12 patients showed more than 2.0-fold higher expression (30%) (Figure 5C). Based on the clinical data and pathological diagnosis (see Supplementary Table 2 in the online version of this article), there is no significant difference in miR-500 expression between hepatitis virus B and C infection (Figure 5D). Importantly, significant difference in miR-500 expression was found between normal liver and liver cirrhosis samples, but not chronic hepatitis (Figure 5E), suggesting that miR-500 expression was upregulated during cirrhosis development. Thus, although only limited samples expressed miR-500 higher, miR-500 might be useful as a biomarker in the early stage of liver cancer.

### *Expression profiling of miR-500 in HCC patient serum*

Recently, it has been reported that miRNAs are circulating in serum (Chim et al. 2008, Gilad et al. 2008) and tumour-derived miRNAs such as miR-155, miR-21, miR-15b, miR-16 and miR-24 are detected in the plasma and serum of tumour patients (Mitchell et al. 2008, Lawrie et al. 2008). In fact, an increased amount of miR-500 was found in the sera of three out of ten HCC patients, which means that liver cancer-specific miRNA such as miR-500 is circulating in the peripheral blood and can be a novel diagnostic marker. To determine whether or not serum levels of miR-500 truly reflect the presence of cancer in the HCC patients, the presence of miR-500 in the sera of three human HCC patients, post- and presurgical treatment, was also assessed. As can be seen in Figure 6, elevated serum levels of miR-500 in the three HCC patients were significantly reduced after surgery and returned to normal levels. These results expect that the miR-500s abundance profile in serum of the HCC patients might reflect physiological and/or pathological conditions.

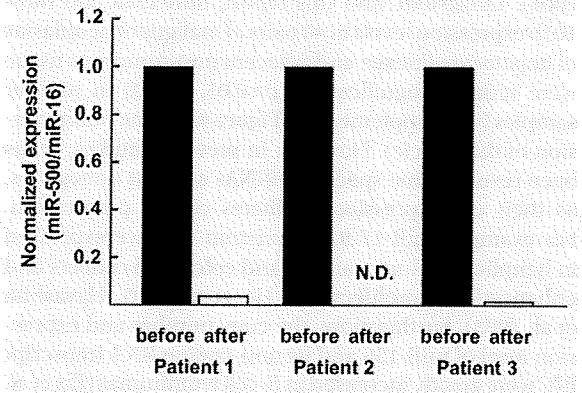


**Figure 6.** Serum levels of miR-500 in hepatocellular carcinoma (HCC) patients. Changes of serum levels of miR-500 in HCC patients ($n=3$) before (preoperation) and after (postoperation within 6 months) surgical removal of the tumour. Expression levels of the miR-500 are normalised to miR-16. N.D., not detected.

## Discussion

Using a global miRNA expression profile in mouse liver development analysed by an LNA-based miRNA microarray, our data indicate that dynamic changes in miRNA expression occur in mouse liver development. However, the number of high-expressed miRNAs was quite limited at all developmental stages of the liver. This finding is also consistent with several reports that dominant miRNA expression is rigidly controlled in a developmental stage-specific and tissue- or cell-type-specific manner (Chen et al 2006, Shan et al. 2007). For example, it has been reported that the expressions of miR-1 and miR-133 are high and specific in adult cardiac and skeletal muscles and modulate skeletal muscle proliferation and differentiation by negatively regulating the histone deacetylase-4 or serum response factor (Chen et al. 2006). On the other hand, expression levels of the general miRNA are low at all stages of liver development. However, our data indicate that the expression pattern of some of the low-expressed miRNAs, including let-7 family, also dramatically change in the process of mouse liver development (Figure 4B). Using this platform, the overall regulation of individual miRNAs of sequential stages of liver development was determined, providing us with a useful baseline for understanding the developmental dynamics of liver miRNA expression.

In this study, we identified a novel cancer biomarker candidate, miR-500, which was designated as an oncofetal miRNA in the early stage of liver cancer, because miR-500 expression is highly expressed in a fetal liver and downregulated in the developmental process and then upregulated in the process of liver cirrhosis. When the expression profile of miR-500 in human tissues was examined, its expression was not specific in the liver and was broadly detected in all tissues (see Supplementary Figure 3 in the online version of this article). However, the expression level of miR-500 is high at the early stages of liver development in mice and humans. Furthermore, miR-500 was abundantly expressed in human liver cancer cell lines (JHH-7, Huh-7, HepG2, Hep3B and Alexander) and liver cancer tissues. Interestingly, six miRNAs (mir-532, 188, 362, 501, 660, 502) in addition to miR-500 make a cluster within a 10-kb distance from miR-500, and their expressions could be modulated by the same transcriptional regulatory unit. However, the levels were not remarkably changed during mouse liver development. Therefore, by analysing these miRNAs together, miR-500 might be a better biomarker in HCC.

We tried to test the effect of miR-500 using liver cancer cell lines. In a knock-down analysis of miR-500 with miR-500 LNA, significant changes in cell proliferation and colony formation were not observed in both Alexander and JHH-7 cells (see Supplementary Figure 5A and B in the online version of this article). Likewise, mature

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 (Johnnidis 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 *HMGA2* 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.



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

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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.**



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

## 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

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

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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 an 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 Silencer® negative control siRNA (Ambion) were transfected with SilentFect (Bio-Rad).

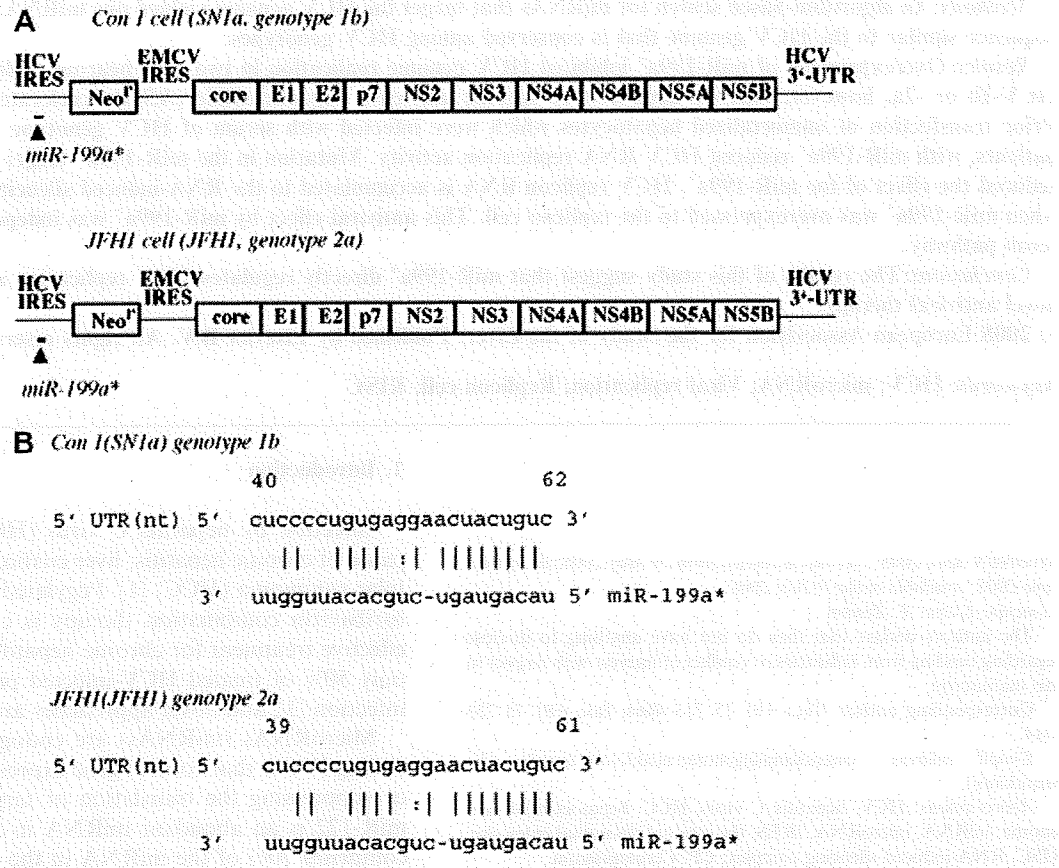


**Fig. 1. (A) Schematic representation of the HCV genome-length replicon. The replicon comprised the HCV 5′-UTR, the HCV IRES, neomycin phosphotransferase gene (Neo^r), EMCV IRES, and the coding region from the core to NS5B and 3′-UTR. An arrowhead points to the target site. (B) miRNA target site sequences 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′-AGCTTTCCAAAAAATACAGTAGTCTGCA CATTGGTTTCTCTTGAAAACCAATGTGCAGA CTACTGTAGG-3′ |
| miR-19a s | 5′-GATCCTGTGCAAATCTATGCAAAACTGAT TCAAGAGATCAGTTTTGCATAGATTTG CACAGGAAA-3′ |
| as | 5′-AGCTTTTCCAAAAAATGTGCAAATCTATGC AAAACTGATCTCTTGAATCAGTTTTGCATAG ATTTGCACAG-3′ |
| Control s | 5′-GATCCCCTTTTTTTTGGAAA-3′ |
| as | 5′-AGCTTTTCCAAAAAAAAGGG-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 $10^5$ HCVRNA 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. Cell 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 ($5 \times 10^3$ cell/well) were transfected into 48-well dishes with DMRIE-C (Invitrogen). 0.25 μg HCV replicon-luciferase RNA, 0.5 μg 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 (IFNα-stimulated response element)-Luciferase was based on the pGL3-Promoter Vector [13]. After seeding cured MH14, HepG2, and Huh-7 cells ($3 \times 10^5$each) in a 6-well plate 24 h before transfection then 2 μg of pISRE-Luc, 2 μg 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 IFNα (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 μg 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, $5 \times 10^6$ 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

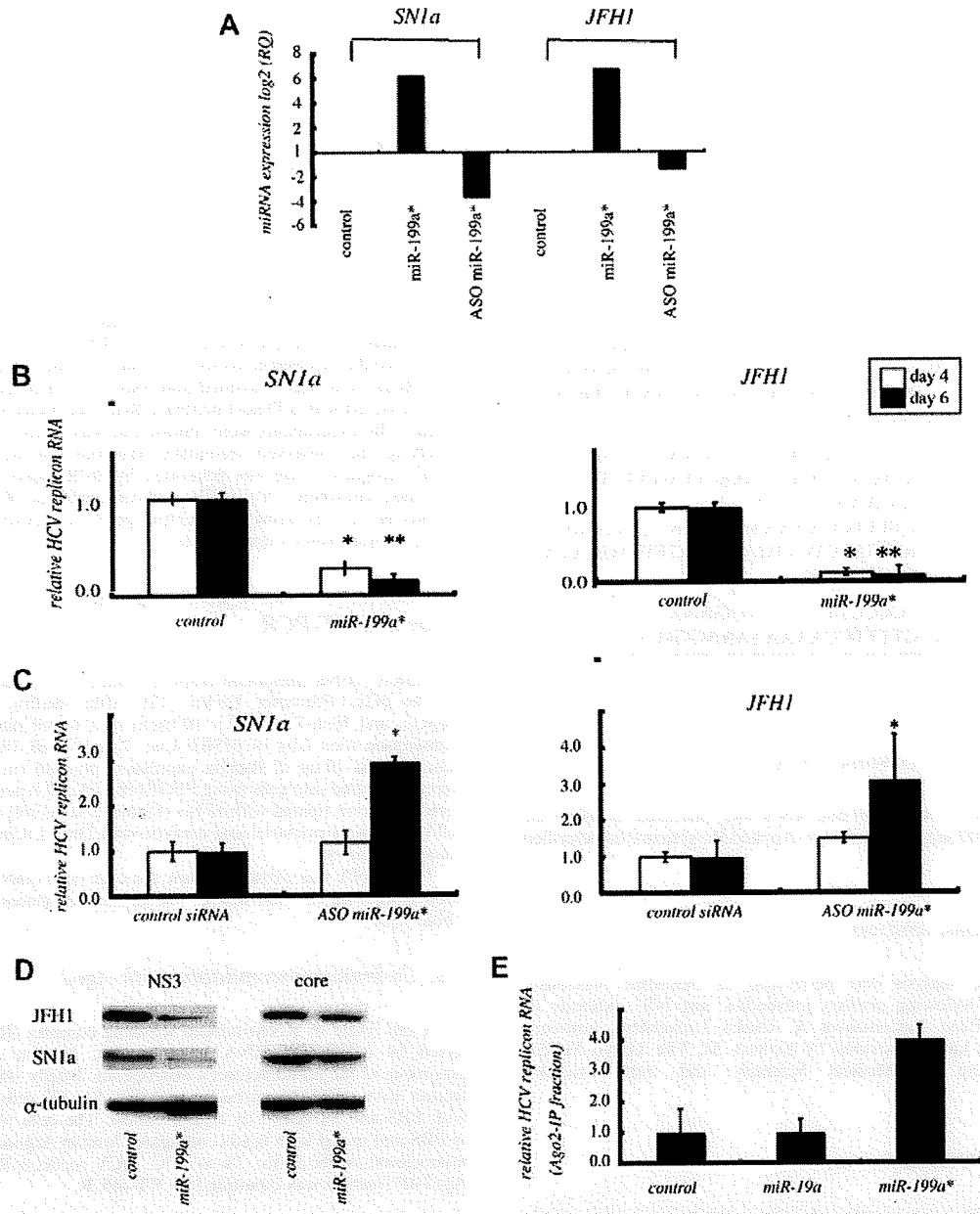


**Fig. 2. Overexpression of miR-199a* decreased HCV replicon RNA levels. (A) Expression levels in SN1a and JFH1 cells after inducing overexpression of miR-199a* by miR-199a* expression vector or suppression of expression of miR-199a* by specific ASO were measured by real-time qPCR. (B) Comparison between 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 ± 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; GeneBank 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

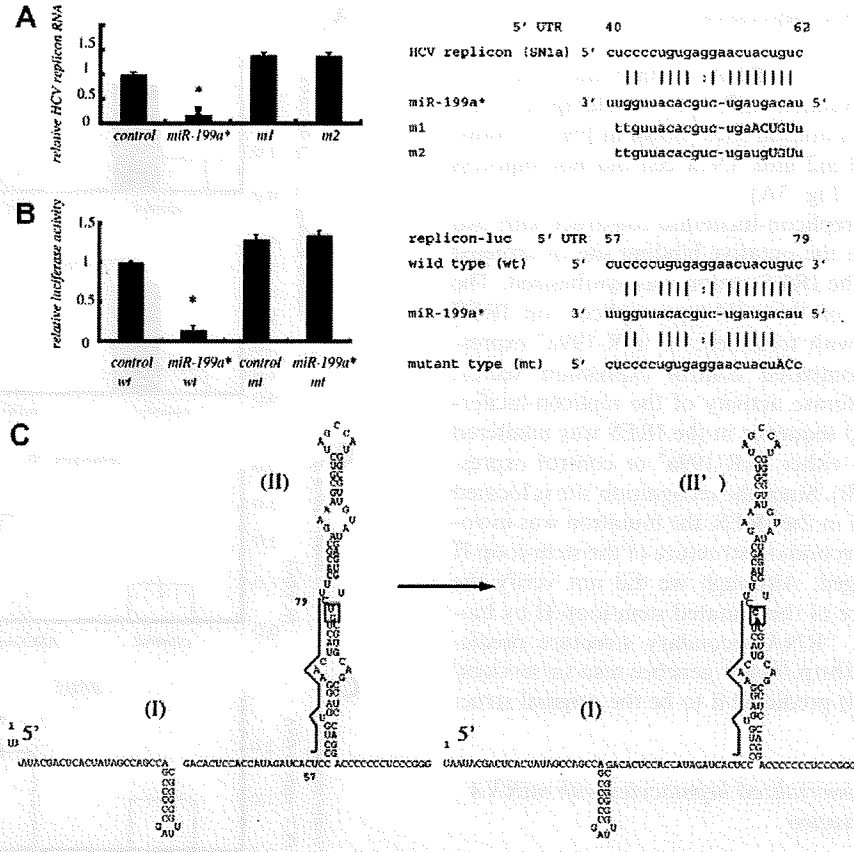


**Fig. 3. Suppression of HCV replicon activity by miR-199a* was abolished by mutations in either the miRNA sequence or the miR-199a* target sequence in the HCV-replicon-luciferase construct. (A) Effect of mutated miR-199a* on HCV replicon activity. Comparison of HCV replicon RNA level in SN1a expressing 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).

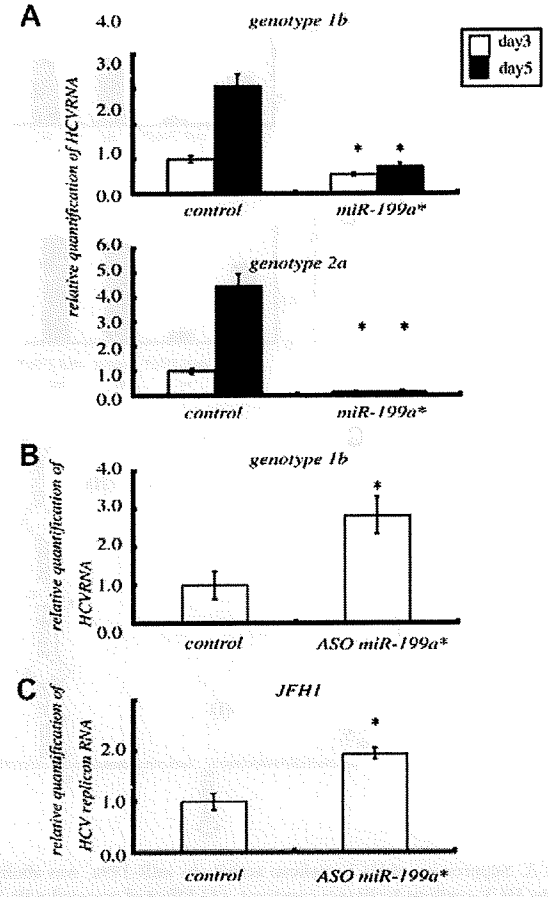


**Fig. 4. Overexpression of miR-199a* reduced HCV replication. (A) Overexpression of miR-199a* reduced the amount of HCV RNA in HuS-E/2 cells infected with serum containing HCV-genotype 1b and 2a, in comparison with the control vector. Each column represents the relative amount of HCV RNA normalized to the vector control sample on post-transfection day 3 and 5. The data shown are means ± 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 ± 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.**

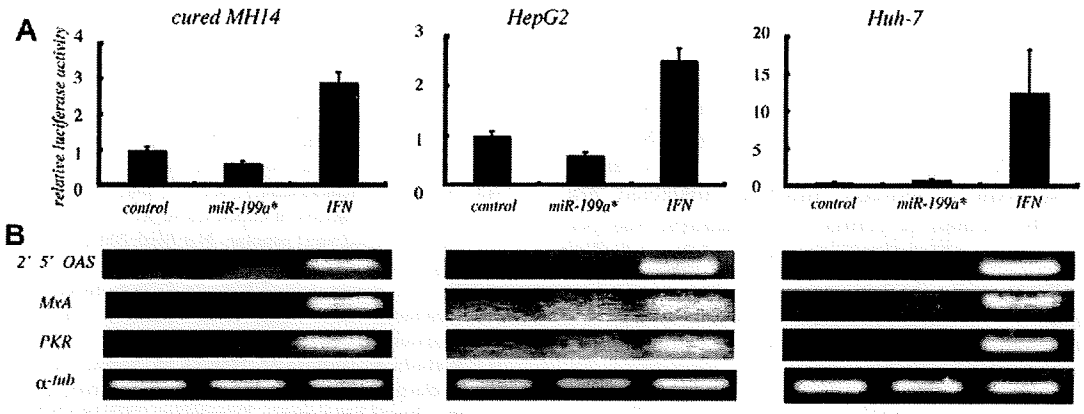


**Fig. 5. Treatment of the cells with miR-199a* did not induce the IFN pathway. (A) Cured MH14 (left), HepG2 (middle) and Huh-7 cells (right) were co-transfected with pISRE-Luc and either miR-199a* expression vector or the control vector. In a control experiment to investigate IFN response, one line of cells treated with IFNα (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 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.

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

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**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-allylaminogeldanamycin (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 ΔNS3 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

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[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.

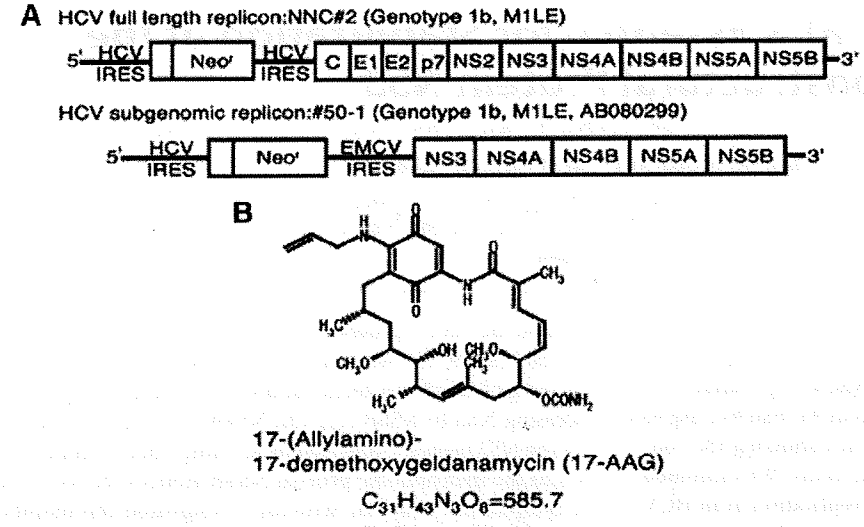


FIGURE 1. **Schematic representation of HCV replicon and structure of 17-AAG.** *A*, structure of the HCV replicon RNAs, comprising the HCV 5′-untranslated region, including the HCV internal ribosome entry site (*IRES*), the neomycin phosphotransferase gene (*Neo'*), the encephalomyocarditis virus (*EMCV*) 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.

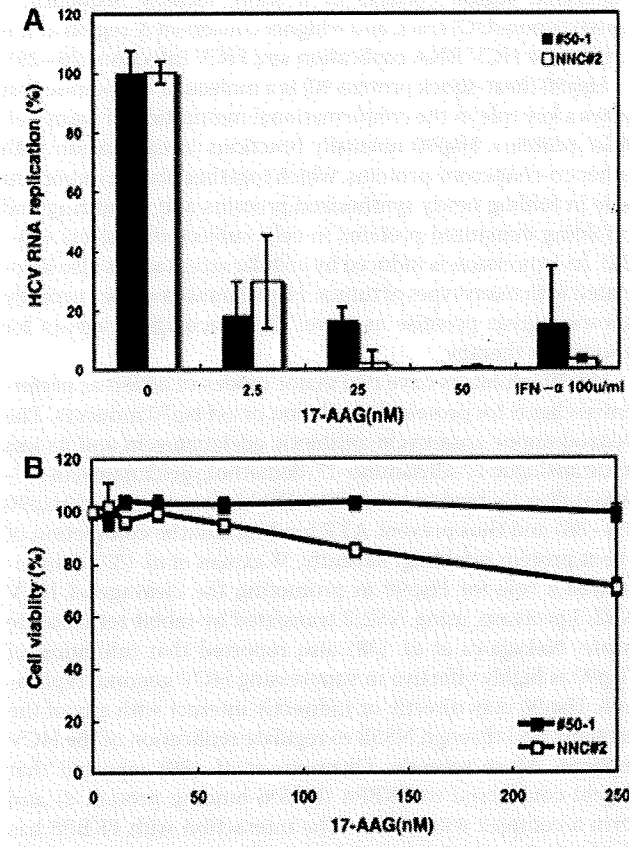


FIGURE 2. **Hsp90 inhibits HCV RNA replication in HCV replicon cells.** *A*, inhibition of HCV replication by 17-AAG in NNC#2 (*white squares*) and #50-1 cells (*black squares*) measured by real time reverse transcription-PCR after 72 h. Interferon-α was used as a positive control. The data are means ± 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 ± 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 μg/ml G418 (Invitrogen) at 37 °C in 5% $CO_2$. 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 μg/ml streptomycin. 17-AAG was purchased from Sigma.

*Measuring HCV RNA by Real Time PCR*—HCV replicon cells were seeded at $1.5 \times 10^5$ 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′-AGTACCACAAGGCCTTTCG-3′; and TaqMan probe (nucleotides 148–168), 5′-CTGCGGAACCG-GTGAGTACAC-3′ (all purchased from Applied Biosystems). The probe sequence was labeled with the reporter dye, 6-carboxyflurorescein, 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× 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), Hsp90α (Calbiochem), Hsp90β (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.

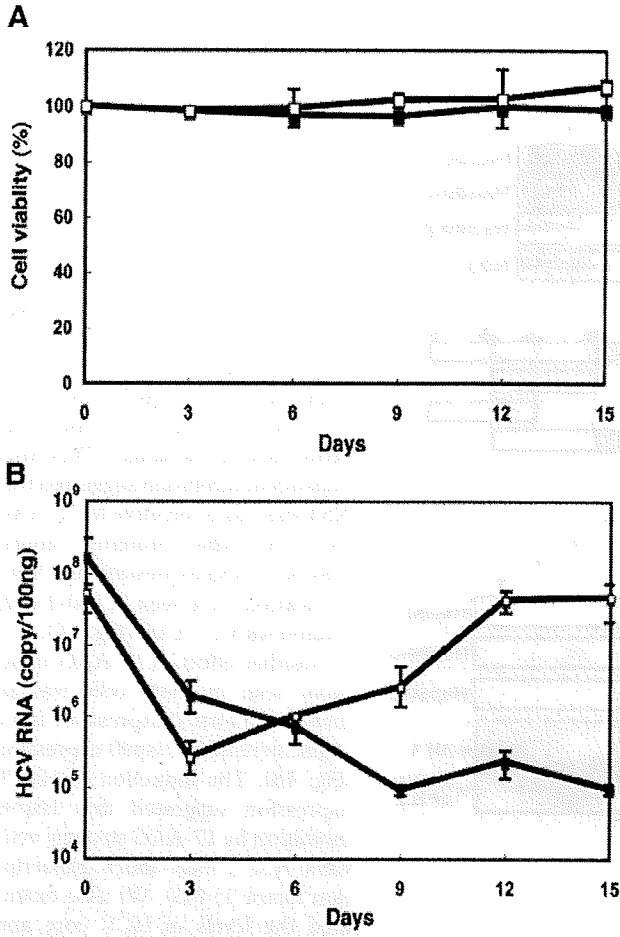


FIGURE 3. **Long term inhibition of HCV replication in NNC#2 cells.** *A*, cytotoxic effect of 17-AAG in NNC#2 cells, shown as the percentage reduction of viable cell numbers assessed by trypan blue staining. NNC#2 cells were treated with 50 nM 17-AAG on day 0 only (*white squares*) or at 3-day intervals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

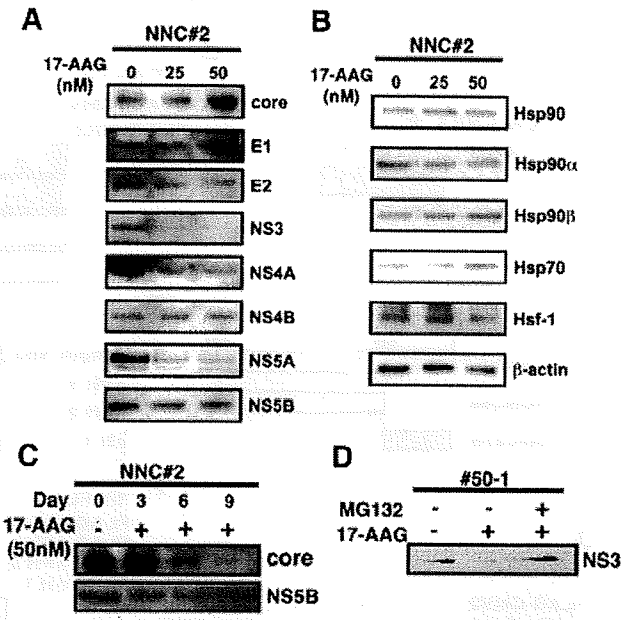


FIGURE 4. **Effect of 17-AAG on HCV NS3 protein levels.** *A*, Western blot analysis of HCV protein expression in NNC#2 or #50-1 cells treated with 17-AAG. NNC#2 or #50-1 cells were treated with 25 and 50 nM 17-AAG for 3 days. Cell lysates were separated by SDS-PAGE, immunoblotted, and probed with antibodies 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.

*[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 $3 \times 10^4$ cells/well in a final culture volume of 100 μl 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) × 100.

*Plasmids and Transfection*—The pFLAG-CMV-NS3 vector was constructed by subcloning a DNA fragment encoding full-length NS3, Δhelicase, Δprotease, ΔPH 1, ΔPH 2, and ΔH 1 into the EcoRI and XbaI sites of the pFLAG-CMV™-2 expression vector (Sigma), so that the amino-terminal FLAG epitope was fused in frame with NS3. The core expression vector was a gift 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. 2*A*). 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. 2*B*). 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.

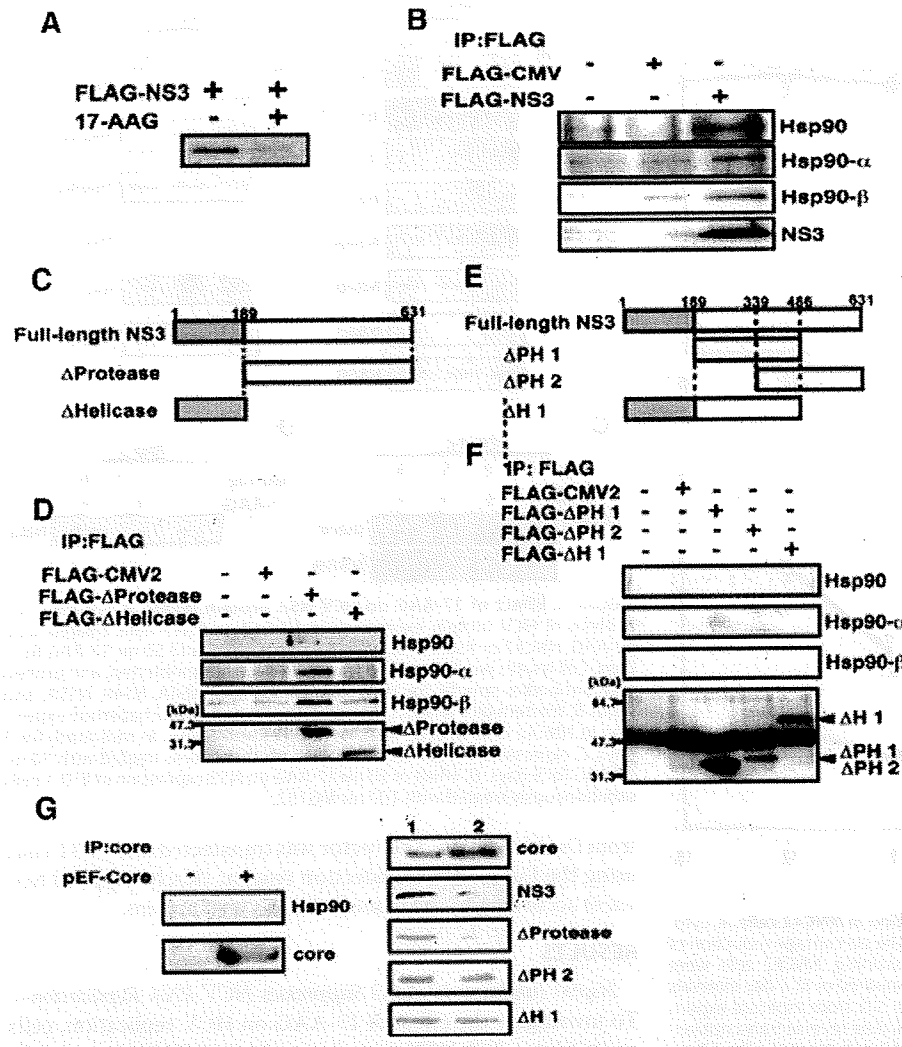


FIGURE 5. **Hsp90 regulates HCV NS3 protein stability.** *A*, Western blot showing the inhibition of NS3 protein expression in 293T cells caused by 17-AAG. Cells were transfected with pFLAG-NS3 in the presence of 250 nM 17-AAG for 48 h. *B*, FLAG-NS3 was expressed in 293T cells and immunoprecipitated (*IP*) from cell lysates with anti-FLAG antibody. Proteins immunoprecipitated were analyzed by Western blotting using anti-Hsp90, anti-Hsp90α, anti-Hsp90β, and anti-FLAG antibodies. The data shown in each *panel* are representative of three independent experiments. *FLAG-CMV*, empty plasmid vector. *C*, schematic representations of HCV NS3 protein and its deletion mutants. *D*, FLAG-NS3, FLAG-Δprotease, and FLAG-Δhelicase were expressed in 293T cells and immunoprecipitated from cell lysates with anti-FLAG antibody. Proteins immunoprecipitated with anti-Hsp90, Hsp90α, Hsp90β, 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-ΔPH 1, FLAG-ΔPH 2, and FLAG-ΔH 1 were expressed in 293T cells and immunoprecipitated from cell lysates with anti-FLAG antibody. Proteins immunoprecipitated with anti-Hsp90, Hsp90α, Hsp90β, 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-Δprotease, FLAG-ΔPH 2, and FLAG-ΔH 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 μM).

3*B*). 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. 3*A*). Our results suggested that 17-AAG has the potential to safely induce long term suppression in HCV replication.

*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. 4*A*) after 3 days, in common with the level of HCV RNA (Fig. 2*A*). 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. 4*A*).

Another effect of 17-AAG treatment seen in these cells was an increase in Hsp70 expression and a slight increase in Hsp90 expression (Fig. 4*B*). 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. 4*C*). 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. 4*D*), 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 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. 5*A*), suggesting that Hsp90 is involved in HCV NS3 degradation, possibly through a physical interaction.

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. 5*B*). NS3 mutants lacking the protease and helicase regions were generated in order to identify the region responsible for the interaction with Hsp90 (Fig. 5*C*). FLAG-NS3, FLAG-NS3-Δhelicase, or FLAG-NS3-Δprotease were transfected into 293T cells, and anti-FLAG antibody immunoprecipitates were analyzed by Western blotting (Fig. 5*D*). Although FLAG-NS3-Δprotease was clearly co-immunoprecipitated with Hsp90, no protein band corresponding to FLAG-NS3-Δhelicase was detected (Fig. 5*D*), suggesting that the NS3 helicase region mediates binding to Hsp90. To confirm this finding, plasmids expressing different NS3 helicase mutants fused with FLAG (ΔPH 1, ΔPH 2, and ΔH 1) were constructed (Fig. 5*E*). 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. 5*F*).

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. 5*G*). The proteins expressed by FLAG-NS3 and FLAG-NS3-Δprotease were degraded in cells pretreated with 17-AAG, whereas no degradation of the ΔPH 2 and ΔH 1 NS3 mutants lacking helicase regions was seen (Fig. 5*G*). 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. 5*G*). Our data demonstrate that 17-AGG destabilizes several binding proteins (NS3 and NS3-Δprotease) to Hsp90 but stablilizes some nonbinding proteins (the ΔPH 2 and ΔH 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 (ΔPH 2 and ΔH 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.

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

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**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 IFNα 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 IFNα 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 μg/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 μg/mL G418 and 2 μg/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 μg/mL G418. Q86R, D320E, Q86R/D320E, and Q86R/I252T/D320E cells were produced by 700 μg/mL G418 selection of Huh7 cells transfected with 5 μg 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 μg/mL G418 for 4 weeks, followed by fixation and staining with crystal violet.

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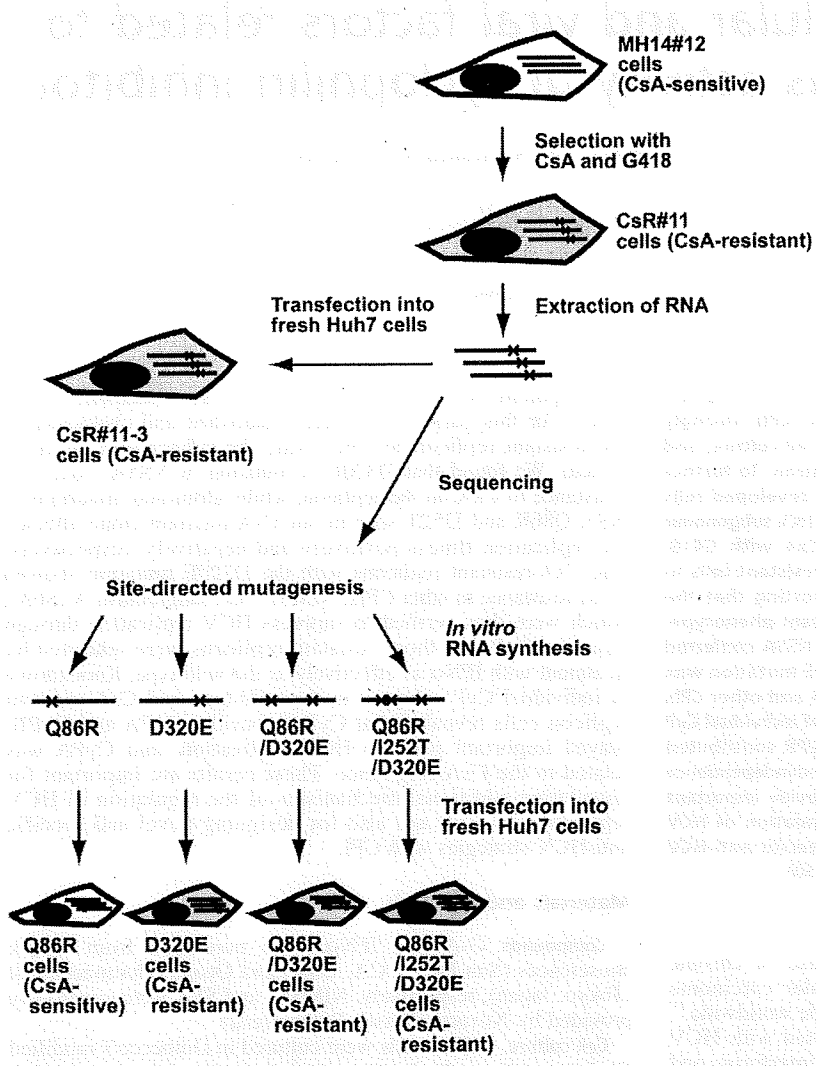


**Fig. 1.** Schematic diagram outlining the production of individual cell clones carrying hepatitis C virus (HCV) subgenomic replicons. MH14#12 cells, carrying wild-type HCV subgenomic repicon, were treated with 2 μg/mL cyclosporin A (CsA) in the presence of 1000 μg/mL G418 and CsR#11 cells were selected. Total RNA was extracted from CsR#11 cells and transduced 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′-AGGACCTCGTCGGCTGGCGGGCGCC-3′ plus Q86R (AS) 5′-GGCGCCCGCCAGCCGACGAGGTCCT-3′, I252T (S) 5′-AACACCAGAACTGGGGTAAGGACCA-3′ plus I252T (AS) 5′-TGGTCCTTACCCCAGTTCTGGTGTT-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 Not-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.** $8 \times 10^6$ 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 μg/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



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