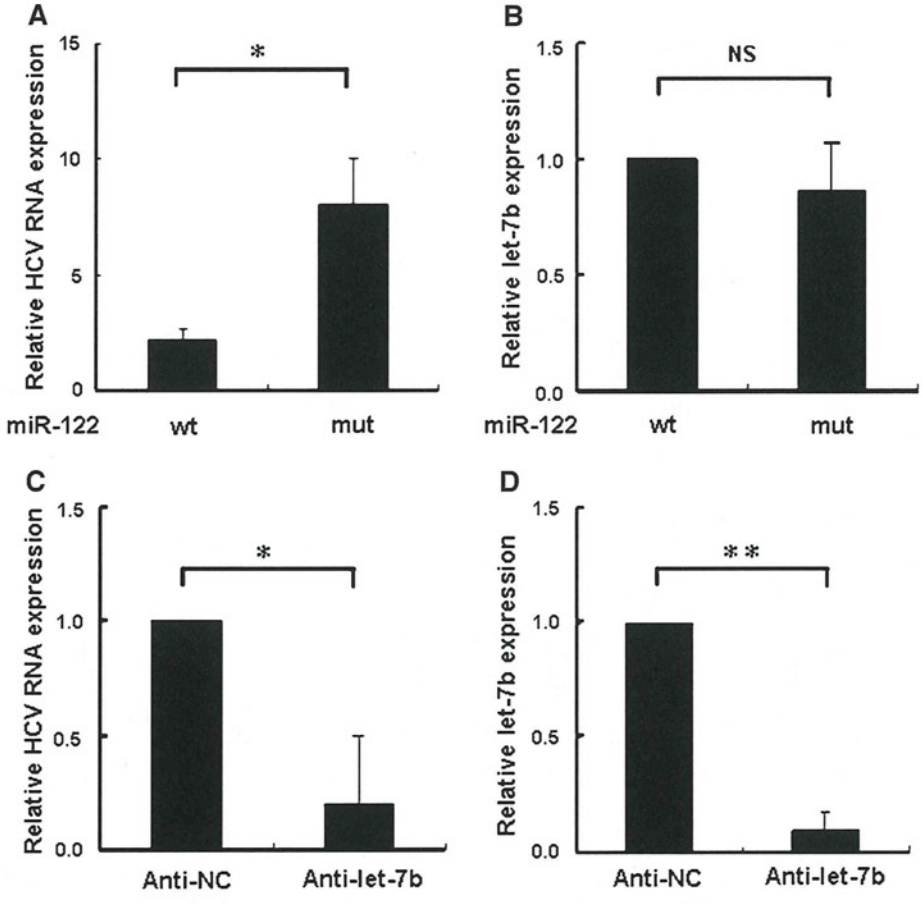

Fig. 4 Let-7b is associated with HCV genome in miRNP complex. **a**, **b** Huh7.5 cells were transfected with 100 pmol of miRNA along with 10 μg of either wild-type (miR-122-wt) or mutant (miR-122-mut) HCV subgenome RNA. The cell extracts were collected to perform co-immunoprecipitation with the anti-Ago2 antibody (Ago2-IP). HCV replicon RNA (*panel* **a**) and let-7b (*panel* **b**) were measured by real-time RT-PCR using total RNA sample from the Ago2-IP fraction. **c** ,**d** For knockdown of endogenous let-7b, Huh7.5 cells were transfected with 100 pmol of the indicated miRNA inhibitors (Anti-NC and Anti-let-7b) for 24 h followed by electroporation of the cells with 10 μg of miR-122-mut and 100 pmol of the indicated miRNA inhibitors. The cell extracts were collected to perform Ago2-IP and the HCV replicon RNA (*panel* **c**) and let-7b (*panel* **d**) were measured by real-time RT-PCR. The relative levels for HCV RNA and let-7b in the Ago2-IP complexes were shown. The data represented the mean ± SD ($n = 3$; $*p < 0.05$, $**p < 0.01$)

Identification of let-7b-responsive elements on the HCV genome

To identify MRE for let-7b, HCV-N genomic sequences (genotype 1b) were subject to bioinformatic prediction using miRanda and RNAhybrid. Several putative MREs for let-7b were revealed (Fig. 5a; Supplementary Tables 3, 4). It is noted that MRE2 (nt 8,745–8,766) and MRE3 (nt 8,977–8,995) that located within the HCV NS5B coding region had the highest prediction score and were selected for analysis. Moreover, the MRE1 (nt 338–365) and MRE4 (nt 9,566–9,603) at the non-coding region that had the lowest minimum free energies (MFE) in NCR region (Table 2) were also subject to further analysis.

To determine whether any of these sequences is the authentic MREs for let-7b, luciferase reporter plasmids with the reported let-7b target sequence (pluc-let-7b) and the putative MREs in HCV genome were constructed (pluc-MRE1, pluc-MRE2, pluc-MRE3, and pluc-MRE4). After co-transfection with let-7b or a negative control miRNA into 293T cells, the luciferase activities for each individual reporter plasmid were measured. Our data revealed that let-7b decreased pluc-MRE1, pluc-MRE2, and pluc-MRE3 luciferase activity by 28, 31, and 37%, respectively (Fig. 5b). No effect was found for pluc-MRE4. These data indicate that the MRE1, MRE2, and MRE3 are the potential let-7b binding sites on HCV genome.

Mutations of the putative let-7b MREs were introduced into the HCV subgenome to exam whether these MREs are responsible for the suppressive effect of let-7b. Silent mutations of MRE2 and MRE3 were designed to avoid amino acid changes while a six-nucleotide substitution mutation was introduced into HCV Rep-Feo subgenome (wild-type) to generate mMRE1, mMRE2, mMRE3, and mMRE2,3, respectively. Structural analysis of these mutations revealed that most of the HCV RNA genome structures were maintained except that mMRE1 appeared

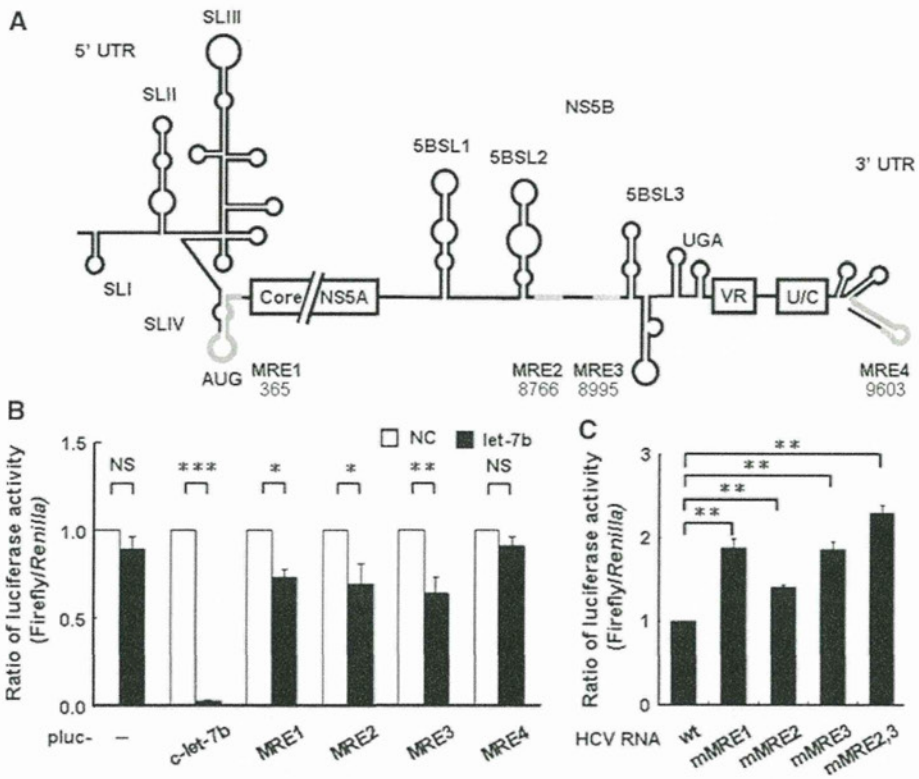

Fig. 5 The MREs of let-7b are located at the NS5B coding sequences and 5′-UTR of HCV genome. **a** Schematic representation for the predicted MREs of let-7b on HCV genome. The *number* corresponds to the first nucleotide of the predictive seed region. **b** The precursor of let-7b or negative control miRNA (NC) was co-transfected with the indicated luciferase reporter plasmid and pRL-TK into 293T cells for 24 h. The luciferase activities were measured and the relative firefly versus Renilla luciferase activity was shown. The plasmid containing perfect complementary sequence of let-7b (c-let-7b) and the vector control reporter plasmid (luciferase activity arbitrarily denoted as one) was used as the positive and negative control, respectively. The data represented the mean ± SD ($n = 3$; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; *NS* no significance). **c** The RNAs for wild-type (wt) HCV genome and the genome with mutations at the indicated MREs regions were obtained by in vitro transcription and were transfected individually into Huh7.5 cells along with let-7b precursor or negative control miRNA (NC) by electroporation. The luciferase activity was determined at 96 h post-transfection. The luciferase activity generated by wild-type HCV genome was arbitrarily denoted as 1 and the luciferase activity derived from each mutant HCV genome normalized by luciferase activity from wild-type was shown. The data represented the mean ± SD ($n = 3$; $**p < 0.01$)

Table 2 Characterization of let-7b predicted binding sites on HCV genome

MRE	Secondary structure	MFE[a]	Score[a]	Position (HCV-N)
MRE1	miRNA 3'-UUGG----UGUGUU--GGAUGAUGGAGU-5' Target 5'-CACCAUGAGCACGAAUCCUAA-ACCUCAA-3'	-26	84	338-365
MRE2	miRNA 3'-UUGGUGUGUUGGAUGAUGGAGU-5' Target 5'-GGCAAAAGGGUGUACUACCUCA-3'	-16.7	156	8745-8766
MRE3	miRNA 3'-UUGGUGUGUUGGAUGAUGGAGU-5' Target 5'-AGCCACUUGAC---CUACCUCA-3'	-21.1	152	8977-8995
MRE4	miRNA 3'-UUGGUGUGUUGG----------AUGA-UGGAGU-5' Target 5'GAGCCGCAUGACUGCAGAGAGUGCUGAUACUGGCCUCU-3'	-28.5	93	9566-9603

[a] MFE was calculated by RNAhybrid while Score was calculated by miRanda

to generate a small stem-loop structure (Supplementary Fig. S1). These mutated HCV RNAs were obtained by in vitro transcription and, together with let-7b, electroporated into Huh7.5 followed by analysis of luciferase activity. Our data revealed that the luciferase activities for HCV subgenome with mMRE1, mMRE2, and mMRE3 were 87, 40, and 86% higher than the wild-type HCV subgenome, respectively (Fig. 5c), implicating that let-7b-mediated suppression of HCV replicon activity is abrogated by mutating the target sequences on HCV genome. Moreover, HCV subgenome with double mutations of MRE2 and MRE3 synergistically enhanced luciferase activity when compared to the single mutant for these two MREs. The RNA structure did not contribute to the loss of let-7b responsiveness because Mfold analysis demonstrated that the wild-type and mutant HCV subgenome had similar RNA structure (Supplement Fig. S1). These data thereby indicate that 5′-UTR and NS5B coding sequences contain let-7b binding sites.

The antiviral effect of let-7b is independent of inhibition of HCV translation

It has been reported that miR-122 enhances HCV replication by stimulating internal ribosome entry site (IRES)-mediated translation in cultured cells [12]. Because MRE1 is located at domain IV of IRES [31], the possibility of let-7b modulating HCV replication through translation was also examined. The replication-deficient J6/JFH (p7-Rlu2A) GNN HCV mutant RNA was transfected along with a capped and polyadenylated FLuc mRNA as an internal control for transfection and translation. The ratio of Renilla luciferase (RLuc) to firefly luciferase (FLuc) activity was used to measure the IRES-directed translation activity. As shown in Fig. 6, miR-122 enhanced HCV IRES activity for approximate threefold, while miR-122 inhibitor resulted in approximate 50% decrease of the activity. However, let-7b or its inhibitor had no effects on HCV translation (Fig. 6). These data indicate that let-7b regulates HCV RNA replication through a mechanism independent of HCV translational regulation.

Let-7b and INFα-2a elicit synergistic anti-HCV activity

We examined further whether there is a synergistic effect between let-7b and IFNα-2a. The Huh7/Rep-Feo cells were treated with different concentrations of IFNα-2a and the luciferase activity for HCV subgenome was measured to determine the optimized dosage of IFNα-2a for synergistic study. As shown in Fig. 7a, the luciferase activity was suppressed by IFNα-2a in a dose-dependent manner with the IC50 equivalent to 1.39 ng/ml. When Huh7/Rep-Feo cells were transfected with let-7b followed by treatment with IFNα-2a, a 60 and 70% decrease in luciferase activity was observed in relative to let-7b or IFNα-2a alone, respectively (Fig. 7b, $p < 0.01$). These data thereby indicate that let-7b and IFNα-2a elicit synergistic inhibitory effect on HCV expression.

Discussion

The interplays between viral infection and miRNA have been demonstrated since the first report unfolding miR-32 as the negative regulator of primate foamy virus RNA accumulation [32]. Subsequently a number of miRNAs

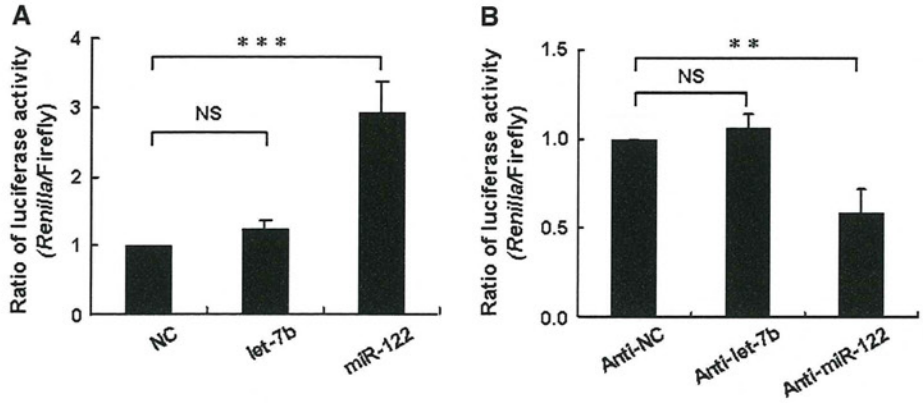

Fig. 6 Let-7b decreases HCV RNA expression independent on translation inhibition. **a, b** Huh7.5 cells were transfected with the indicated miRNAs (*panel* **a**) or miRNA inhibitors (*panel* **b**). Twenty-four hour later, HCV RNAs carrying GND mutation and Renilla luciferase coding sequence were transfected with a capped and polyadenylated firefly luciferase mRNA. At 4 h after transfection, the cell lysates were subject to dual luciferase activity assays. The relative firefly versus Renilla luciferase activity is shown. The data represented the mean ± SD ($n = 3$; $^{**}p < 0.01$; $^{***}p < 0.001$; *NS* no significance)

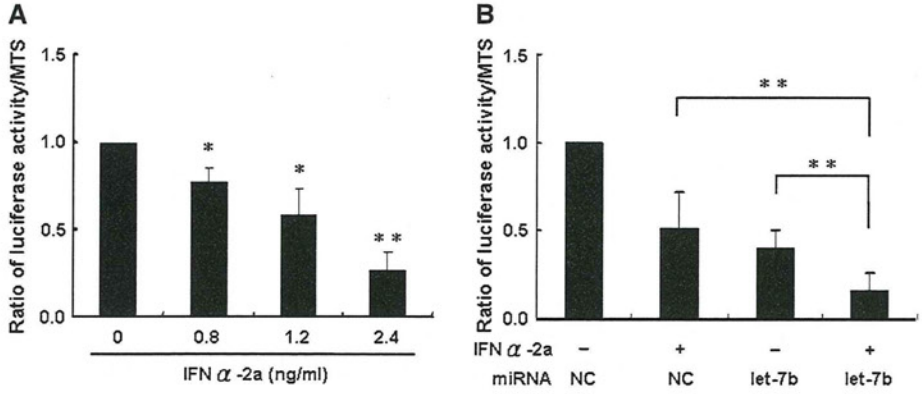

Fig. 7 Let-7b and IFNα-2a elicit synergistic inhibitory effects on HCV RNA accumulation. **a** Huh7/Rep-Feo cells (3×10^4) were treated with the indicated doses of IFN-2α for 72 h and the luciferase activity and cell viability were determined. **b** Huh7/Rep-Feo cells were transfected with 100 nM of let-7b or negative control miRNA (NC) by RNAiMAX for 4 h followed by treatment with INF-2α or medium control for additional 72 h. The luciferase activity and cell viability were determined. The data represented the mean ± SD ($n = 3$) with the luciferase activity normalized by the cell viability. ($^{*}p < 0.05$; $^{**}p < 0.01$)

were found to elicit anti-HCV activity [11, 13, 14]. In this study, bioinformatic tools and virological analyses are employed to unveil novel cellular miRNAs associated with HCV infection. We demonstrate that, in addition to miR-122 that has been reported to augment HCV infection, let-7b targets the HCV genome leading to a decrease in HCV RNA accumulation and viral production. This study thereby represents the first report to identify let-7b as a negative regulator of HCV infection.

Let-7b is the first known human miRNA [33] that is closely associated with the status of cellular differentiation and is usually down-regulated in cancers [34, 35]. Experimental evidence we present in this study unveils the role of let-7b in the control of HCV pathogenesis. Let-7b expression is irreversibly correlated with HCV infectivity in the cell-based systems; the cell lines bearing replicated HCV genome that are permissive for HCV replication (such as Huh7.5 and Con1) usually have low levels of let-7b expression. Moreover, let-7b is associated with HCV genome in Ago2 miRNP complex. Cellular study further reveals that let-7b diminishes luciferase reporter gene expression in Huh7/Rep-Feo subgenome replicon (1b genotype), the viral protein expression in ConI replicon (1b genotype) and HCV RNA accumulation and viral production upon HCVcc infection (2a genotype). These findings not only indicate that let-7b plays a role in the host antiviral response, but also reveal the universal effects of let-7b on different HCV genotypes.

The molecular basis for the anti-HCV effect of let-7b is also elucidated in this study. Our data support the notion that let-7b directly interacts with the HCV genome and modulates virus production. Although a number of miRNAs have been reported to regulate HCV replication and pathogenesis, only miR-122 and miR199a* were

demonstrated to directly target on HCV genome [11, 13]. Let-7b thereby represents the third cellular miRNA that elicits a direct effect on HCV genome and modulates HCV replication. In contrast to miR-122 and miR-199a*, of which the target sequences mapped to 5′-UTR [11, 13], one of the unique features for let-7b is that two of the let-7b target sequences, MRE2 and MRE3, are located within the coding region of NS5B. Although it is not common, miRNA has been shown to affect gene expression by interacting with mRNA coding regions [36, 37]. For example, miR-148 and miR-24 repress DNA methyltransferase 3b and p16 expression, respectively, primarily through the coding region recognition site [38, 39]. Let-7b targets the coding sequence of Dicer and establishes a miRNA/Dicer autoregulatory negative feedback loop. While the advantages for let-7b targeting the HCV coding sequence remain to be elucidated, let-7b symbolizes the first cellular miRNA with recognition sequences in the coding region of the HCV genome.

Although our data indicate that let-7b acts on the HCV genome leading to a decrease in HCV expression, we cannot rule out that the host factors regulated by let-7b may also play a role in the regulation of HCV expression. Several host factors are down-regulated by let-7b, including HMGA2 [40–42]. Moreover, TargetScan prediction reveals at least 79 cellular target genes regulated by let-7b have some associations with HCV infection. Despite that knockdown of HMGA2 does not have any effect of HCV protein expression, whether the other host factors mediating let-7b effects on HCV expression remains to be investigated.

While only let-7b meets our preset selection criteria, bioinformatic prediction data reveal that three other family members of let-7, including let-7a, let-7c, and let-7f, are also liver-abundant (Supplementary Table 1). Because let-7 family members differ by only one to a few nucleotides [43], let-7a, let-7c, and let-7f were also tested for their potential effects on HCV expression. As shown in Supplementary Fig. S2, let-7a and let-7f were expressed at a lower level in HCV-sensitive cell lines while let-7c was expressed at a higher level. In addition, these three miRNAs can also reduce HCV activity on subgenome replicon cells and reporter virus. However, let-7b exhibits more prominent suppressive effects than the others. Hence, it is likely that let-7 family members may act on HCV in a similar way to let-7b with various regulations.

In addition to controlling multiple cellular events, miRNA has been proposed as a therapeutic regimen for various diseases [44, 45]. Recently, a locked nucleic acid-modified oligonucleotide complementary to miR-122 exhibits a long-lasting suppression of HCV viremia in chronically infected chimpanzees [46]. Small-molecule inhibitors and activators of miR-122 have been developed to reduce HCV viral replication [47]. In this study, we found that let-7b plays a role in host defense to combat HCV infection and reduces HCV infectivity. The synergistically inhibitory effect of let-7b and IFNα-2a on HCV replication further implies that let-7b is a good candidate for developing an adjuvant regimen for IFNα-2a in a clinical setting.

In conclusion, we demonstrate for the first time that let-7b inhibits HCV expression and replication by targeting the conserved HCV 5′UTR and coding region. Furthermore, let-7b and IFNα-2a elicit synergistically inhibitory effect on HCV infection. This study thereby contributes to our understanding for let-7b on the control of HCV pathogenesis and offers new insight for developing novel anti-HCV therapeutic approaches.

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Conflict of interest None.

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Association of Gene Expression Involving Innate Immunity and Genetic Variation in Interleukin 28B With Antiviral Response

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Innate immunity plays an important role in host antiviral response to hepatitis C viral (HCV) infection. Recently, single nucleotide polymorphisms (SNPs) of *IL28B* and host response to peginterferon α (PEG-IFNα) and ribavirin (RBV) were shown to be strongly associated. We aimed to determine the gene expression involving innate immunity in *IL28B* genotypes and elucidate its relation to response to antiviral treatment. We genotyped *IL28B* SNPs (rs8099917 and rs12979860) in 88 chronic hepatitis C patients treated with PEG-IFNα-2b/RBV and quantified expressions of viral sensors (*RIG-I, MDA5*, and *LGP2*), adaptor molecule (*IPS-1*), related ubiquitin E3-ligase (*RNF125*), modulators (*ISG15* and *USP18*), and *IL28* (*IFNλ*). Both *IL28B* SNPs were 100% identical; 54 patients possessed rs8099917 TT/rs12979860 CC (*IL28B* major patients) and 34 possessed rs8099917 TG/rs12979860 CT (*IL28B* minor patients). Hepatic expressions of viral sensors and modulators in *IL28B* minor patients were significantly up-regulated compared with that in *IL28B* major patients (≈3.3-fold, $P < 0.001$). However, expression of *IPS-1* was significantly lower in *IL28B* minor patients (1.2-fold, $P = 0.028$). Expressions of viral sensors and modulators were significantly higher in nonvirological responders (NVR) than that in others despite stratification by *IL28B* genotype (≈2.6-fold, $P < 0.001$). Multivariate and ROC analyses indicated that higher *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* expression ratio were independent factors for NVR. IPS-1 down-regulation in *IL28B* minor patients was confirmed by western blotting, and the extent of IPS-1 protein cleavage was associated with the variable treatment response. *Conclusion:* Gene expression involving innate immunity is strongly associated with *IL28B* genotype and response to PEG-IFNα/RBV. Both *IL28B* minor allele and higher *RIG-I* and *ISG15* expressions and *RIG-I/IPS-1* ratio are independent factors for NVR. (HEPATOLOGY 2012;55:20-29)

Infection with hepatitis C virus (HCV) is a common cause of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma in many patients.[1] Pegylated interferon α (PEG-IFNα) and ribavirin (RBV) combination therapy has been used to treat chronic hepatitis C (CH-C) to alter the natural course of this disease. However, 20% patients are nonvirological responders (NVR) whose HCV-RNA does not become negative during the 48 weeks of PEG-IFNα/RBV combination therapy.[2] In a recent genome-wide association study, single nucleotide polymorphisms (SNPs) located near interleukin 28B

Abbreviations: CH-C, chronic hepatitis C; γ-GTP, γ-glutamyl transpeptidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HMBS, hydroxymethylbilane synthase; IL28, interleukin 28; IPS-1, IFNβ promoter stimulator 1; ISG15, interferon-stimulated gene 15; MDA5, melanoma differentiation associated gene 5; NVR, nonvirological responders; PEG-IFNα, pegylated interferonα; SNP, single nucleotide polymorphism; RIG-I, retinoic acid-inducible gene I; RBV, ribavirin; RNF125, ring-finger protein 125; ROC, receiver operator characteristic; SVR, sustained viral responder; TVR, transient virological responder; USP18, ubiquitin-specific protease 18; VR, virological responder.

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(*IL28B*) that encodes for type III IFNλ3 were shown to be strongly associated with a virological response to PEG-IFNα/RBV combination therapy.[3-5] In particular, the rs8099917 TG and GG genotypes were shown to be strongly associated with a null virological response to PEG-IFNα/RBV.[3] However, mechanisms involving resistance to PEG-IFNα/RBV have not been completely elucidated.

The innate immune system has an essential role in host antiviral defense against HCV infection.[6] The retinoic acid-inducible gene I (RIG-I), a cytoplasmic RNA helicase, and related melanoma differentiation associated gene 5 (MDA5) play essential roles in initiating the host antiviral response by detecting intracellular viral RNA.[7,8] The IFNβ promoter stimulator 1 (IPS-1)—also called the caspase-recruiting domain adaptor inducing IFNβ, mitochondrial antiviral signaling protein, or virus-induced signaling adaptor—is an adaptor molecule. IPS-1 connects RIG-I sensing to downstream signaling, resulting in *IFNβ* gene activation.[9-12] RIG-I sensing of incoming viral RNA has been shown to be modified by LGP2,[8,13] a helicase related to RIG-I and MDA5 lacking caspase-recruiting domain. The ubiquitin ligase ring-finger protein 125 (RNF125) has been shown to conjugate ubiquitin to RIG-I, MDA5, and IPS-1 and this suppresses the functions of these proteins.[14] Further, these molecules are ISGylated by the IFN-stimulated gene 15 (ISG15), a ubiquitin-like protein,[15] and ISG15 is specifically removed from ISGylated protein by ubiquitin-specific protease 18 (USP18) to regulate the RIG-I/IPS-1 system.[16,17] Moreover, the NS3/4A protease of HCV specifically cleaves IPS-1 as part of its immune-evasion strategy.[9,18] Therefore, the RIG-I/IPS-1 system and its regulatory systems have essential roles in the innate antiviral response.

Recently, we demonstrated that baseline intrahepatic gene expression levels of the RIG-I/IPS-1 system were prognostic biomarkers of the final virological outcome in CH-C patients who were treated with PEG-IFNα/RBV combination therapy.[19] We found that up-regulation of *RIG-I* and *ISG15* and a higher expression ratio of *RIG-I/IPS-1* could predict NVR for subsequent treatment with PEG-IFNα/RBV combination therapy.[19] However, association of gene expression involving innate immunity and genetic variation of *IL28B* has not yet been elucidated. Hence, the aim of this study was to determine gene expression involving the innate immune system in different genetic variations of *IL28B* and elucidate the relation of gene expression to final virological outcome of PEG-IFNα/RBV combination therapy in CH-C patients.

Patients and Methods

Patients. Among histologically proven CH-C patients admitted at the Musashino Red Cross Hospital, 88 patients with HCV genotype 1b and a high viral load (>5 log IU/mL by TaqMan HCV assay; Roche Molecular Diagnostics, Tokyo, Japan) were included in the present study (Table 1). Patients with decompensated liver cirrhosis, autoimmune hepatitis, or alcoholic liver injury were excluded. No patient had tested positive for hepatitis B surface antigen or anti-human immunodeficiency virus antibody or had received immunomodulatory therapy before enrollment. Forty-two patients had been enrolled in a previous study that determined hepatic gene expression involving innate immunity.[19] Written informed consent was obtained from all patients and the study was approved by the Ethical Committee of Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

Treatment Protocol. The patients were administered subcutaneous injections of PEG-IFNα-2b (PegIntron, MSD, Whitehouse Station, NJ) at a dose of 1.5 $\mu g\ kg^{-1}\ week^{-1}$ for 48 weeks. RBV (Rebetol, MSD) was administered concomitantly over this treatment period, administered orally twice daily at 600 mg/day for patients who weighed less than 60 kg and 800 mg/day for patients who weighed between 60-80 kg. The dose of PEG-IFNα-2b was reduced to 0.75 $\mu g\ kg^{-1}\ week^{-1}$ when either neutrophil count was less than $750/mm^3$ or platelet count was less than $80 \times 10^3/mm^3$. The dose of RBV was reduced to 600 mg/day when the hemoglobin concentration decreased to 10 g/dL. More than 80% adherence was achieved in all patients.

Measurement of Hepatic Gene Expression. Liver biopsy was performed immediately before initiating

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