

miR-500 were transfected into Li-7 cells, which did not express miR-500 and we found there are no significant differences in cell proliferation (see Supplementary Figure 5C in the online version of this article). Although our data indicated that miR-500 did not affect cell proliferation in liver cancer cell lines, there might be a close association between tissue development and carcinogenesis in the fields of miRNA. For detailed analysis of function of miR-500, we await for generation of miR-500 knockout mice.

As several groups have reported that levels of certain circulating miRNA are associated with clinical characteristics in diseases (Gilad et al. 2008, Lawrie et al. 2008), our data suggest that miR-500 was circulating in the sera of the HCC patients and miR-500 levels in sera of the HCC patients returned to normal after the surgery. Although our results are promising for miRNA-based HCC screening, there are several limitations in this study and we suggest: (1) as the sample size is quite small, further validation that miR-500 could be a reliable marker for HCC in a large cohort is necessary; (2) use of better controls to determine whether or not serum miR-500 levels are changed due to the trauma of surgery; (3) it is desirable to examine whether serum miR-500 levels change in patients with chronic hepatitis and liver cirrhosis; (4) it is necessary to compare if serum miR-500 could be better than earlier diagnostic methods such as serum α -fetoprotein.

The differential expression patterns of miR-101b, miR-122a, miR-142-5p, miR-223 and miR-451 were determined by miRNA microarray and real-time PCR analysis. The specific expression of miR-122 in the liver has previously been described by several research groups. Esau et al. (2006) reported that miR-122 was a key regulator of lipid metabolism in the liver, regulating increased hepatic fatty acid oxidation, a decrease in hepatic fatty acid and cholesterol synthesis rates by reductions of several lipogenic genes. Interestingly, two groups demonstrated evidence that the hepatitis C virus genome has predicted binding sites of miR-122 and that miR-122 positively regulated the replication hepatitis C virus in human liver (Jopling et al. 2005, Randall et al. 2007). In addition to miR-122a, we found that miR-101b expression was upregulated in mouse liver development. Furthermore, upregulation of miR-101b and miR-122a expression was observed in the *in vitro* cultured of fetal hepatocytes treated with OsM and Dex (see Supplementary Figure 4A-C in the online version of this article). It has been reported that miR-101 is related to the immune system and megakaryocytopoiesis (Yu D et al. 2007, Garzon et al. 2006); however, the role of miR-101 in the liver has not yet been examined.

During early development in mice, haematopoietic stem cells emerge in the aorta/gonado/mesonephros

region and then the stem cells migrate and expand in the fetal liver before haematopoiesis takes place in the bone marrow by the time of birth. Although most of the miRNAs that we observe in the liver developmental process are constitutively expressed, specific miRNAs are enriched at distinct stages of haematopoietic development. We found that the expression of miR-142-5p, miR-223 and miR-451 was downregulated in the process of liver maturation. As it has been reported that miR-142-5p and miR-142-3p are highly expressed in all haematopoietic tissues (Chen et al. 2004), miR-142 may thus play a critical role at the early stage of haematopoiesis. The expression of miR-223 was mainly detected in bone marrow and negatively regulated myeloid progenitor proliferation and granulocytic differentiation and activation (Johndidis et al. 2008). In addition, miR-451 expression was upregulated during erythroid differentiation, and gain- and loss-of-function studies disclosed that miR-451 was related to erythroid maturation (Zhan et al. 2007).

Recent studies have indicated that a decrease of mature miRNA expression by impaired miRNA processing accelerates tumorigenesis and that a global reduction of miRNAs is observed in human cancers, suggesting that the role of overall miRNAs is to guard against oncogenic transformation (Kumar et al. 2007, Lu et al. 2005). In particular, the let-7 family is broadly known as a tumour suppressor. It has been reported that a decrease of let-7 expression was observed in human lung cancer and that let-7 negatively regulates the expression of H-ras and *HMG2* oncogenes in breast cancer cells (Johnson et al. 2005, Yu F et al. 2007, Takamizawa et al. 2004). In addition, miR-16 was also reported as a tumour suppressor by inducing apoptosis mediated by Bcl-2 and modulating the cell cycle (Cimmino et al. 2005, Linsley et al. 2007). In a study of liver carcinogenesis, a decrease of miR-122 expression was observed in rat liver tumour (Kutay et al. 2006). Consistent with this report, miR-122a and miR-101b expression levels in 40 pairs of malignant neoplasias of hepatocyte lineage and adjacent non-tumorous tissue were reduced significantly ($p < 0.05$, $n = 40$) in tumour samples (see Supplementary Figure 4D in the online version of this article). However, in previous studies, it has been revealed that specific miRNAs acted as oncogenes, as their overexpression facilitates cancer progression. For example, miR-17-92 polycistron was overexpressed in lymphomas, lung cancers and colorectal cancers and enhanced cell proliferation (He et al. 2005, Hayashita et al. 2005). Furthermore, the copy number and expression level of miR-155 and its non-coding RNA transcript BIC were greatly increased in B-cell lymphomas (Eis et al. 2005). Our data show that the expression profile of oncogenic miRNAs was downregulated and, vice versa, the expression of tumour-suppressor miRNAs was upregulated in the process of liver development (Figure 4). This suggests that elevated oncogenic miRNAs are important

at the early developmental stage of the liver because, in this period, cell proliferation is frequent; in contrast, upregulation of tumour suppressor miRNAs is essential for preventing abnormal cell proliferation at the late stage of liver development. Therefore, our data suggest that the tight regulation of expression of cancer-related miRNAs (both oncogenic miRNAs and tumour suppressor miRNAs) occurred during normal liver development.

Finally, we have documented dynamic changes in miRNA expression that were found in the process of mouse liver development and some of them behaved as an oncofetal miRNA in HCC. Although little is known about the expression regulations, targets or roles of miRNAs in the liver, the expression profiles of miRNA in development could be informative with respect to the elucidation of the process of the development and diagnosis of cancer because the expression of some of the cancer-related miRNAs dramatically changed. Further studies on the differential expression of miRNA in liver development could contribute to a better understanding of the process of liver development and embryonic haematopoiesis and could facilitate the discovery of candidate miRNAs for cancer diagnosis and therapeutic targets in liver cancer.

Acknowledgements

We thank Dr Lim Chun Ren and Dr Kenichi Matsubara at DNA Chip Research Inc. for supporting the processing of microarray data. We thank Ms Ayako Inoue and Ms Nachi Namatame for their excellent technical assistance. This work was supported in part by a Grant-in-Aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control; Health Science Research Grants for Research on the Hepatitis C Virus from the Ministry of Health, Labour, Welfare of Japan; the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio); a Grant for Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

References

- Ambros V. (2004). The functions of animal microRNAs. *Nature* 431:350-5.
- Bartel DP. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116:281-97.
- Chen CZ, Li L, Lodish HF, et al. (2004). MicroRNAs modulate hematopoietic lineage differentiation. *Science* 303:83-6.
- Chen JF, Mandel EM, Thomson JM, et al. (2006). The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat Genet* 38:228-33.
- Chim SS, Shing TK, Hung EC, et al. (2008). Detection and characterization of placental microRNAs in maternal plasma. *Clin Chem* 54:482-90.
- Cimmino A, Calin GA, Fabbri M, et al. (2005). miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A* 102:13944-9.
- Eis PS, Tam W, Sun L, et al. (2005). Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc Natl Acad Sci U S A* 102:3627-32.
- Esau C, Davis S, Murray SE, et al. (2006). miR-122 regulation of lipid metabolism revealed by *in vivo* antisense targeting. *Cell Metab* 3:87-98.
- Esquela-Kerscher A, Slack FJ. (2006). Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer* 6:259-69.
- Fu H, Tie Y, Xu C, et al. (2005). Identification of human fetal liver miRNAs by a novel method. *FEBS Lett* 579:3849-54.
- Garzon R, Pichiorri F, Palumbo T, et al. (2006). MicroRNA fingerprints during human megakaryocytopoiesis. *Proc Natl Acad Sci U S A* 103:5078-83.
- Gilad S, Meiri E, Yogeve Y, et al. (2008). Serum microRNAs are promising novel biomarkers. *PLoS ONE* 3:e3148.
- Gramantieri L, Ferracin M, Fornari F, et al. (2007). Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. *Cancer Res* 67:6092-9.
- Han J, Lee Y, Yeom KH, et al. (2006). Molecular basis for the recognition of primary microRNAs by the Droscha-DGCR8 complex. *Cell* 125:887-901.
- Hayashita Y, Osada H, Tatematsu Y, et al. (2005). A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. *Cancer Res* 65:9628-32.
- He L, He X, Lim LP, et al. (2007). A microRNA component of the p53 tumour suppressor network. *Nature* 447:1130-4.
- He L, Thomson JM, Hemann MT, et al. (2005). A microRNA polycistron as a potential human oncogene. *Nature* 435:828-33.
- Johnnidis JB, Harris MH, Wheeler RT, et al. (2008). Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. *Nature* 451:1125-9.
- Johnson SM, Grosshans H, Shingara J, et al. (2005). RAS is regulated by the let-7 microRNA family. *Cell* 120:635-47.
- Jopling CL, Yi M, Lancaster AM, et al. (2005). Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* 309:1577-81.
- Ketting RE, Fischer SE, Bernstein E, et al. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes Dev* 15:2654-9.
- Kumar MS, Lu J, Mercer KL, et al. (2007). Impaired microRNA processing enhances cellular transformation and tumorigenesis. *Nat Genet* 39:673-7.
- Kutay H, Bai S, Datta J, et al. (2006). Downregulation of miR-122 in the rodent and human hepatocellular carcinomas. *J Cell Biochem* 99:671-8.
- Lawrie CH, Gal S, Dunlop HM, et al. (2008). Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. *Br J Haematol* 141:672-5.
- Lee Y, Kim M, Han J, et al. (2004). MicroRNA genes are transcribed by RNA polymerase II. *Embo J* 23:4051-60.
- Linsley PS, Schelter J, Burchard J, et al. (2007). Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression. *Mol Cell Biol* 27:2240-52.
- Li QJ, Chau J, Ebert PJ, et al. (2007). miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell* 129:147-61.
- Lu J, Getz G, Miska EA, et al. (2005). MicroRNA expression profiles classify human cancers. *Nature* 435:834-8.
- Lund E, Guttinger S, Calado A, et al. (2004). Nuclear export of microRNA precursors. *Science* 303:95-8.
- Mitchell PS, Parkin RK, Kroh EM, et al. (2008). Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* 105:10513-18.
- Nelson PT, Hatzigeorgiou AG, Mourelatos Z. (2004). miRNP:mRNA association in polyribosomes in a human neuronal cell line. *RNA* 10:387-94.

Randall G, Panis M, Cooper JD, et al. (2007). Cellular cofactors affecting hepatitis C virus infection and replication. *Proc Natl Acad Sci U S A* 104:12884-9.

Shan Y, Zheng J, Lambrecht RW, et al. (2007). Reciprocal effects of micro-RNA-122 on expression of heme oxygenase-1 and hepatitis C virus genes in human hepatocytes. *Gastroenterology* 133:1166-74.

Takamizawa J, Konishi H, Yanagisawa K, et al. (2004). Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res* 64:3753-6.

Yu D, Tan AH, Hu X, et al. (2007). Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. *Nature* 450:299-303.

Yu F, Yao H, Zhu P, et al. (2007). Let-7 regulates self renewal and tumorigenicity of breast cancer cells. *Cell* 131:1109-23.

Zhan M, Miller CP, Papayannopoulou T, et al. (2007). MicroRNA expression dynamics during murine and human erythroid differentiation. *Exp Hematol* 35:1015-25.

Zhao Y, Ransom JE, Li A, et al. (2007). Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. *Cell* 129:303-17.

Regulation of the hepatitis C virus genome replication by miR-199a[☆]

Yoshiki Murakami^{1,*}, Hussein H. Aly¹, Atsushi Tajima², Ituro Inoue²,
Kunitada Shimotohno^{3,4}

¹Center for Genomic Medicine, Kyoto University, Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606-8507, Japan

²Department of Molecular Life Science, Tokai University School of Medicine, Isehara, Kanagawa 259-1192, Japan

³Center for Integrated Medical Research, Keio University, Shinanomachi, Shinjuku, Tokyo 160-8582, Japan

⁴Chiba Institute for Technology, Tsudanuma, Chiba 275-0016, Japan

See Editorial, pages 441–444

Background/Aims: Hepatitis C virus (HCV) infection causes chronic hepatitis and hepatocellular carcinoma. Current anti-HCV therapies are based on interferon therapy, which is insufficiently effective. microRNAs (miRNAs) are non-coding RNAs that regulate gene expression, and they have recently been shown to play an important role in viral replication.

Methods: An algorithm-based search for miRNAs that target the HCV genome yielded one miRNA, miR-199a^{*}, with a sequence similar to the HCV genome that is conserved among HCV genotypes.

Results: Overexpression of miR-199a^{*} inhibited HCV genome replication in two cells bearing replicons (replicon cell) HCV-1b or -2a, however, miRNA inhibition by specific antisense oligonucleotide (ASO) accelerated viral replication. Prior transfection of immortalized hepatocytes which were infected with serum of HCV genotype 1b and 2a-infected patients, with miR-199a^{*} reduced HCV RNA replication activity. Mutation in the miR-199a^{*} target site in the replicon reduced the effect of the miR-199a^{*}. HCV replicon RNA is accumulated to the RNA-induced silencing complex (RISC) when miR-199a^{*} was overexpressed to the replicon cell. This antiviral effect by miR-199a^{*} was independent of the interferon pathway.

Conclusions: The results of this study suggest that miR-199a^{*} directly regulates HCV replication and may serve as a novel antiviral therapy.

© 2008 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

Keywords: HCV; microRNA; Viral replication; Replicon cell; RISC

Received 9 April 2008; received in revised form 29 May 2008; accepted 3 June 2008; available online 9 July 2008

Associate Editor: F. Zoulim

[☆] The authors declare that they do not have anything to disclose regarding funding from industries or conflict of interest with respect to this manuscript.

* Corresponding author. Tel.: +81 75 753 4661; fax: +81 75 753 9314.

E-mail address: ymurakami@genome.med.kyoto-u.ac.jp (Y. Murakami).

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; miRNA, microRNA; IRES, the internal ribosomal entry site; RISC, RNA-induced silencing complex; CP, Ceruloplasmin.

1. Introduction

Infection by hepatitis C virus (HCV) infection is a cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. Pegylated interferon (IFN)- α /ribavirin combination therapy is currently the most effective treatment for chronic hepatitis C [2]. However, only 50% of treated HCV-infected patients clear HCV infection [3], and novel approaches are urgently needed.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that control gene expression by degrading or suppressing the translation of target mRNAs [4,5]. miR-122 is an abundant miRNA in the liver, where it comprises 70% of the miRNA in the liver [6]. Both the

5'- and 3'-UTRs of the HCV genome contain sequences that are partially complementary to miR-122. Administration of miR-122 accelerates HCV replication [7]. Several factors related to the RNAi machinery were recently found to reduce HCV production and HCV RNA level [8]. Targeting multiple viral and cellular elements with RNAi may increase the potency of antiviral gene therapies [9–12]. siRNA-based antiviral strategies are expected to have clinical applications as a means of eradicating persistent infection. It has been hypothesized that several miRNAs play pivotal roles in viral replication and proliferation and it is possible that miRNAs control HCV replication.

In this study, we used a miRNA target search algorithm and succeeded in identifying a miRNA that targets the HCV genome. We demonstrated that specific miRNA effectively acts as an RNA silencing-based antiviral response during HCV replication with miRNA specific machinery. The present study suggests that miRNA-mediated HCV inhibition and it may be possible to apply to novel anti-HCV therapies.

2. Materials and methods

2.1. Cell preparation, plasmid construction, liver tissue and transfection

Huh-7, HepG2, and cured MH14 cells [13] were cultured in D-MEM (Invitrogen) with 10% fetal bovine serum and nonessential amino acids (Invitrogen). SN1a [14] and JFH1 cells [15], which carry the full genome replicon of HCV genotype 1b and 2a, respectively, (Fig. 1A) were cultured in the above medium supplemented with 0.5–1.0 mg/ml G418 (Invitrogen). HuS-E/2 immortalized hepatocytes were cultured as described previously [16]. We obtained normal liver tissue from the Liver Transplantation Unit of Kyoto University after receiving the approval of the Ethical Committee of Kyoto University. To express miRNAs, plasmids containing the miR-199a*, miR-122, miR-19a, or a control sequence were constructed by ligating annealed oligonucleotides encoding each miRNA into the pSilencerH1-puro vector (Ambion) (Table 1). Cells were plated the day before transfection and grown to 50% confluence in 6-well plates. miRNA expression constructs (2 µg) were transfected into cells with FuGENE 6 (Roche). Cells were selected by puromycin (1.25 µg/ml) two days after transfection, and they were harvested more than two or four days later. For miRNA suppression, 50 pmol of 2'-O-methylated antisense oligonucleotide (ASO) (Hokkaido System Science) and Silence[®] negative control siRNA (Ambion) were transfected with SilentFect (Bio-Rad).

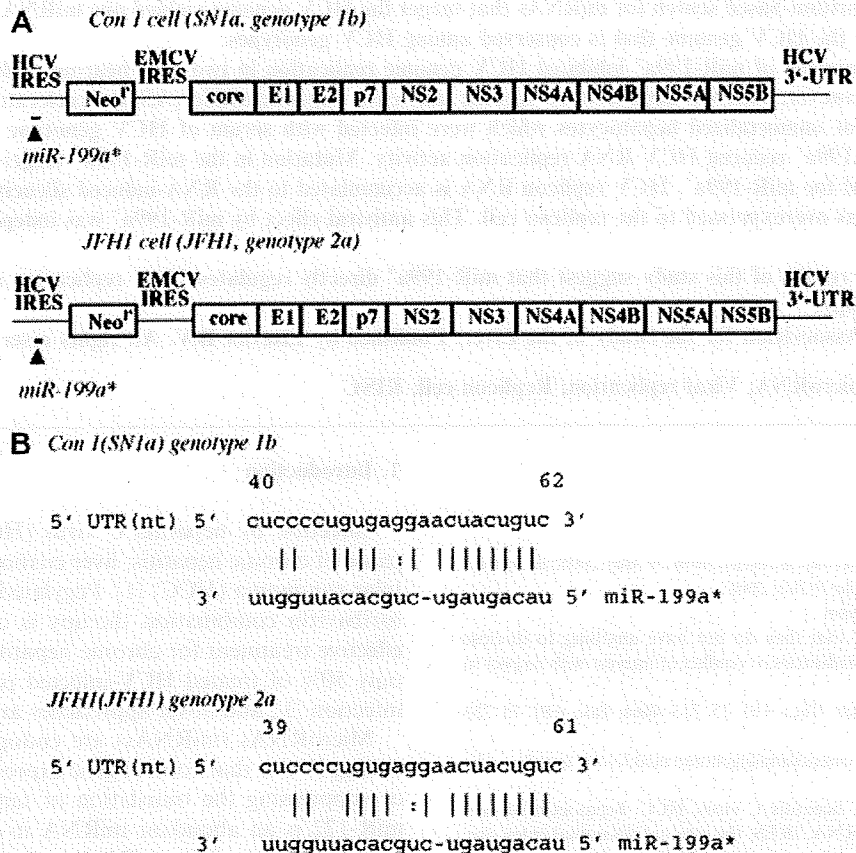


Fig. 1. (A) Schematic representation of the HCV genome-length replicon. The replicon comprised the HCV 5'-UTR, the HCV IRES, neomycin phosphotransferase gene (Neo^r), EMCV IRES, and the coding region from the core to NS5B and 3'-UTR. An arrowhead points to the target site. (B) miRNA target site sequences in the IRES of HCV genotype 1b and 2a. Numbers above sequences of 5'-UTR show nucleotide from 5' end. Vertical bars indicated the complementary bases between the HCV replicon genome and miRNA. The G:U wobble pair also was shown as colon.

Table 1
Part of list of oligonucleotide of miRNA expression vector from as to miR-199a* as from as to miR-19 as from as to Control as.

(a) Primer sequence	
MxA-s	5'-GCTACACACCGTGACGGATATGG-3'
MxA-as	5'-CGAGCTGGATTGGAAAGCCC-3'
PKR-s	5'-GCCTTTTCATCCAAATGGAATTC-3'
PKR-as	5'-GAAATCTGTTCTGGGCTCATG-3'
2'-5'-OAS-s	5'-TCAGAAGAGAAGCCAACGTGS-3'
2'-5'-OAS-as	5'-CGGAGACAGCGAGGGTAAAT-3'
(b) List of oligonucleotide for miRNA expression vector	
miR-199a* s	5'-GATCCGTACAGTAGTCTGCACATTG GTTTTCAAGAGAAACCAATGTGCAGACT ACTGTATTCAAGAGTTTTTTGGAAA-3'
as	5'-AGCTTTCCAAAAATACAGTAGTCTGCA CATTGGTTTCTCTTGGAAAACCAATGTGCAGA CTACTGTAGG-3'
miR-19a s	5'-GATCCTGTGCAAATCTATGCAAAACTGAT TCAAGAGATCAGTTTTGCATAGATTTG CACAGGAAA-3'
as	5'-AGCTTTTCCAAAAAATGTGCAAATCTATGC AAAACGTATCTCTTGAATCAGTTTTGCATAG ATTTGCACAG-3'
Control s	5'-GATCCCCTTTTTTTTGGAAA-3'
as	5'-AGCTTTTCCAAAAAAGGG-3'

2.2. Quantitative real-time PCR

The 5'-UTR of HCV genomic RNA was measured by using the ABI PRISM 7700 sequence detector (Applied Biosystems) as described previously [17].

2.3. Immunoblot analysis

Immunoblot analysis was performed as described previously [18], using the following primary antibodies: anti-NS3 antibody (a generous gift from Takamizawa A, Osaka University), anti-core antibody (32-1; kindly provided by Kohara M, The Tokyo Metropolitan Institute of Medical Science), and anti α -tubulin (Oncogene).

2.4. In vitro HCV infection

HuS-E/2 cells were transfected with miRNA expression vector by Effectene transfection reagent (Qiagen) and control siRNA or ASO was electroporated into HuS-E/2 cells 24 h prior to infection (Bio-Rad). *In vitro* HCV infection was performed as described previously [19]. HuS-E/2 cells were inoculated with serum prepared from an HCV positive blood donor or with concentrated JFH1 medium (in a titer equivalent to 10^5 HCV RNA copies). Twenty-four hours post-infection, the cells were washed three times with phosphate buffered saline (PBS) and then maintained in growth medium.

2.5. Quantification of miRNA expression level

On day 4 after transfection the TaqMan[®] microRNA assay (Applied Biosystems) was used to quantify miR-199a* expression by using U18 as an internal control.

2.6. Transfection type of miRNA in experiments of effects of mutated miRNA sequences

SN1a cells were plated in 6-well plates the day before transfection and grown to 50% confluence. Cells were transfected with 50 pmol of miR-199a* (Hokkaido System Science), two types of mutated miR-199a* (PROLIGO), and control siRNA, by using SilentFect. Cells were harvested at day 4.

2.7. Transient transfection and luciferase assay

A luciferase assay monitored HCV replicon-luciferase as described previously [13]. Briefly, cured MH14 cells (5×10^3 cell/well) were transfected into 48-well dishes with DMRIE-C (Invitrogen). 0.25 μ g HCV replicon-luciferase RNA, 0.5 μ g miRNA expression vector, and 10 ng Renilla expression vector (internal control) after 96 h later the transfected cells were harvested and lysed, and their luciferase activity was measured with a Dual-Luciferase Reporter Assay System kit (Promega). The experiments were repeated at least three times. An IRES retaining the conserved secondary structure but lacking the miR-199a* recognition site was generated by PCR-based mutagenesis as described elsewhere [20]. HCV replicon-luciferase RNA was transcribed *in vitro* by using a MEGAscript T7 kit (Ambion) according to the manufacturer's instructions.

2.8. Interferon-related-gene response test by luciferase assay and RT-PCR

pISRE (IFN α -stimulated response element)-Luciferase was based on the pGL3-Promoter Vector [13]. After seeding cured MH14, HepG2, and Huh-7 cells (3×10^5 each) in a 6-well plate 24 h before transfection then 2 μ g of pISRE-Luc, 2 μ g of miR-199a* expression plasmid, and 10 ng of Renilla expression plasmid (internal control) were transfected into cells using FuGENE 6. At 90 h post-transfection, these cells were treated with IFN α (Sigma; 500 IU/ml) for 6 h. Whole cell lysates were prepared and assayed with Dual-Luciferase Reporter Assay System kit.

Total RNA was extracted from the cells, and cDNA was synthesized from 5 μ g of total RNA. The primer sequences are shown in Table 1 [21].

2.9. Co-immunoprecipitation with Ago2

A cell lysate of RNA-induced silencing complex (RISC) was collected by using microRNA Isolation Kit, Human Ago2 (Wako) according to the manufacturer's instructions. Briefly, 48 h after transfection with miRNA expression plasmid, 5×10^6 cells were washed with PBS and harvested by trypsinization. The cells were suspended in PBS and mixed with beads conjugated human Argonaute2 (hAgo2) monoclonal antibody for 2 h at 4 °C. HCV replicon RNA from the Ago2-IP fraction was quantified by RT-qPCR.

2.10. Statistical analyses

Data were statistically analyzed by the Student's *t*-test; and *p* values less than 0.05 were considered statistically significant.

3. Results

3.1. The HCV genome contains a putative miRNA target sequence

To search for an miRNA with complementarity to the HCV genome, we performed *in silico* analysis with

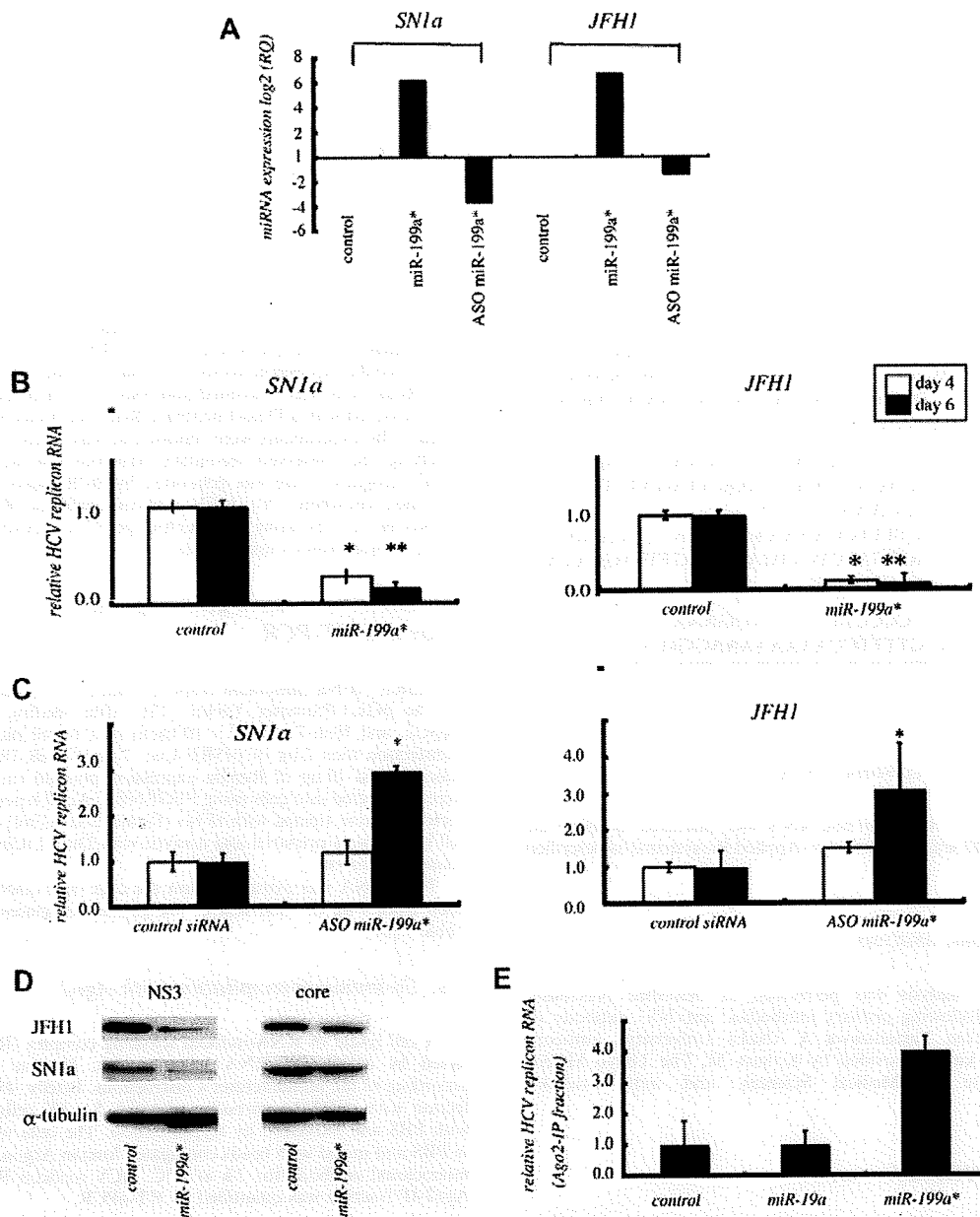


Fig. 2. Overexpression of miR-199a* decreased HCV replicon RNA levels. (A) Expression levels in SN1a and JFH1 cells after inducing overexpression of miR-199a* by miR-199a* expression vector or suppression of expression of miR-199a* by specific ASO were measured by real-time qPCR. (B) Comparison between HCV replicon RNA levels in SN1a (left) and JFH1 cell (right) before and after miRNA expression. The relative amounts of HCV replicon RNA per 50 ng of total RNA were measured by real-time qPCR at 4 and 6 days after transfection of miR-199a* and control vector. The data were normalized by the respective RNA levels in the controls. The data shown are as means \pm SD of four independent experiments. **Significant differences from the control at $p < 0.05$ and $p < 0.01$, respectively. (C) ASO with complementarity to miR-199a* stimulated HCV replicon RNA replication (left: SN1a cells, right: JFH1 cells). Relative amounts of HCV replicon RNA per 50 ng of total RNA were measured at day 4 and 6. (D) Immunoblot analysis of HCV core, NS3, and α -tubulin (internal control) in whole cell lysates from SN1a and JFH1 cells treated with miR-199a* expression or control vector. Fifty micrograms of whole cell lysates harvested 6 days after transfection was analyzed. (E) HCV replicon RNA is accumulated in RISC after miR-199a* expression. HCV replicon RNA was measured by real-time qPCR in 50 ng sample of total RNA from the Ago2-immunoprecipitation fraction.

ViTa algorithms [22]. miR-199a* was identified as having a target sequence in the internal ribosomal entry site (IRES) of both the genotype 1b replicon (Con 1; GeneBank Accession No. AJ238799) and 2a (JFH1; Gene-

Bank Accession No. AB047639) (Fig. 1). Endogenous expression levels of miR-199a* and 122 in HeLa, Huh-7, SN1a (containing Con 1 replicon (genotype 1b)) cells, and normal liver tissue were shown (Supplementary

Fig. 1). Endogenous expression level of miR-122 was abundant in Huh-7, SN1a, and normal liver tissue [6] and endogenous expression level of miR-199a* in Huh-7 and SN1a was more than in normal liver tissue.

3.2. miR-199a* overexpression suppresses the replication of HCV replicon

This study showed that the expression level of miR-199a* was modified by the expression vector and by ASO complementary to miR-199a* in cells bearing either replicon, SN1a or JFH1 (Fig. 2A). Overexpression markedly restricted replicon replication in both cell

lines (Fig. 2B). To determine whether miR-199a* has specific anti-viral effects, we inhibited miR-199a* activity with ASO. HCV replicon replication significantly increased in both cell lines upon treatment with ASO on day 4 and 6 (Fig. 2C). Immunoblot analysis showed good concordance with the result of real-time qPCR (Fig. 2D).

3.3. Ago2 co-immunoprecipitates target mRNA

To show that miR-199a* is associated with HCV genome physiologically [23], we performed an Ago2-co-immunoprecipitation (Ago2-IP) analysis to see whether

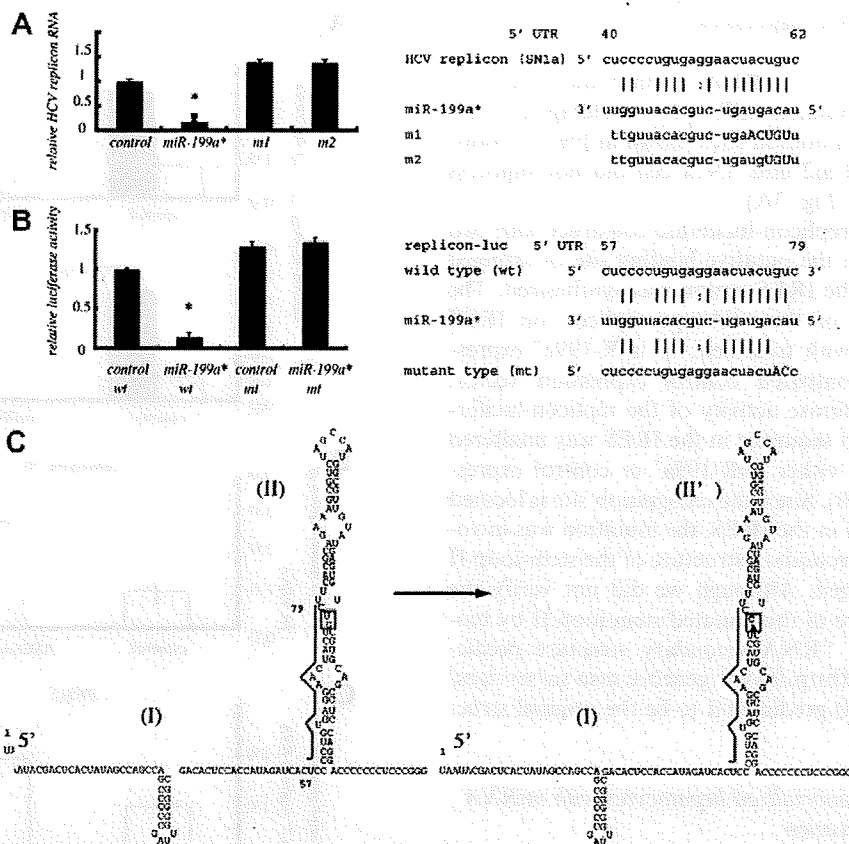


Fig. 3. Suppression of HCV replicon activity by miR-199a* was abolished by mutations in either the miRNA sequence or the miR-199a* target sequence in the HCV-replicon-luciferase construct. (A) Effect of mutated miR-199a* on HCV replicon activity. Comparison of HCV replicon RNA level in SN1a expressing ectopically with wild-type and mutated miRNAs measured by real-time qPCR. Complementarity among HCV replicon genome (upper number corresponds to the 5'-UTR start codon) and miR-199a* and two types of mutated miR-199a*: m1 and m2 are shown. Mutated nucleotides in m1 and m2 sequences are shown in capital letters. Additional nucleotides added to stabilize m1 and m2 are represented by tt. Relative HCV replicon RNA levels were normalized to the control siRNA level and are expressed as means ± SD of four independent experiments. *Significant difference at $p < 0.05$. (B) Effect of the miR-199a* on the wild-type and mutant-type HCV replicon activities. Comparison between the level of replicon activity in cured MH14 cell lysate after co-transfected of either miR-199a* expression vector or control vector, and either wild-type or mutant-type HCV replicon-luciferase construct. Sequences of wild-type (upper) and mutant-type (lower) HCV-replicon-luciferase in the IRES region are shown. Vertical bars and horizontal bars indicate complementary bases and gaps in complementarity between miR-199a* and the HCV-replicon-luciferase [13]. Mutated nucleotides are shown in capital letters. Nucleotide numbers begin at the start codon in the IRES. Mutated HCV-replicon-luciferase (mt) reduced the inhibitory effect of miR-199a*. Each column represents luciferase values standardized to the control vector co-transfected with the wild-type HCV-replicon-luciferase. The data shown are means ± SD of three replicates. (C) The target region of miR-199a* in the stem-loop II in IRES and the upstream stem-loop I together with its flanking region is shown. Small numbers indicate nucleotide positions from the 5' end [28]. The thick bar indicates the target site. The mutated sequence is shown in II' with the mutated residues boxed.

RISC could retain HCV genome when miR-199a* was over-produced. SN1a cells were transfected with either miR-199a*, miR-19a expression vector, or a control vector and used to prepare respective Ago2-IP fractionated cell lysates. Since miR-19a* does not recognize either HCV genome 1b or 2a, we used miR-19a as a negative control. The HCV-replicon RNA in the Ago2-IP fraction (IP RNA) was quantified by real-time qPCR. The concentration of HCV-replicon RNA was higher in the IP-RNA obtained by treatment with miR-199a* than by treatment with the control expression vector or miR-19a expression vector (Fig. 2E).

3.4. Effects of mutated miRNA sequences and target site sequences on miRNA suppression

We synthesized two miRNAs, with 5 and 3 bases in the sequence of mutated miR-199a*, yielding m1, and m2. We chose the mutation sites shown in Fig. 3. Transfection of m1 and m2 into SN1a cell did not suppress HCV RNA levels (Fig. 3A).

Mutated HCV-replicon-luciferase construct with two point mutations in the putative binding site of targeted by miR-199a* in the IRES region was synthesized. The luciferase activity of the wild-type replicon on IRES region decreased with treatment of miR-199a* expression vector as compared control expression vector. However, the luciferase activity of the replicon-luciferase with a mutated sequence in the IRES was unaltered by treatment with either miR-199a* or control expression vector (Fig. 3B). Since the recognition site is located in the stem-loop II in the IRES, the mutation was introduced so that the secondary structure of the stem-loop II would be unchanged. Although we did not verify the secondary structure of the mutated stem-loop II by biochemical analysis, "RNA secondary structure prediction" software (http://www.genebee.msu.su/services/rna2_reduced.html) predicted it to be the original structure (Fig. 3C).

3.5. Pretreating immortalized hepatocytes with miRNA reduces HCV replication

Immortalized HuS-E/2 cells ectopically expressing miR-199a* were infected with serum from an HCV-1b or HCV-2a-infected patients. The HCV replication was significantly lower in cells expressing miR-199a*, irrespective of the HCV genotype (Fig. 4A). In contrast, treatment of the cells before HCV infection with miR-199a* ASO caused to accumulate HCV RNA (Fig. 4B). We then performed an *in vitro* infection study with the infectious recombinant HCV-JFH1 virus. HuS-E/2 cells were inoculated with concentrated JFH1 medium. In HuS-E/2 cells after treatment with miR-199a* ASO, we found an increase in JFH1-RNA (Fig. 4C).

3.6. The anti-viral effect of miR-199a* is independent of the interferon (IFN) pathway

Cured MH14, HepG2, and Huh-7 cells co-transfected with the ISRE-luciferase reporter and either miR-199a* expression vector or the control vector were harvested and analyzed for luciferase activity. There was no significant difference in luciferase activity between the control and the miR-199a*-treated group (Fig. 5A) [24]. Moreover, no expression of the IFN-induced genes; MxA, PKR, and 2'-5'-OAS was induced by treatment of miR-199a* (Fig. 5B).

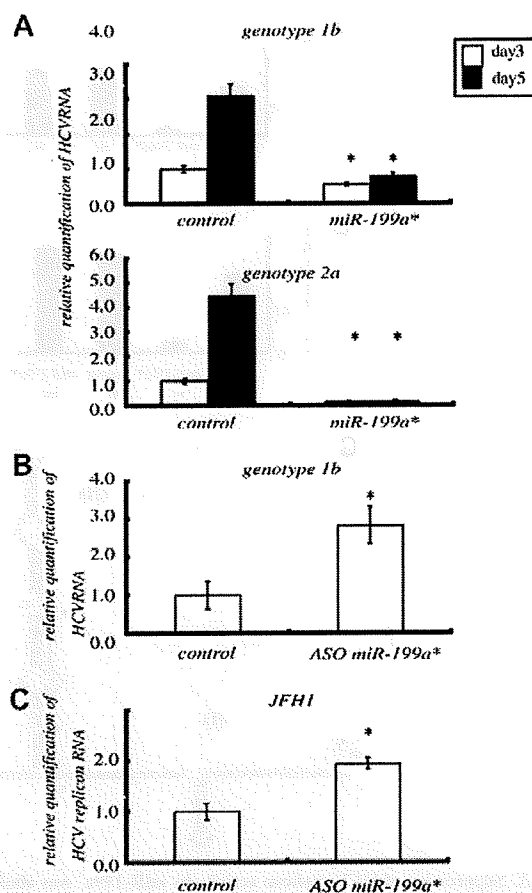


Fig. 4. Overexpression of miR-199a* reduced HCV replication. (A) Overexpression of miR-199a* reduced the amount of HCV RNA in HuS-E/2 cells infected with serum containing HCV-genotype 1b and 2a, in comparison with the control vector. Each column represents the relative amount of HCV RNA normalized to the vector control sample on post-transfection day 3 and 5. The data shown are means \pm SD of four independent experiments. **Significant difference at $p < 0.05$ and $p < 0.01$, respectively. (B) Prior transfection of HuS-E/2 cells with miR-199a* ASO accelerated HCV-1b replication activity on day 3. HCV RNA levels are expressed as means \pm SD of four independent experiments. (C) miR-199a* ASO prior to *in vitro* infection with JFH1 replicon RNA accelerated JFH1 replicon RNA. Experimental procedure is described in Section 2.

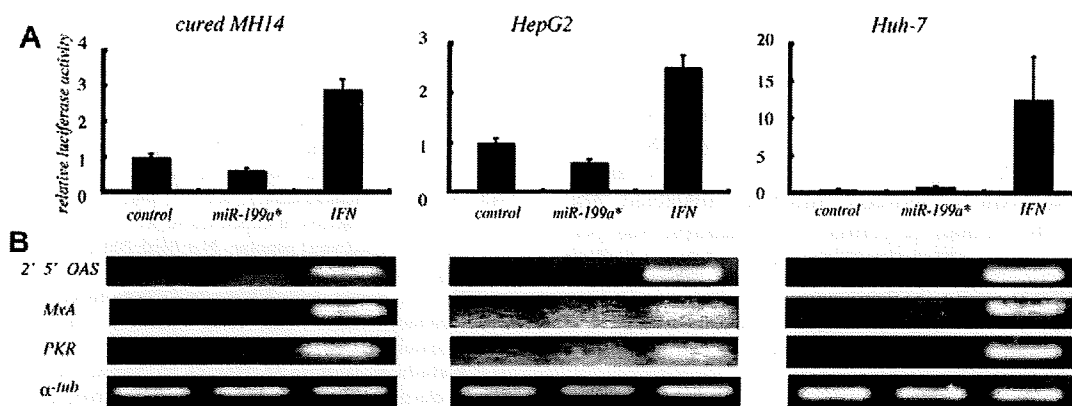


Fig. 5. Treatment of the cells with miR-199a* did not induce the IFN pathway. (A) Cured MH14 (left), HepG2 (middle) and Huh-7 cells (right) were co-transfected with pISRE-Luc and either miR-199a* expression vector or the control vector. In a control experiment to investigate IFN response, one line of cells treated with IFN α (500 IU/ml) for 6 h at 90 h after transfection of the control vector. The cellular luciferase activity was measured 96 h after transfection and normalized to luciferase activity of the cells treated with the control vector treatment. Each column represents the luciferase activity, normalized to the control vector. (B) 2'-5'-OAS, MxA, and PKR expression was measured by semi-quantitative RT-PCR on day 4 after transfection.

4. Discussion

This study demonstrates that HCV replication can be controlled by miRNA. Viruses use non-coding RNAs, such as miRNAs, to inhibit intrinsic anti-viral immunity in mammalian cells. For example, herpesvirus encodes viral miRNAs that dampen host antiviral immunity [25], and many mammalian viruses usurp or divert host mRNA silencing machinery to their advantage. By contrast, some host-encoded miRNAs have anti-viral functions [26]. RNA silencing-based antiviral responses may work in concert with innate and acquired antiviral systems [27].

miR-199a* has a target sequence in domain II of the IRES region in the HCV 5'-UTR, a region that is highly conserved across all HCV genotypes [28] and is crucial for viral replication. Introduction of miR-199a* ASO into replicon cells had the opposite effect of increasing viral replication. Mutagenesis analyses showed that the inhibitory effect of miR-199a* on HCV replication was highly dependent on complementarity between the viral and host sequences. Because HCV-replicon RNA is concentrated in the RISC by overexpression of miR-199a*, miR-199a* and HCV-replicon RNA seem to be complexed in cells under certain pathophysiological conditions. Our study indicates that the sequence-dependent interactions contribute to the anti-HCV activities of miR-199a*.

However, miRNAs often recognize target genes with incomplete complementarity, which allows them to recognize many target-candidate genes. To determine whether the anti-viral effect of the miRNA is mediated not only by mechanism that directly targets the HCV genome but by other mechanism, such as modulation of cellular genes, we analyzed the mRNA expression profiles of miR-199a*-transfected cells by microarray.

Based on the results of the microarray analysis, as shown in Supplementary Fig. 2, we identified several genes whose expression level changed at least twofold after overexpression. Ceruloplasmin (CP) has already been reported to be involved in HCV replication [29]. Although CP is not contained in a list of miRase targets (<http://microrna.sanger.ac.uk/target/v5/>) for miR-199a*, it will be necessary to determine whether CP is controlled by miR-199a* as miRNA machinery and whether CP is capable of participating in HCV replication.

Chronic HCV infection causes various liver diseases, from chronic hepatitis to hepatocellular carcinoma. Previously we demonstrated that miRNA expression profiles change with the degree of liver fibrosis and pathological differentiation of HCC [18]. In the present study we demonstrated that miR-199a* can control viral replication. miRNAs may have future application as efficient, safe, and specific means of antiviral therapy.

Acknowledgments

We thank R. Bartenschlager at Heidelberg University for providing the Con 1 strain and Kaku Goto for technical assistance. Y.M. and K.S. were supported by the Ministry of Health, Labour and Welfare of Japan; grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Appendix A. Supplementary data

Microarray analysis. Fluorescent (cyanine 3-CTP)-labeled cRNA was synthesized from 500 ng of total RNA with a Low RNA Input Fluorescent Linear

Amplification Kit (Agilent Technologies), and hybridized to a Human 1A(v2) Oligo microarray (Agilent Technologies). The signal intensity per spot was analyzed from scanned images using Feature Extraction Software ver8.5 (Agilent Technologies). To compare expression profiles between miRNA-transfected and control cells, median percentile normalization was performed using GeneSpring GX 7.3 (Agilent Technologies).

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2008.06.010.

References

- [1] Wasley A, Alter MJ. Epidemiology of hepatitis C: geographic differences and temporal trends. *Semin Liver Dis* 2000;20:1–16.
- [2] Foster GR. Past, present, and future hepatitis C treatments. *Semin Liver Dis* 2004;24:97–104.
- [3] McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, et al. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *New Engl J Med* 1998;339:1485–1492.
- [4] Pillai RS. MicroRNA function: multiple mechanisms for a tiny RNA? *RNA* 2005;11:1753–1761.
- [5] Zamore PD, Haley B. Ribo-gnome: the big world of small RNAs. *Science* 2005;309:1519–1524.
- [6] Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. *Curr Biol* 2002;12:735–739.
- [7] Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* 2005;309:1577–1581.
- [8] Randall G, Panis M, Cooper JD, Tellinghuisen TL, Sukhodolets KE, Pfeffer S, et al. Cellular cofactors affecting hepatitis C virus infection and replication. *Proc Natl Acad Sci USA* 2007;104:12884–12889.
- [9] Henry SD, van der Wegen P, Metselaar HJ, Tilanus HW, Scholte BJ, van der Laan LJ. Simultaneous targeting of HCV replication and viral binding with a single lentiviral vector containing multiple RNA interference expression cassettes. *Mol Ther* 2006;14:485–493.
- [10] Kapadia SB, Brideau-Andersen A, Chisari FV. Interference of hepatitis C virus RNA replication by short interfering RNAs. *Proc Natl Acad Sci USA* 2003;100:2014–2018.
- [11] Wang Y, Kato N, Jazag A, Dharel N, Otsuka M, Taniguchi H, et al. Hepatitis C virus core protein is a potent inhibitor of RNA silencing-based antiviral response. *Gastroenterology* 2006;130:883–892.
- [12] Yokota T, Sakamoto N, Enomoto N, Tanabe Y, Miyagishi M, Maekawa S, et al. Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep* 2003;4:602–608.
- [13] Murata T, Ohshima T, Yamaji M, Hosaka M, Miyanari Y, Hijikata M, et al. Suppression of hepatitis C virus replicon by TGF-beta. *Virology* 2005;33:407–417.
- [14] Ishii N, Watashi K, Hishiki T, Goto K, Inoue D, Hijikata M, et al. Diverse effects of cyclosporine on hepatitis C virus strain replication. *J Virol* 2006;80:4510–4520.
- [15] Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005;11:791–796.
- [16] Aly HH, Watashi K, Hijikata M, Kaneko H, Takada Y, Egawa H, et al. Serum-derived hepatitis C virus infectivity in interferon regulatory factor-7-suppressed human primary hepatocytes. *J Hepatol* 2007;46:26–36.
- [17] Watashi K, Hijikata M, Hosaka M, Yamaji M, Shimotohno K. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. *Hepatology* 2003;38:1282–1288.
- [18] Murakami Y, Yasuda T, Saigo K, Urashima T, Toyoda H, Okanoue T, et al. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. *Oncogene* 2006;25:2537–2545.
- [19] Kato N, Ikeda M, Mizutani T, Sugiyama K, Noguchi M, Hirohashi S, et al. Replication of hepatitis C virus in cultured non-neoplastic human hepatocytes. *Jpn J Cancer Res* 1996;87:787–792.
- [20] Ohshima T, Shimotohno K. Transforming growth factor-beta-mediated signaling via the p38 MAP kinase pathway activates Smad-dependent transcription through SUMO-1 modification of Smad4. *J Biol Chem* 2003;278:50833–50842.
- [21] MacQuillan GC, Mamotte C, Reed WD, Jeffrey GP, Allan JE. Upregulation of endogenous intrahepatic interferon stimulated genes during chronic hepatitis C virus infection. *J Med Virol* 2003;70:219–227.
- [22] Hsu PW, Lin LZ, Hsu SD, Hsu JB, Huang HD. ViTa: prediction of host microRNAs targets on viruses. *Nucleic Acids Res* 2007;35:D381–D385.
- [23] Karginov FV, Conaco C, Xuan Z, Schmidt BH, Parker JS, Mandel G, et al. A biochemical approach to identifying microRNA targets. *Proc Natl Acad Sci USA* 2007;104:19291–19296.
- [24] Naganuma A, Nozaki A, Tanaka T, Sugiyama K, Takagi H, Mori M, et al. Activation of the interferon-inducible 2'-5'-oligoadenylate synthetase gene by hepatitis C virus core protein. *J Virol* 2000;74:8744–8750.
- [25] Umbach JL, Kramer MF, Jurak I, Karnowski HW, Coen DM, Cullen BR. MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. *Nature* 2008;454:780–783.
- [26] Cullen BR. Viruses and microRNAs. *Nat Genet* 2006;38:S25–S30.
- [27] Reherrmann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. *Nat Rev* 2005;5:215–229.
- [28] Honda A, Arai Y, Hirota N, Sato T, Ikegaki J, Koizumi T, et al. Hepatitis C virus structural proteins induce liver cell injury in transgenic mice. *J Med Virol* 1999;59:281–289.
- [29] Fillebeen C, Muckenthaler M, Andriopoulos B, Bisaiillon M, Mounir Z, Hentze MW, et al. Expression of the subgenomic hepatitis C virus replicon alters iron homeostasis in Huh7 cells. *J Hepatol* 2007;47:12–22.

Heat-shock Protein 90 Is Essential for Stabilization of the Hepatitis C Virus Nonstructural Protein NS3*

Received for publication, August 20, 2008, and in revised form, December 22, 2008. Published, JBC Papers in Press, January 16, 2009, DOI 10.1074/jbc.M806452200

Saneyuki Ujino[‡], Saori Yamaguchi[‡], Kunitada Shimotohno^{§¶}, and Hiroshi Takaku^{¶||}

From the [‡]Department of Life and Environmental Sciences, ^{||}High Technology Research Center, and [§]Research Institute, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino, Chiba 275-0016, Japan and the [¶]Center for Integrated Medical Research, School of Medicine, Keio University, Shinanomachi, Tokyo 160-8582, Japan

The hepatitis C virus (HCV) is a major cause of chronic liver disease. Here, we report a new and effective strategy for inhibiting HCV replication using 17-allylamino geldanamycin (17-AAG), an inhibitor of heat-shock protein 90 (Hsp90). Hsp90 is a molecular chaperone with a key role in stabilizing the conformation of many oncogenic signaling proteins. We examined the inhibitory effects of 17-AAG on HCV replication in an HCV replicon cell culture system. In HCV replicon cells treated with 17-AAG, we found that HCV RNA replication was suppressed in a dose-dependent manner, and interestingly, the only HCV protein degraded in these cells was NS3 (nonstructural protein 3). Immunoprecipitation experiments showed that NS3 directly interacted with Hsp90, as did proteins expressed from Δ NS3 protease expression vectors. These results suggest that the suppression of HCV RNA replication is due to the destabilization of NS3 in disruption of the Hsp90 chaperone complex by 17-AAG.

Infection by the hepatitis C virus (HCV)² is a major public health problem, with 170 million chronically infected people worldwide (1, 2). The current treatment by combined interferon-ribavirin therapy fails to cure the infection in 30–50% of cases (3, 4), particularly those with HCV genotypes 1 and 2. Chronic infection with HCV results in liver cirrhosis and can lead to hepatocellular carcinoma (5, 6). Although an effective combined interferon- α -ribavirin therapy is available for about 50% of the patients with HCV, better therapies are needed, and preventative vaccines have not yet been developed.

HCV is a member of the *Flaviviridae* family and has a positive strand RNA genome (7, 8) that encodes a large precursor polyprotein, which is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, E1 (envelope 1), E2, p7, NS2 (nonstructural protein 2), NS3, NS4A, NS4B, NS5A, and NS5B (9, 10). NS2 and the amino terminus of NS3

comprise the NS2-3 protease responsible for cleavage between NS2 and NS3 (9, 11), whereas NS3 is a multifunctional protein consisting of an amino-terminal protease domain required for processing NS3 to NS5B (12, 13). NS4A is a cofactor that activates the NS3 protease function by forming a heterodimer (14–17), and the hydrophobic protein NS4B induces the formation of a cytoplasmic vesicular structure, designated the membranous web, which is likely to contain the replication complex of HCV (18, 19). NS5A is a phosphoprotein that appears to play an important role in viral replication (20–23), and NS5B is the RNA-dependent RNA polymerase of HCV (24, 25). The 3'-untranslated region consists of a short variable sequence, a poly(U)-poly(UC) tract, and a highly conserved X region and is critical for HCV RNA replication and HCV infection (26–29).

Hsp90 (heat-shock protein 90) is a molecular chaperone that plays a key role in the conformational maturation of many cellular proteins. Hsp90 normally functions in association with other co-chaperone proteins, which together play an important role in folding newly synthesized proteins and stabilizing and refolding denatured proteins in cells subjected to stress (30–34). Its expression is induced by cellular stress and is also associated with many types of tumor. Hsp90 inhibitors are currently showing great promise as novel pharmacological agents for anticancer therapy.

Hsp90 inhibitors have two major modes of action as preferential clients for protein degradation or as Hsp70 inducers. The benzoquinone ansamycin antibiotic geldanamycin and its less toxic analogue 17-allylamino-17-demethoxygeldanamycin (17-AAG) directly bind to the ATP/ADP binding pocket of Hsp90 (34–36) and thus prevent ATP binding and the completion of client protein refolding. Recently, Waxman *et al.* (37) demonstrated a role for Hsp90 in promoting the cleavage of HCV NS2/3 protease, using NS2/3 translated by rabbit reticulocyte lysate. Nakagawa *et al.* (38) also reported that inhibition of Hsp90 is highly effective in suppressing HCV genome replication. Hsp90 may directly or indirectly interact with any of the proteins NS3 through NS5B to regulate replication of the HCV replicon. More recently, Okamoto *et al.* (39) reported that Hsp90 could bind to FKBP8 (FK506-binding protein 8) and form a complex with NS5A. The interaction with FKBP8 has also been shown to be the mechanism by which Hsp90 regulates HCV RNA replication, a process in which Hsp90 clearly plays an important role.

In this study, we have demonstrated that NS3 also forms a complex with Hsp90, which is critical for HCV replication. On the basis of the findings that treating HCV replicon cells with

* This work was supported by a grant-in-aid for HCV research from the Ministry of Health, Labor, and Welfare of Japan and by a grant-in-aid for high technology research from the Ministry of Education, Science, Sports, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ To whom correspondence should be addressed: Dept. of Life and Environmental Science and High Technology Research Center, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino-shi, Chiba 275-0016, Japan. Tel.: 81-47-478-0407; Fax: 81-47-471-8764; E-mail: hiroshi.takaku@it-chiba.ac.jp.

² The abbreviations used are: HCV, hepatitis C virus; 17-AAG, 17-allylamino-17-demethoxygeldanamycin.

Stabilization of the HCV NS3 by Hsp90

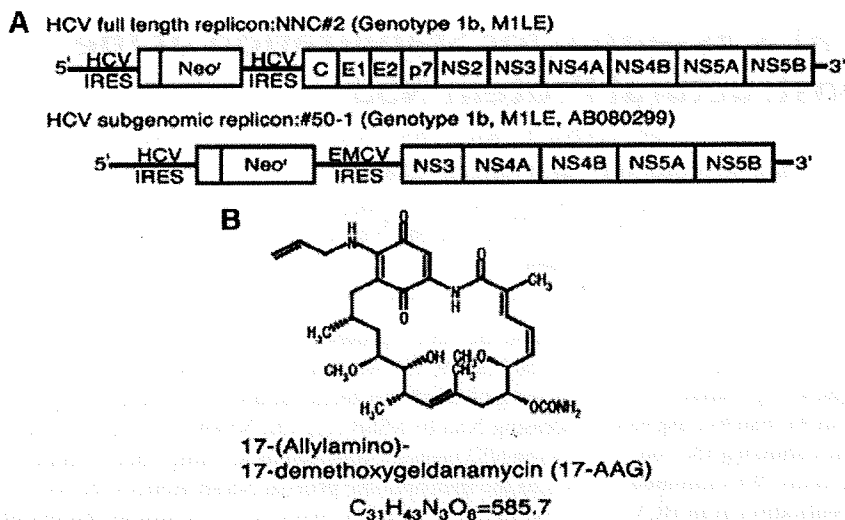


FIGURE 1. Schematic representation of HCV replicon and structure of 17-AAG. *A*, structure of the HCV replicon RNAs, comprising the HCV 5'-untranslated region, including the HCV internal ribosome entry site (IRES), the neomycin phosphotransferase gene (Neo^r), the encephalomyocarditis virus (EMCV) IRES or HCV IRES, and the coding region for HCV proteins NS3 to NS5B (in the HCV subgenomic replicon) or core to NS5B (in the HCV full-length replicon). *B*, structures of the Hsp90 inhibitor, 17-AAG.

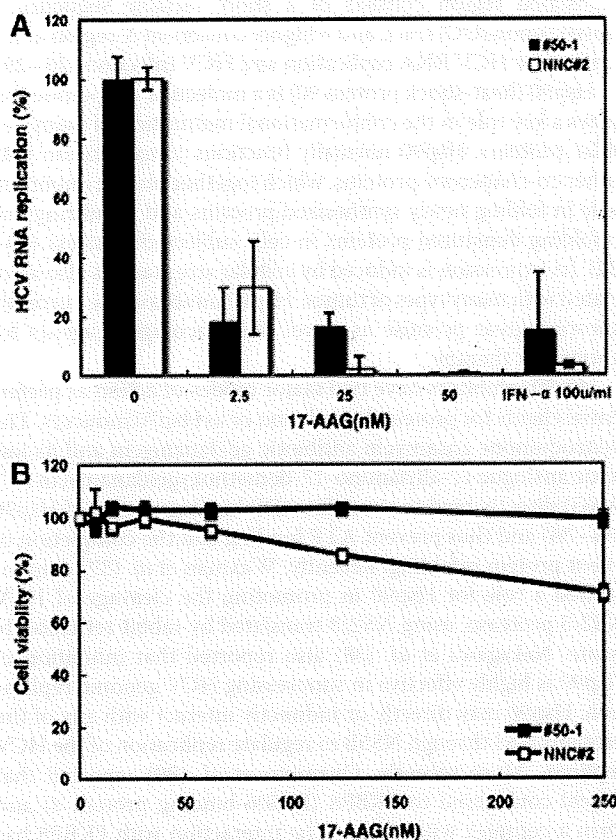


FIGURE 2. Hsp90 inhibits HCV RNA replication in HCV replicon cells. *A*, inhibition of HCV replication by 17-AAG in NNC#2 (white squares) and #50-1 cells (black squares) measured by real time reverse transcription-PCR after 72 h. Interferon- α was used as a positive control. The data are means \pm S.D. from triplicate experiments. *B*, cytotoxic effects of 17-AAG in NNC#2 (white squares) and #50-1 (black squares), shown as the percentage reduction in viable cell numbers in an [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (inner salt)] assay. The data are means \pm S.D. from triplicate experiments.

the Hsp90 inhibitor, 17-AAG, suppressed HCV RNA replication, and that the only HCV protein degraded in these cells was NS3, we suggest a crucial role for Hsp90-NS3 protein complexes in the HCV life cycle.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The HCV replicon cell lines #50-1 (NN/1b/SG) (40), which carries a subgenomic replicon, and NNC#2 (NN/1b/FL) (41), which carries a full genome replicon, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, nonessential amino acids, L-glutamine, penicillin/streptomycin, and 300–1,000 μ g/ml G418 (Invitrogen) at 37 °C in 5% CO₂. The human embryonic kidney-derived cell line 293T was grown in Dulbecco's modified

Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. 17-AAG was purchased from Sigma.

Measuring HCV RNA by Real Time PCR—HCV replicon cells were seeded at 1.5×10^5 cells in 24-well plates and cultured for 72 h. Total RNA was then isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. HCV RNA was quantified by real time reverse transcription-PCR using an ABI 7700 sequence detector (PerkinElmer Life Sciences) and the following primers and TaqMan probes located in the 5'-untranslated region: forward primer (nucleotides 130–146), 5'-CGGGAGAGCCATAGTGG-3'; reverse primer (nucleotides 272–290), 5'-AGTACCACAAGGCCTTCG-3'; and TaqMan probe (nucleotides 148–168), 5'-CTGCGGAACCGGTGAGTACAC-3' (all purchased from Applied Biosystems). The probe sequence was labeled with the reporter dye, 6-carboxyfluorescein, at the 5'-end and with the quencher dye TAMRA at the 3'-end (42).

Western Blotting and Immunoprecipitation Analyses—Cells were lysed in 1 \times CAT enzyme-linked immunosorbent assay buffer (Roche Applied Sciences). Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes, and these were blocked with 5% skimmed milk. The primary antibodies used were monoclonal or polyclonal antibody against FLAG-M5 (Sigma), Hsp70 (Sigma), Hsp90 (Cell Signaling Technologies, Danvers, MA), Hsp90 α (Calbiochem), Hsp90 β (Calbiochem), and Hsf-1 (Calbiochem). Core, NS4A, and NS4B were a gift from Dr. M. Kohara (Tokyo Metropolitan Institute of Medical Science). E1, E2, NS3, NS5A, and NS5B were a gift from Prof. Y. Matsuura (Osaka University, Japan). Immunoprecipitation from cell lysates was carried out using anti-FLAG M5 antibody (Sigma) and the Protein G immunoprecipitation kit (Sigma), according to the manufacturer's instructions, and the immunoprecipitates were analyzed by Western blotting.

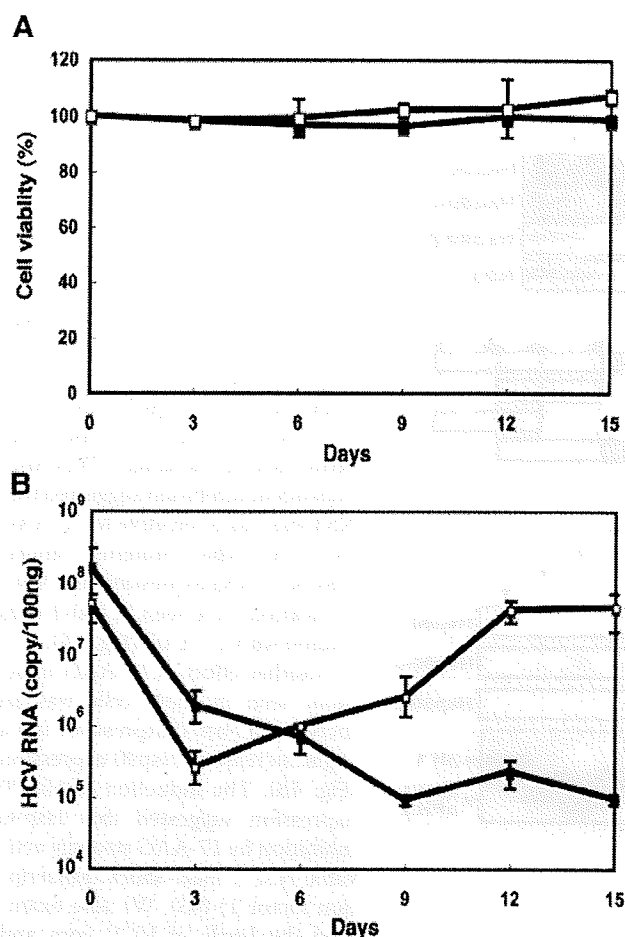


FIGURE 3. Long term inhibition of HCV replication in NNC#2 cells. A, cytotoxic effect of 17-AAG in NNC#2 cells, shown as the percentage reduction of viable cell numbers assessed by trypan blue staining. NNC#2 cells were treated with 50 nM 17-AAG on day 0 only (white squares) or at 3-day intervals for 15 days (black squares). The data are means \pm S.D. from triplicate experiments. B, measurement of HCV replication by real time reverse transcription-PCR. Inhibition of HCV RNA replication in NNC#2 cells treated with 50 nM 17-AAG on day 0 only (white squares) or at 3-day intervals for 15 days (black squares). Day 0, mock. The data are means \pm S.D. from triplicate experiments.

[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt Assay—HCV replicon cells were seeded in 96-well plates at 3×10^4 cells/well in a final culture volume of 100 μ l for 72 h before the addition of increasing concentrations of 17-AAG. After incubation for 3 days, viable cell numbers were determined using the Celltiter 96 Aqueous nonradioactive cell proliferation assay (Promega Corp., Madison, WI). The value of the background absorbance at 490 nm (A_{490}) of wells without cells was subtracted. The percentages of viable cells were then calculated using the formula, (A_{490} of 17-AAG-treated sample/ A_{490} of untreated cells) \times 100.

Plasmids and Transfection—The pFLAG-CMV-NS3 vector was constructed by subcloning a DNA fragment encoding full-length NS3, Δ helicase, Δ protease, Δ PH 1, Δ PH 2, and Δ H 1 into the EcoRI and XbaI sites of the pFLAG-CMVTM-2 expression vector (Sigma), so that the amino-terminal FLAG epitope was fused in frame with NS3. The core expression vector was a gift

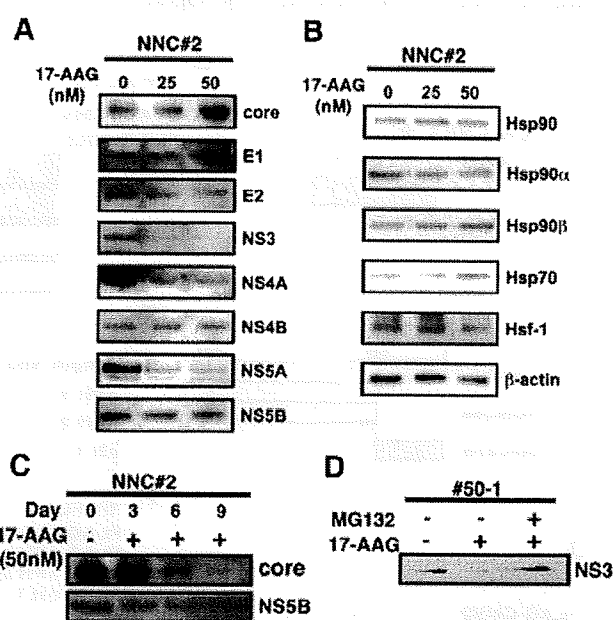


FIGURE 4. Effect of 17-AAG on HCV NS3 protein levels. A, Western blot analysis of HCV protein expression in NNC#2 or #50-1 cells treated with 17-AAG. NNC#2 or #50-1 cells were treated with 25 and 50 nM 17-AAG for 3 days. Cell lysates were separated by SDS-PAGE, immunoblotted, and probed with antibodies specific for HCV core, E1, E2, NS3, NS4A, NS4B, NS5A, and NS5B. B, Western blot analysis of Hsp90, Hsp70, and other chaperone expression in NNC#2 cells treated with 17-AAG (25 and 50 nM, as indicated) for 3 days. C, expression of HCV core and NS5B protein in cells treated with 50 nM 17-AAG for 9 days. D, effect of 50 nM 17-AAG on NS3 expression in #50-1 cells simultaneously treated with 100 nM MG132.

from Dr. M. Kohara. The vector was transfected into 293T cells using the FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's instructions.

RESULTS

Hsp90 Inhibitor 17-AAG Suppresses HCV RNA Replication—To investigate the effect of 17-AAG on HCV replication, cells containing a full HCV genome replicon (NNC#2) or a subgenomic replicon (#50-1) were treated with 17-AAG (Fig. 1, A and B). Both of the HCV replicon cell lines were treated for 72 h with different concentrations of 17-AAG or with DMSO as a control. In cells treated with 50 nM 17-AAG, HCV RNA replication was suppressed by 99% in both of the HCV replicon cell lines, and the inhibition of RNA replication occurred in a dose-dependent manner (Fig. 2A). The half-maximal inhibitory concentration (IC_{50}) values of 17-AAG for HCV replication were 0.3 nM in NNC#2 cells and 0.1 nM in #50-1 cells. Furthermore, we used a tetrazolium-based [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay to determine the viability of NNC#2 and #50-1 cells in the presence of 17-AAG. 17-AAG showed no toxicity to NNC#2 and #50-1 cells at 50 nM, (Fig. 2B). These results suggested that 17-AAG had a greater inhibitory effect on HCV RNA replication than 100 units/ml interferon- α .

Long Term Suppression of HCV RNA Replication—We next examined the effect of 17-AAG on HCV replication over time. When NNC#2 cells were cultured with 50 nM 17-AAG only on day 0 (white squares), the level of HCV RNA was reduced by 2 log on day 3 but had increased to control levels by day 12 (Fig.

Stabilization of the HCV NS3 by Hsp90

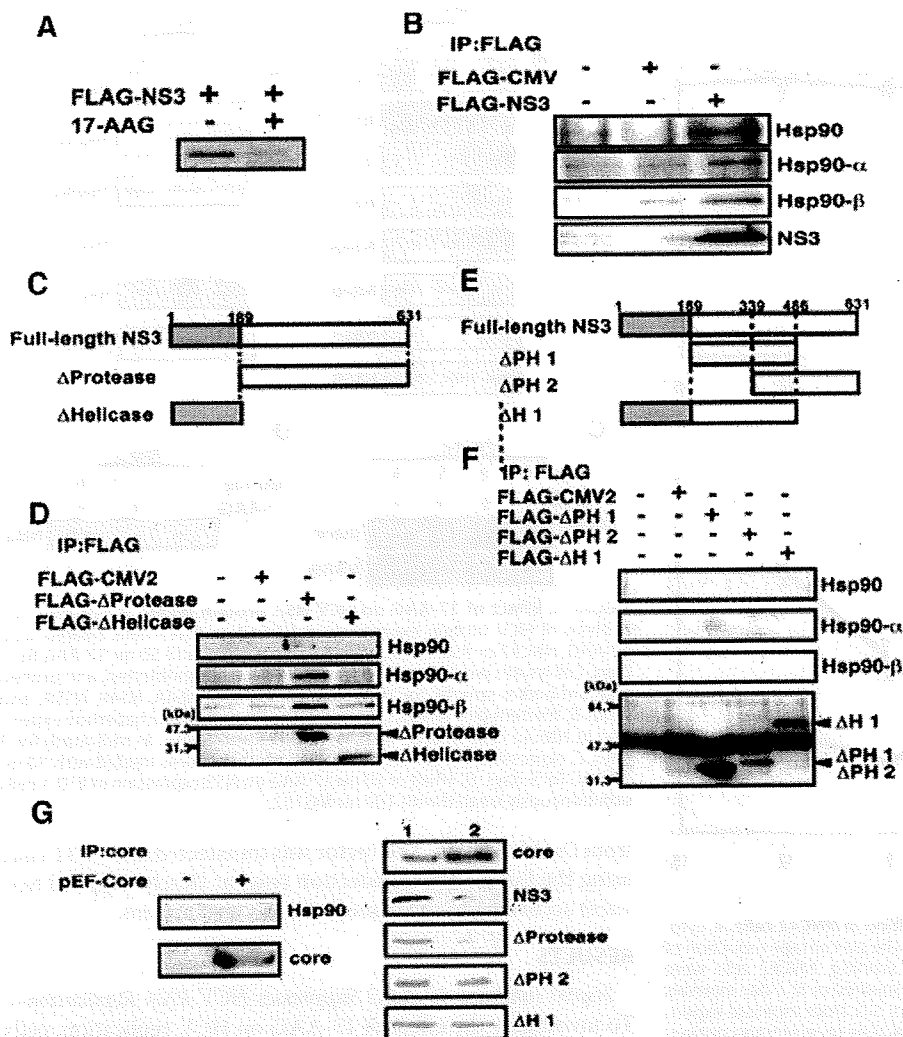


FIGURE 5. Hsp90 regulates HCV NS3 protein stability. A, Western blot showing the inhibition of NS3 protein expression in 293T cells caused by 17-AAG. Cells were transfected with pFLAG-NS3 in the presence of 250 nM 17-AAG for 48 h. B, FLAG-NS3 was expressed in 293T cells and immunoprecipitated (IP) from cell lysates with anti-FLAG antibody. Proteins immunoprecipitated were analyzed by Western blotting using anti-Hsp90, anti-Hsp90 α , anti-Hsp90 β , and anti-FLAG antibodies. The data shown in each panel are representative of three independent experiments. FLAG-CMV2, empty plasmid vector. C, schematic representations of HCV NS3 protein and its deletion mutants. D, FLAG-NS3, FLAG- Δ protease, and FLAG- Δ helicase were expressed in 293T cells and immunoprecipitated from cell lysates with anti-FLAG antibody. Proteins immunoprecipitated with anti-Hsp90, Hsp90 α , Hsp90 β , and FLAG antibodies were analyzed by Western blotting. The data shown in each panel are representative of three independent experiments. E, schematic representations of HCV NS3 protein and further deletion mutants. F, FLAG- Δ PH 1, FLAG- Δ PH 2, and FLAG- Δ H 1 were expressed in 293T cells and immunoprecipitated from cell lysates with anti-FLAG antibody. Proteins immunoprecipitated with anti-Hsp90, Hsp90 α , Hsp90 β , and FLAG antibodies were analyzed by Western blotting. The data shown in each panel are representative of three independent experiments. G, FLAG-NS3, pEF-Core, FLAG- Δ protease, FLAG- Δ PH 2, and FLAG- Δ H 1 were expressed in 293T cells treated with 17-AAG. Proteins immunoprecipitated with anti-core, Hsp90 antibody were analyzed by Western blotting. 17-AAG-treated cell lysates were analyzed on Western blots, using the specific antibodies shown to the right of the panels. Lane 1, control; lane 2, 17-AAG (1 μ M).

3B). However, when 50 nM 17-AAG was added to the cells at 3-day intervals for 15 days (black squares), the observed significant reduction in HCV RNA (by 3 log) was sustained from day 3 to day 15. We used trypan blue staining to check that long term treatment with 17-AAG did not induce cellular toxicity (Fig. 3A). Our results suggested that 17-AAG has the potential to safely induce long term suppression in HCV replication.

dependent on the proteasome system (44, 45).

Protein Folding in Hsp90-NS3 Interaction—To investigate the role of Hsp90 in HCV NS3 activation, the FLAG-NS3 protein was transfected into 293T cells, with or without 17-AAG, and the cell lysates were analyzed by Western blotting. The expression of NS3 from FLAG-NS3 was reduced in the presence of 17-AAG (Fig. 5A), suggesting that Hsp90 is involved in HCV NS3 degradation, possibly through a physical interaction.

Reduced Expression of NS3 Protein in 17-AAG-treated HCV Replicon Cells—To investigate the mechanism by which 17-AAG inhibited HCV replication, we analyzed the expression of HCV core, E1, E2, NS3, NS4A, NS4B, NS5A, and NS5B proteins by Western blotting. NNC#2 cells treated with increasing doses of 17-AAG showed a marked reduction in the expression of NS3 (Fig. 4A) after 3 days, in common with the level of HCV RNA (Fig. 2A). However, levels of the other proteins were unchanged. This dose-dependent inhibition suggested that NS3 was more sensitive to 17-AAG than the other proteins. Similar effects on NS3 expression and RNA replication were seen in #50-1 cells treated with 17-AAG (Fig. 4A).

Another effect of 17-AAG treatment seen in these cells was an increase in Hsp70 expression and a slight increase in Hsp90 expression (Fig. 4B). The induction of Hsp70 expression suggested that Hsp90 inhibition by 17-AAG strongly activated HSF-1 (heat-shock transcription factor 1) (43). We also examined the levels of HCV core and NS5B protein expression in NNC#2 cells treated with 50 nM 17-AAG. Reduced levels of these proteins were seen in NNC#2 cells on day 6, and both HCV core and NS5B protein were undetectable on day 9 (Fig. 4C). To determine whether 17-AAG promoted the degradation of NS3, we next looked at the effect of 17-AAG on #50-1 cells in which proteasomal degradation was also inhibited. Although 17-AAG treatment still induced a reduction in the NS3 protein level in #50-1 cells (Fig. 4D), the degradation of NS3 was completely blocked in the presence of the proteasome inhibitor, MG132. This suggested that the pharmacological effect of 17-AAG was

We confirmed this specific interaction by immunoprecipitating 293T cell lysates with anti-FLAG antibody. This clearly showed that FLAG and Hsp90 co-precipitated, suggesting that NS3 was bound to the chaperone complex formed with Hsp90 (Fig. 5B). NS3 mutants lacking the protease and helicase regions were generated in order to identify the region responsible for the interaction with Hsp90 (Fig. 5C). FLAG-NS3, FLAG-NS3- Δ helicase, or FLAG-NS3- Δ protease were transfected into 293T cells, and anti-FLAG antibody immunoprecipitates were analyzed by Western blotting (Fig. 5D). Although FLAG-NS3- Δ protease was clearly co-immunoprecipitated with Hsp90, no protein band corresponding to FLAG-NS3- Δ helicase was detected (Fig. 5D), suggesting that the NS3 helicase region mediates binding to Hsp90. To confirm this finding, plasmids expressing different NS3 helicase mutants fused with FLAG (Δ PH 1, Δ PH 2, and Δ H 1) were constructed (Fig. 5E). Expressing these NS3 helicase mutants in 293T cells and analyzing their immunoprecipitates with anti-FLAG antibody by Western blotting showed that, although all of the NS3 helicase mutant proteins were immunoprecipitated by anti-FLAG-antibody, no Hsp90 was co-precipitated (Fig. 5F).

We also confirmed that the NS3 helicase region mediated the specific interaction with Hsp90 by transfecting FLAG-NS3 and FLAG-NS3 deletion mutants into 293T cells pretreated with 17-AAG (Fig. 5G). The proteins expressed by FLAG-NS3 and FLAG-NS3- Δ protease were degraded in cells pretreated with 17-AAG, whereas no degradation of the Δ PH 2 and Δ H 1 NS3 mutants lacking helicase regions was seen (Fig. 5G). Further, when pEF-core was expressed in 293T cells, core was unable to co-immunoprecipitate Hsp90, and no degradation of core protein was observed (Fig. 5G). Our data demonstrate that 17-AGG destabilizes several binding proteins (NS3 and NS3- Δ protease) to Hsp90 but stabilizes some nonbinding proteins (the Δ PH 2 and Δ H 1 NS3 mutants lacking helicase regions and core) to Hsp90. In previous reports (46), similar effects were observed when wild-type and mutated p53 were translated in the presence of geldanamycin. These results further supported the hypothesis that Hsp90 has a role in folding the NS3 helicase domain and that this has an important role in stabilizing the full-length NS3 protein. A protein complex that includes NS3 and Hsp90 is therefore implicated in the control of HCV replication.

DISCUSSION

The Hsp90 inhibitor, 17-AAG, is known to have highly selective effects on tumor cells that are a result of its high affinity for Hsp90 client oncoproteins, which are incorporated into the Hsp90-dependent multichaperone complex, thereby increasing their binding affinity for 17-AAG more than 100-fold (47). This high selectivity effectively minimizes the toxic side effects of 17-AAG so that it is a good candidate for clinical application, especially in treating neurodegenerative diseases. In this study, we observed the inhibitory effects of 17-AAG on the replication of an HCV subgenomic replicon that lacked NS2. On the other hand, Waxman *et al.* (37) demonstrated a role for Hsp90 in promoting the cleavage of HCV NS2/3 protein using NS2/3 translated in rabbit reticulocyte lysate and expressed in Jurkat cells. Because the replicon cells used in our study genetically

lacked NS2, our results suggest that Hsp90 may directly interact with the NS3 protein in the HCV replicon.

In cell lines in which 17-AAG was a potent inhibitor of HCV replication, with IC_{50} values of 3–10 nM, we also found strong evidence that the association between HCV Hsp90 and NS3, but not other NS proteins, was the essential mechanism controlling the preferential degradation of NS3 after 17-AAG treatments. Furthermore, we showed that NS3 interacted with Hsp90 through the NS3 helicase domain. It was also clear that the expression of NS3 protein with helicase activity in 293T cells pretreated with 17-AAG was reduced, but the expression of NS3 mutants lacking the helicase regions (Δ PH 2 and Δ H 1) was not. The role of Hsp90 in achieving and/or stabilizing the NS3 protein was suggested by the fact that only 17-AGG bound to Hsp90 was capable of affecting NS3. The use of Hsp90 inhibitors represents a novel strategy for the development of anti-HCV therapies.

Acknowledgments—We are grateful to M. Sato, R. Tobita, and Y. Katamura for excellent technical assistance.

REFERENCES

- Alter, H. J., Purcell, R. H., Shih, J. W., Melpolder, J. C., Houghton, M., Choo, Q. L., and Kuo, G. (1989) *N. Engl. J. Med.* **321**, 1494–1500
- Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M. (1989) *Science* **244**, 359–362
- McHutchison, J. G., Gordon, S. C., Schiff, E. R., Shiffman, M. L., Lee, W. M., Rustgi, V. K., Goodman, Z. D., Ling, M. H., Cort, S., and Albrecht, J. K. (1998) *N. Engl. J. Med.* **339**, 1485–1492
- Glue, P., Rouzier-Panis, R., Raffanel, C., Sabo, R., Gupta, S. K., Salfi, M., Jacobs, S., and Clement, R. P. (2000) *Hepatology* **32**, 647–653
- Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., and Ohta, Y. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 6547–6549
- Seeff, L. B. (1997) *Hepatology* **26**, 21S–28S
- Bartenschlager, R., and Lohmann, V. (2001) *Antiviral Res.* **52**, 1–17
- Taylor, D. R., Shi, S. T., Romano, P. R., Barber, G. N., and Lai, M. M. (1999) *Science* **285**, 107–110
- Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M., and Rice, C. M. (1993) *J. Virol.* **67**, 1385–1395
- Hijikata, M., Mizushima, H., Akagi, T., Mori, S., Kakiuchi, N., Kato, N., Tanaka, T., Kimura, K., and Shimotohno, K. (1993) *J. Virol.* **67**, 4665–4675
- Grakoui, A., McCourt, D. W., Wychowski, C., Feinstone, S. M., and Rice, C. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 10583–10587
- Bartenschlager, R., Ahlborn-Laake, L., Mous, J., and Jacobsen, H. (1993) *J. Virol.* **67**, 3835–3844
- Grakoui, A., McCourt, D. W., Wychowski, C., Feinstone, S. M., and Rice, C. M. (1993) *J. Virol.* **67**, 2832–2843
- Bartenschlager, R., Lohmann, V., Wilkinson, T., and Koch, J. O. (1995) *J. Virol.* **69**, 7519–7528
- Failla, C., Tomei, L., and De Francesco, F. (1995) *J. Virol.* **69**, 1769–1777
- Lin, C., Thomson, J. A., and Rice, C. M. (1995) *J. Virol.* **69**, 4373–4380
- Tanji, Y., Hijikata, M., Satoh, S., Kaneko, T., and Shimotohno, K. (1995) *J. Virol.* **69**, 1575–1581
- Egger, D., Wolk, B., Gosert, R., Bianchi, L., Blum, H. E., Moradpour, D., and Bienz, K. (2002) *J. Virol.* **76**, 5974–5984
- Gosert, R., Egger, D., Lohmann, V., Bartenschlager, R., Blum, H. E., Bienz, K., and Moradpour, D. (2003) *J. Virol.* **77**, 5487–5492
- Blight, K. J., Kolykhalov, A. A., and Rice, C. M. (2000) *Science* **290**, 1972–1974
- Guo, J. T., Bichko, V. V., and Seeger, C. (2001) *J. Virol.* **75**, 8516–8523
- Krieger, N., Lohmann, V., and Bartenschlager, R. (2001) *J. Virol.* **75**, 4614–4624

Stabilization of the HCV NS3 by Hsp90

23. Lohmann, V., Hoffmann, S., Herian, U., Penin, F., and Bartenschlager, R. (2003) *J. Virol.* **77**, 3007–3019
24. Behrens, S. E., Tomei, L., and De Francesco, R. (1996) *EMBO J.* **15**, 12–22
25. Lohmann, V., Korner, F., Herian, U., and Bartenschlager, R. (1997) *J. Virol.* **71**, 8416–8428
26. Friebe, P., and Bartenschlager, R. (2002) *J. Virol.* **76**, 5326–5338
27. Kolykhalov, A. A., Mihalik, K., Feinstone, S. M., and Rice, C. M. (2000) *J. Virol.* **74**, 2046–2051
28. Yanagi, M., St. Claire, M., Emerson, S. U., and Purcell Bukh, J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 2291–2295
29. Yi, M., and Lemon, S. M. (2003) *J. Virol.* **77**, 3557–3568
30. Picard, D. (2002) *Cell Mol. Life Sci.* **59**, 1640–1648
31. Wegele, H., Muller, L., and Buchner, J. (2004) *Rev. Physiol. Biochem. Pharmacol.* **151**, 1–44
32. Pratt, W. B., and Toft, D. O. (2003) *Exp. Biol. Med.* **228**, 111–133
33. Smith, D. F., Whitesell, L., and Katsanis, E. (1998) *Pharmacol. Rev.* **50**, 493–514
34. McClellan, A. J., and Frydaman, J. (2001) *Nat. Cell Biol.* **3**, E1–E3
35. Grenert, J. P., Sullivan, W. P., Fadden, P., Haystead, T. A., Clark, J., Mimmnaugh, E., Krutzsch, H., Ochel, H. I., Schulte, T. W., Sausville, E., Neckers, L. M., and Toft, D. O. (1997) *J. Biol. Chem.* **272**, 23843–23850
36. Supko, J. G., Hickman, R. L., Grever, M. R., and Malspeis, L. (1995) *Cancer Chemother. Pharmacol.* **36**, 305–315
37. Waxman, L., Whitney, M., Pollok, B. A., Kuo, L. C., and Darke, P. L. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 13931–13935
38. Nakagawa, S., Umehara, T., Matsuda, C., Kuge, S., Sudoh, M., and Kohara, M. (2007) *Biochem. Biophys. Res. Commun.* **353**, 882–888
39. Okamoto, T., Nishimura, Y., Ichimura, T., Suzuki, K., Miyamura, T., Suzuki, T., Moriishi, K., and Matsuura, Y. (2006) *EMBO J.* **25**, 5015–5025
40. Kishine, H., Sugiyama, K., Hijikata, M., Kato, N., Takahashi, H., Noshi, T., Nio, Y., Hosaka, M., Miyanari, Y., and Shimotohno, K. (2002) *Biochem. Biophys. Res. Commun.* **290**, 993–999
41. Ishii, N., Watashi, K., Hishiki, T., Goto, K., Inoue, D., Hijikata, M., Wakita, T., Kato, N., and Shimotohno, K. (2006) *J. Virol.* **80**, 4510–4520
42. Takeuchi, T., Katsume, A., Tanaka, T., Abe, A., Inoue, K., Tsukiyama-kohara, K., Kawaguchi, R., Tanaka, S., and Kohara, M. (1999) *Gastroenterology* **111**, 636–642
43. Sittler, A., Lurz, R., Ueder, G., Priller, J., Lehrach, H., Hayer-Hartl, M. K., Hartl, F. U., and Wanker, E. E. (2001) *Hum. Mol. Genet.* **10**, 1307–1315
44. Bonvini, P., Dalla Rosa, H., Vignes, N., and Rosolen, A. (2004) *Cancer Res.* **64**, 3256–3264
45. Mimnaugh, E. G., Chavany, C., and Neckers, L. (1996) *J. Biol. Chem.* **271**, 22796–22801
46. Blagosklonny, M. V., Toretsky, J., Bohan, S., and Neckers, L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 8379–8383
47. Kamal, A., Thao, L., Sensintaffar, J., Zhang, L., Boehm, M. F., Fritz, L. C., and Burrows, F. J. (2003) *Nature* **425**, 357–359

Identification of cellular and viral factors related to anti-hepatitis C virus activity of cyclophilin inhibitor

Kaku Goto,¹ Koichi Watashi,^{1,2} Daisuke Inoue,¹ Makoto Hijikata¹ and Kunitada Shimotohno^{1,3,4}

¹Laboratory of Human Tumor Viruses, Department of Viral Oncology, Institute for Virus Research, Kyoto University, Kyoto, Japan; ²Molecular Virology Section, Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA; ³Research Center, Chiba Institute of Technology, Chiba, Japan

(Received April 25, 2009/Revised June 8, 2009/Accepted June 16, 2009/Online publication July 30, 2009)

We have so far reported that an immunosuppressant cyclosporin A (CsA), a well-known cyclophilin (CyP) inhibitor (CPI), strongly suppressed hepatitis C virus (HCV) replication in cell culture, and that CyPB was a cellular cofactor for viral replication. To further investigate antiviral mechanisms of CPI, we here developed cells carrying CsA-resistant HCV replicons, by culturing the HCV subgenomic replicon cells for 4 weeks in the presence of CsA with G418. Transfection of total RNA from the isolated CsA-resistant cells to naïve Huh7 cells conferred CsA resistance, suggesting that the replicon RNA itself was responsible for the resistant phenotype. Of the identified amino acid mutations, D320E in NS5A conferred the CsA resistance. The replicon carrying the D320E mutation was sensitive to interferon- α , but was resistant to CsA and other CPIs including NIM811 and sanglifehrin A. Knockdown of individual CyP subtypes revealed CyP40, in addition to CyPA and CyPB, contributed to viral replication, and CsA-resistant replicons acquired independence from CyPA for efficient replication. These data provide important evidence on the mechanisms underlying the regulation of HCV replication by CyP and for designing novel and specific anti-HCV strategies with CPIs. (*Cancer Sci* 2009; 100: 1943–1950)

Hepatitis C virus (HCV) is a leading cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC), and affects an estimated 170 million people worldwide.⁽¹⁾ The current standard therapy for patients infected with HCV is the combination treatment with pegylated interferon and ribavirin.^(2,3) However, approximately half of individuals infected with HCV are unable to reach sustained virological response following such treatment. In addition, several side effects have been reported, which hinder continued treatment and impair the regimen efficacy. Thus, the development of novel anti-HCV strategies is essential for the treatment of infected individuals.

We have previously reported that a well-known immunosuppressant cyclosporin A (CsA) strongly suppressed the replication of HCV *in vitro*, in a manner independent of the interferon (IFN) signal transduction pathway.⁽⁴⁾ Cyclophilin B (CyPB), a cellular target of CsA, was subsequently revealed to facilitate viral replication via the regulation of the RNA binding ability of NS5B.⁽⁵⁾ Thus CyP, in addition to viral proteins including NS3 protease and NS5B polymerase, can also prove useful as a molecular target for antiviral strategies. Indeed, the non-immunosuppressive CsA analogs NIM811, DEBIO-025, and SCY635 have been observed to exert strong inhibitory effects on HCV replication, and these compounds are now in clinical trial.^(6–8) Thus, it is crucial to deepen understanding of the anti-HCV actions of cyclophilin inhibitor (CPI) in order to maximize the efficacy of the agent. CPIs also need to face challenges such as side effects and drug resistance, which was observed as barrier to successful treatment in cases of human immunodeficiency virus (HIV),^(9–12) and further clarification of the mechanism of CPI's anti-HCV activities is vital for the

development of stronger and more specific therapeutic drug types. For this purpose, we here established and characterized the resistant replicon to CPIs using the subgenomic replicon system. We found that D320E, a mutation in NS5A, conferred resistance to CsA on the replicon, while additional mutations in NS3, Q86R and I252T seen in our CsA-resistant clone affected the replication fitness positively and negatively, respectively. The CsA-resistant replicons with the D320E mutation showed cross-resistance to other CPIs, NIM811 and sanglifehrin A (SFA), which were thus verified to suppress HCV replication through targeting CyP, and those resistant replicons were inhibited by treatment with IFN α as effectively as the wild type. Knockdown of individual CyP subtypes in the wild-type and CsA-resistant replicon cells revealed that CyP40, besides CyPA and CyPB, played important roles in HCV replication, and CyPA was related to the CsA-resistance. These results are important for elucidating additional mechanisms of the regulation of HCV replication by CyP and also for designing novel and specific anti-HCV strategies with CPI.

Materials and Methods

Compounds. CsA and IFN α were purchased from Merck Biosciences (San Diego, CA, USA) and Otsuka Pharmaceutical (Tokyo, Japan), respectively. NIM811 and SFA were generously provided by Novartis (Basel, Switzerland).

Cell culture. MH14 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum, nonessential amino acids (Invitrogen), and L-glutamine (Invitrogen) in the presence of 700 μ g/mL G418 (Invitrogen).

Establishment of cell clones. We established each cell clone along with the outline shown in Figure 1. CsR#4, CsR#10, and CsR#11 cells were established through the selection of MH14#12 cell colonies in the presence of 1000 μ g/mL G418 and 2 μ g/mL CsA. CsR#11-2 and CsR#11-3 cells were established from Huh7 cells transfected with total RNAs extracted from CsR#11 cells in the presence of 700 μ g/mL G418. Q86R, D320E, Q86R/D320E, and Q86R/I252T/D320E cells were produced by 700 μ g/mL G418 selection of Huh7 cells transfected with 5 μ g RNA transcribed from pMH14 carrying the individual mutations Q86R in NS3 and D320E in NS5A, double mutations Q86R in NS3 and D320E in NS5A, and triple mutations Q86R in NS3, I252T in NS3, and D320E in NS5A, respectively.

Colony formation assay. MH14 cells were treated with either CsA or NIM811 in the presence of 700 μ g/mL G418 for 4 weeks, followed by fixation and staining with crystal violet.

*To whom correspondence should be addressed.
E-mail: kunitada.shimoto@it-chiba.ac.jp

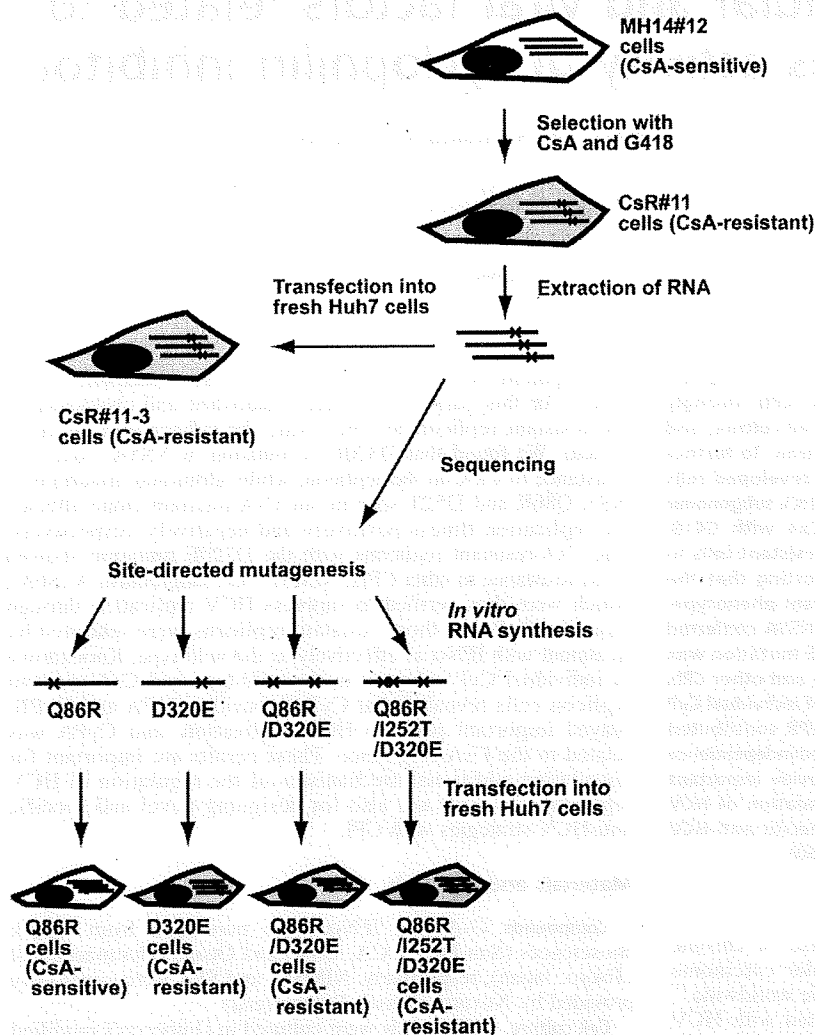


Fig. 1. Schematic diagram outlining the production of individual cell clones carrying hepatitis C virus (HCV) subgenomic replicons. MH14#12 cells, carrying wild-type HCV subgenomic replicon, were treated with 2 $\mu\text{g}/\text{mL}$ cyclosporin A (CsA) in the presence of 1000 $\mu\text{g}/\text{mL}$ G418 and CsR#11 cells were selected. Total RNA was extracted from CsR#11 cells and transfected into naïve Huh7 cells to select CsR#11-3 cells, and sequencing of the replicon RNA in CsR#11 cells identified mutations, Q86R in NS3, I252T in NS3, and D320E in NS5A. Site-directed mutagenesis followed by *in vitro* RNA synthesis generated HCV replicon RNA carrying Q86R, D320E, Q86R/D320E, and Q86R/I252T/D320E mutations. Transduction of the RNA into naïve Huh7 cells resulted in the production of Q86R, D320E, Q86R/D320E, and Q86R/I252T/D320E cells. The sensitivity of each replicon clone to CsA is presented as 'CsA-resistant' or 'CsA-sensitive'.

Real-time RT-PCR analysis. The 5'-non-translated region of HCV-RNA was quantified using an ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, CA, USA), as previously described.⁽⁴⁾

Replicon sequencing. Total RNA from replicon cells was extracted with sepaSol-RNA I Super (Nacalai Tesque, Kyoto, Japan) and subjected to RT-PCR reaction using super script III (Invitrogen). The products were then amplified by dividing the whole HCV region into approximately 300 bp using appropriate primer sets, and the sequence of the entire region encoding non-structural proteins was determined.

Plasmid construction. The Q86R and I252T mutations in NS3 and the D320E mutation in NS5A were generated via site-directed mutagenesis using the following primer sets: Q86R (S) 5'-AGGACCTCGTCCGCTGGCGGGCGCC-3' plus Q86R (AS) 5'-GGCGCCCGCAGCCGACGAGGTCCCT-3', I252T (S) 5'-AACACCAGAACTGGGGTAAGGACCA-3' plus I252T (AS) 5'-TGGTCCTTACCCAGTTCTGGTGTT-3', and D320E (S) 5'-GAGTATAATCCTCCACTGCTAGAGC-3' plus D320E (AS) 5'-GCTCTAGCAGTGGAGGATTATACTC-3', respectively. The PCR products carrying either Q86R in NS3, I252T in NS3, or D320E in NS5A were inserted into the NotI-MluI and MluI-XbaI sites of pMH14, respectively. The resultant plasmids were termed pMH14 (Q86R), pMH14 (I252T), and pMH14 (D320E)

respectively. The double mutant carrying both Q86R and D320E mutations was produced by exchanging the MluI-XbaI region of pMH14 (Q86R) with that of pMH14 (D320E), and termed pMH14 (Q86R/D320E). The triple mutant carrying Q86R, I252T, and D320E was produced by exchanging the NotI-MluI region of pMH14 (D320E) with the fragments amplified by the primer set, I252T (S) plus I252T (AS), using pMH14 (Q86R) as templates for the PCR reaction. Sequence analysis of the resultant plasmids was also undertaken for confirmation of the mutations.

***In vitro* RNA synthesis.** Wild-type and mutant RNA of pMH14 was prepared by *in vitro* transcription using the MEGAscript T7 kit (Ambion, Austin, TX, USA), as described previously.⁽¹³⁾

Electroporation and colony formation. 8×10^6 cells suspended in 400 μL of cytomix buffer (120 mM KCl, 0.15 mM CaCl_2 , 10 mM K_2HPO_4 , 25 mM HEPES, 2 mM EGTA, and 5 mM MgCl_2 , together with 2 mM ATP, 5 mM reduced form of glutathione, and 1.25% DMSO) were electroporated at 250 V, 950 μF with either 100 μg total RNA extracted from replicon cells or 5 μg RNA transcribed *in vitro* from the HCV replicon construct cDNA. Cells were then treated with 1000 $\mu\text{g}/\text{mL}$ G418 for 4 weeks following electroporation.

RNAi. Validated siRNAs against CyPB were purchased from Invitrogen. siRNA duplexes against CyPA (siCyPA161, 5'-UCUGUGAAAGCAGGAACCCUU-3'; siCyPA285, 5'-GAUG

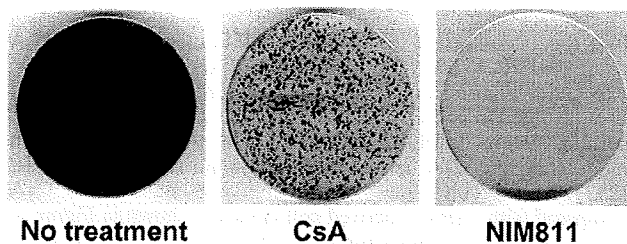


Fig. 2. Cyclophilin (Cp) inhibitor (CPI)-resistant colony emergence. MH14#12 cells were treated either without (left panel) or with 2 $\mu\text{g}/\text{mL}$ CPis, cyclosporin A (CsA) (middle panel), and NIM811 (right panel), in the presence of 700 $\mu\text{g}/\text{mL}$ G418. Following 4 weeks in culture, cells were fixed and stained with crystal violet.

CCAGGACCCGUAUGCUU-3'; siCyPA459, 5'-CUUCUUG CUGGUCUUGCCAUU-3') were synthesized (Yahima Pure Chemicals, Osaka, Japan). siRNAs against CyP40 were purchased from Invitrogen (siCyP40-3) and from Ambion (siCyP40-4). Pre-designed siRNAs, siCyPC, siCyPE, siCyPF, and siCyPG were obtained from Ambion. Transfection was performed using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) with 20 nM siRNAs in the absence of CsA according to the manufacturer's protocol.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. RT-PCR was performed as described previously⁽⁴⁾ using the following primer sets: 5'-TGTTCCTTCGACATTGCCGTC-3' and 5'-CAGTCTTGGCAGTGCAGATG-3' to detect mRNA for CyPA, 5'-TCTCCGAACGCAACATGAAG-3' and 5'-CTGCCGATGATCACATCCTTC-3' to detect mRNA for CyPB, 5'-GGCGCACTTGTTTCTTC-3' and 5'-TGCCATAGTCTTCAGCTTG-3' to detect mRNA for CyPC, 5'-TTTCGTGCACTGTGTACAGG-3' and 5'-TTGGCTCTATCTGCTGTCTC-3' to detect mRNA for CyP40, 5'-AGAGGAAGTGGACGACAAAG-3' and 5'-GATGTCCATGTACACCTGAG-3' to detect mRNA for CyPE, 5'-TGGAGCTGAAGGCAGATGTC-3' and 5'-ACGTGACCGAACACAACATG-3' to detect mRNA for CyPF, 5'-GAGTTGTCTCTTTCACAGAG-3' and 5'-AACTGAGTATCCGTACCTCC-3' to detect mRNA for CyPG, and 5'-ATGGGGGAAGGTGAA GGTCGG-3' and 5'-TGGAGGGATCTCGCTCCTGG-3' to detect glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

Results

Resistance emergence against individual CPis. We have previously demonstrated the robust anti-HCV activities of CPis, and it was reported that CPI significantly decreased HCV viral load in HCV-infected patients.^(14,15) The problem of the drug-resistant HCV variants, hence, should be assessed *in vitro*, considering that practical efficacies of these inhibitors with long-term effectiveness are required in patients. In the first step of this study, we investigated the emergence of drug resistant replicon against CPis. We treated MH14#12 cells, Huh7 cells carrying wild-type MH14 replicon with 2 $\mu\text{g}/\text{mL}$ CsA, or the non-immunosuppressive analog NIM811 in the presence of 700 $\mu\text{g}/\text{mL}$ G418 for 4 weeks. To visualize the appearance of drug-resistant clones, we stained cells after the selection. We observed colonies resistant to CsA, while we obtained few colonies under the treatment with the same concentration of NIM811 (Fig. 2).

Isolation and characterization of replicon cells resistant to CsA. To characterize the CsA-resistant HCV, we isolated the resistant clones following selection with 2 $\mu\text{g}/\text{mL}$ CsA and 1000 $\mu\text{g}/\text{mL}$ G418 for 4 weeks. We obtained several clones (named CsR cells), and examined their CsA responses. In contrast to the wild-type MH14#12 replicon cell, which showed an approximately 2-log

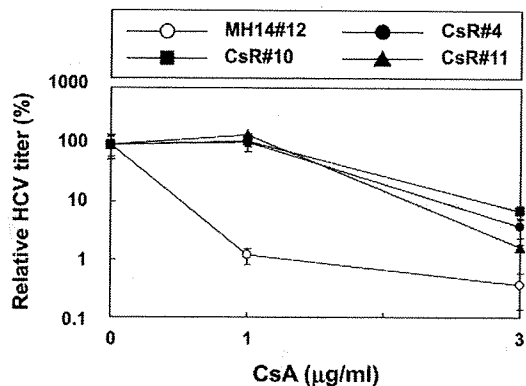


Fig. 3. Cyclosporin A (CsA) responses of the hepatitis C virus (HCV) replicon clones surviving the selection with CsA and G418. MH14#12 cells and three MH14#12-derived cell clones that survived double selection with G418 and CsA, CsR#4, CsR#10, and CsR#11, were treated with 1 and 3 $\mu\text{g}/\text{mL}$ CsA for 7 days, and the HCV-RNA titers were measured by real-time RT-PCR. The data represent the percentage of HCV-RNA level in cells either untreated or treated with CsA, and the dots represent the means of three independent experiments.

reduction of HCV-RNA level by treatment with 1 $\mu\text{g}/\text{mL}$ CsA for 7 days, all the clones isolated (the results of three representative clones, CsR#4, CsR#10, and CsR#11 cells are shown here) demonstrated resistant phenotypes against CsA with no significant reduction of HCV-RNA by CsA treatment at 1 $\mu\text{g}/\text{mL}$ (Fig. 3). The resistance of these clones was thought to arise as a result of (1) mutations on the HCV-RNA genome or (2) alterations in cellular factors. To test the first possibility, we investigated whether HCV-RNA itself in CsR#11 could induce the CsA resistance to naïve cells. Fresh Huh7 cells were transfected with total RNA, including HCV replicon RNA, extracted from CsR#11 cells or MH14#12 cells as controls and cultured for 3 weeks in the presence of G418 (Fig. 1). The resulting colonies were isolated and propagated individually (named cell clones from total RNA of wild-type MH14#12, MH14#12-1, MH14#12-4, and MH14#12-5 cells, and those from CsR#11, CsR#11-2, CsR#11-3, and CsR#11-5 cells). The HCV-RNA titer in MH14#12-derived cells was reduced approximately to 100th by treatment with 1 $\mu\text{g}/\text{mL}$ CsA for 7 days (Fig. 4). In contrast, cell clones generated from CsR#11 cells retained a normal HCV titer level after treatment with CsA, indicating that they had lost their sensitivity to CsA. Thus, it was suggested that the CsA-resistant profile in CsR#11 cells was attributed to its HCV-RNA.

D320E mutation in NS5A confers HCV replicon resistance to CsA. In order to identify the mutation in the HCV genome that resulted in the resistance to CsA, HCV subgenomic RNA isolated from CsR#11 cells was sequenced across the subgenomic region encoding non-structural proteins. We found three specific base changes that resulted in amino acid alteration including changes from glutamine to arginine, and isoleucine to threonine at positions 86 (Q86R) and 252 (I252T) in NS3, respectively, and a change from aspartic acid to glutamic acid at position 320 (D320E) in NS5A. Given that all these three mutations, Q86R, I252T, and D320E, were retained in every replicon in CsR#11-2, CsR#11-3, and CsR#11-5 cells, it is likely that they are inherited from CsR#11 cells and are associated with the acquired CsA-resistant phenotype. To examine this possibility, we synthesized replicon RNA carrying all these three mutations and established cells carrying these replicons. The resultant cell clones were named Q86R/I252T/D320E-1 and -3 cells. Treatment of these cells with 1 $\mu\text{g}/\text{mL}$ CsA decreased the HCV titer only