**SPT assay.** To examine the effect of NA255 on SPT inhibitory activity, we prepared human recombinant SPT encoding two different genes, *LCB1* and *LCB2*. These cDNAs were generated from a liver cDNA library by RT-PCR. Human embryonic kidney (HEK) 293 cells were transiently transfected with His-tagged LCB1 and LCB2 in a dual expression vector, pBudCE4.1 (Invitrogen), for 72 h and then harvested. The cells were lysed in lysis buffer (50 mM $NaH_2PO_4$, 30 mM NaCl, 10 mM imidazole and 0.1% sucrose monolaurate) and sonicated 30 times with short pulses. After centrifugation at 1,200*g* for 5 min, the supernatant was incubated with Ni-NTA agarose (QIAGEN), washed with wash buffer (50 mM $NaH_2PO_4$, 30 mM NaCl, 20 mM imidazole and 0.1% sucrose monolaurate) and eluted with elution buffer (50 mM $NaH_2PO_4$, 30 mM NaCl, 250 mM imidazole and 0.1% sucrose monolaurate). The eluted solution was concentrated by using Amicon Ultra-4 (Millipore) and exchanged into buffer (10 mM HEPES buffer, pH 7.4, containing 250 mM sucrose and 0.1% sucrose monolaurate). The purified SPT fraction was stored at −20 °C until use. Purified human SPT was added to 0.1 ml of a reaction mixture containing 200 mM HEPES buffer (pH 8.0), 5 mM EDTA, 10 mM DTT, 0.05 mM pyridoxal 5-phosphate, 0.2 mM palmitoyl-CoA, 0.1 mM L-serine and 1 μCi [$^3$H]serine (Amersham) in the presence of NA255. After a 15-min incubation at 37 °C, the reaction was stopped by the addition of 0.25 ml of 0.5 M $NH_4OH$ and 0.75 ml of chloroform/methanol (1:2, v/v). The products were extracted and the organic phases were then washed twice with water, followed by measurement of the radioactivity by lipid scintillation counting.

**Detection of cellular sphingolipids.** Cells were incubated for 18 h with [$^{14}$C]serine (0.2 μCi $ml^{-1}$) in Opti-MEM (Gibco BRL) and washed with PBS after treatment with NA255. After cells were lysed with 0.3 ml of 0.1% SDS, and the lysate was suspended by pipetting. Total lipids were extracted with 0.9 ml of chloroform/methanol (1:2 v/v), and then 0.3 ml chloroform and 0.3 ml PBS were added and mixed well. The extracts were spotted on Silica Gel 60 thin-layer chromatography (TLC) plates (Merck) and chromatographed with chloroform/methanol (10:1, v/v) or methyl acetate/1-propanol/chloroform/methanol/ 0.25% KCl (25:25:25:10:9, v/v). Radioactive spots were evaluated using a bio-imager (BAS 1000, Fuji Photo Film).

**Accession codes.** Protein Data Bank accession codes: 1QUV, HCV- NS5B; 1CE4, HIV-1 gp120 V3 loop peptide. International Patent Organism Depositary of the National Institute of Advanced Industrial Science and Technology (Japan) accession code: FERM BP-8920, strain F1476, *Fusarium* sp. BIND identifiers (http://bind.ca): 334490–334492.

Additional methods are available as **Supplementary Methods** online.

*Note: Supplementary information is available on the Nature Chemical Biology website.*

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# Modulation of host metabolism as a target of new antivirals ☆

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**Abstract**

The therapy for chronic hepatitis C (CH–C) started with interferon (IFN) monotherapy in the early 1990s and this therapy was considered effective in about 10% of cases. The present standard therapy of pegylated IFN with ribavirin achieves a sustained virologic response in about 50% of patients. However, about half of the CH–C patients are still at risk of fatal liver cirrhosis and hepatocellular carcinoma. The other significant event in hepatitis C virus (HCV) research has been the development of a cell culture system. The subgenomic replicon system enables robust HCV RNA replication in hepatoma cells. And recently, the complete life cycle of HCV has been achieved using a genotype 2a strain, JFH1. These hallmarks have provided much information about the mechanisms of HCV replication, including information on the host molecules required for the replication. Anti-HCV reagents targeting HCV proteins have been developed, and some of them are now in clinical trials. However, the RNA-dependent RNA polymerase frequently causes mutations in the HCV genome, which lead to the emergence of drug-resistant HCV mutants. Some of the cellular proteins essential for HCV RNA replication have already been discovered using the HCV cell culture system. These host molecules are also candidate targets for antivirals. Here, we describe the recent progress regarding the anti-HCV reagents targeting host metabolism.
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*Keywords:* Hepatitis C virus; Replicon; Antiviral; Interferon; Host metabolism; Statin

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*Abbreviations:* HCV, hepatitis C virus; CH, chronic hepatitis; HCC, hepatocellular carcinoma; IFN, interferon; SVR, sustained virological response; PEG-IFN, pegylated-IFN; GBV-B, GB virus B; uPA-SCID, urokinase plasminogen activator-severe combined immunodeficiency; NS, nonstructural; RdRp, RNA dependent RNA polymerase; CyPB, cyclophilin B; CsA, cyclosporine A; HSP90, heat shock protein 90; La, La auto antigen; PTB, polypyrimidine tract-binding protein; ALT, alanine aminotransferase; Neo, neomycin phosphotransferase; EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site; ORF, open reading frame; FKBP8, FK-506-binding protein 8; GGPP, geranylgeranyl pyrophosphate; FPP, farnesyl pyrophosphate; FTase, farnesyltransferase; GGTase-I, geranylgeranyl-transferase type I; GGTI, GGTase-I inhibitor; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LOV, lovastatin; ATV, atorvastatin; FLV, fluvastatin; PRV, pravastatin; SMV, simvastatin; $EC_{50}$, 50%; effective concentration to inhibit HCV RNA replication; PTV, pitavastatin; RSV, respiratory syncytial virus; CMV, cytomegarovirus; HIV, human immunodeficiency virus; ICAM-1, integrin intercellular adhesion molecule 1; LFA-1, lymphocyte function associated antigen-1; DRM, detergent resistant membrane; SPT, serine palmitoyltransferase; ER, endoplasmic reticulum; GSL, glycosphingolipid; SBD, sphingolipid-binding domain; IMPDH, inosine monophosphate dehydrogenase; XMP, xanthosine 5′ ; monophosphate; MPA, mycophenolic acid; RMP, ribavirin monophosphate; RDP, ribavirin diphosphate; RTP, ribavirin triphosphate; GTP, guanosine triphosphate; SARS, severe acute respiratory syndrome; HBV, hepatitis B virus; VLP, virus-like particle; PIAS1, protein inhibitor of activated STAT1; PRMT1, protein arginine methyltransferase 1; PP2Ac, catalytic subunit of protein phosphatase 2A; AdoMet, S-adenosyl-L-methionine; PUFAs, polyunsaturated fatty acids; AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid.

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## 1. Introduction

Hepatitis C virus (HCV) was discovered in 1989 [1] as the causative agent of chronic hepatitis C (CH–C), liver cirrhosis and hepatocellular carcinoma (HCC) [2]. It is estimated that 170 million people worldwide are infected with HCV [3]. The ultimate goal of both clinical and basic HCV studies is the suppression of liver-related death caused by HCV infection. With respect to clinical studies, interferon (IFN) has played a major role in the treatment of patients with CH–C. IFN therapy started with IFN monotherapy in the early 1990s, and a sustained virologic response (SVR) was obtained in about 10% of patients [4]. IFN therapy was developed by the hepatologists, and the current therapy of pegylated IFN (PEG-IFN) with ribavirin has improved the SVR to about 50% [4]. Therefore, the next stage of the therapy for CH–C is to develop new anti-HCV reagents to improve the SVR.

During the development of IFN therapy, the most striking discovery in the basic research was the development of a cell culture system for robust HCV RNA replication. In 1999, Lohmann et al. [5] achieved subgenomic HCV RNA replication in a human hepatoma cell line, HuH-7. The advantages of this novel system (known as the replicon system) were that it provided not only a way to screen for anti-HCV reagents but also information about the mechanism of HCV RNA replication. This cell culture system has been further improved, and recently the complete life cycle of HCV was achieved using a genotype 2a HCV strain, JFH1 [6–8]. This newest system has extended the targets of the anti-HCV therapy to the virus infection and release.

The effects of anti-HCV reagents selected from the cell culture-based screening should be evaluated using an animal model system for HCV infection before they can be released to clinical trial. Chimpanzees were the only animal model in the early HCV studies [9]. However, the use of chimpanzees is limited for ethical and financial reasons. In addition to chimpanzees, a study using tree shrews (*Tupaia belangeri chinensis*) has been reported [10]. A different approach to the study of HCV using animal models was achieved using the related GB virus B (GBV-B). GBV-B belongs to the *Flaviviridae* family and can be transmitted to tamarins and marmosets [11,12]. These animal models may be valuable surrogate models for HCV study. Another approach was demonstrated in a study using urokinase plasminogen activator-severe combined immunodeficiency (uPA-SCID) mice transplanted with human hepatocytes [13]. This chimeric mouse model can support chronic HCV viremia under the circumstance without immune system. Mass screening for anti-HCV reagents using cell culture systems will become a more powerful tool when combined with small animal model systems to evaluate the antiviral effects of selected reagents before clinical trial.

In considering a new strategy for CH–C to be used in place of or in combination with IFN, the main targets are HCV proteins and HCV RNA. With respect to the HCV proteins, two of these, nonstructural (NS) 3-4A and NS5B, have been well-characterized as protease and RNA-dependent RNA polymerase (RdRp), respectively [14,15]. Several reagents have been reported to be inhibitors of NS3-4A serine protease, including SCH6 [16,17], SCH503034 [18], VX-950 [19,20], and BILN-2061 [21]. Valopicitabine (NM283) was reported to inhibit NS5B RdRp [22]. HCV RNA itself is also a target of antivirals, and recent RNA interference technologies using siRNA or shRNA have targeted HCV RNA [23–25]. As RdRp lacks proofreading activity, the high mutation rate of RdRp allows the virus to escape from the reagents targeting HCV proteins and HCV RNA. These anti-HCV reagent-targeting viral proteins and genome will be reviewed in another section.

Other targets are the cellular proteins essential for HCV RNA replication and infection. The expression of HCV proteins is thought to affect the host cells' gene expression profiles and vise versa [26]. The interaction of the specific cellular proteins with HCV proteins is essential for HCV replication (Table 1). Cyclosporine A (CsA) is one of the best characterized inhibitors targeting the cellular proteins required for HCV replication [27–36]. The interaction of cyclophilin B (CyPB) with NS5B is required for HCV RNA replication [28]. CsA inhibits HCV RNA replication by interrupting the interaction between NS5B and CyPB. Heat shock protein 90 (HSP90) has also been reported to be an essential cellular protein for HCV RNA replication [37–39]. Knockdown or inhibition of HSP90 has been shown to result in the anti-HCV activity in cell culture and in uPA-SCID mouse systems [37].

Table 1
Host molecules as targets of anti-HCV

| Target molecules | Reagents | References |
|---|---|---|
| HMG–CoA reductase | Statin | [68–71] |
| Serine palmitoyltransferase | NA255 | [81] |
| | Myriocin | [82] |
| IMP dehydrogenase | VX-497 | [98] |
| | Ribavirin | [74,86] |
| | Mizoribin | [74] |
| | MPA | [97] |
| Protein arginin methyltransferase | AdoMet, Betaine | [102] |
| α-Glucosidase | Deoxynojirimycin | [101] |
| Cyclophilins | CsA | [27–36] |
| | NIM811 | [27,33] |
| | DEBIO-025 | [34] |
| HSP90 | Geldanamycin, Radicicol | [37–39] |
| FKBP8 | | [38] |
| Unknown | PUFAs | [70,103,108] |

FKBP8, a member of the FK506-binding protein family, specifically interacts with NS5A and forms a complex with HSP90 [38]. The La autoantigen (La) and polypyrimidine tract-binding protein (PTB) are also candidate cellular proteins for the inhibition of HCV RNA replication [40], although no inhibitors for these proteins have been reported to date. Thus, inhibition of the metabolism has recently been reported as a target of the new antivirals. Here, we survey the recent progress on enzyme inhibitors of the cholesterol, sphingolipid, and guanosine triphosphate (GTP) synthesis pathways, as well as other metabolic pathways.

## 2. Current standard therapy for chronic hepatitis C

HCV was discovered to be the causative agent of non-A, non-B hepatitis by the Chiron Corporation in 1989 [1]. However, a treatment for patients with non-A, non-B hepatitis was established before the discovery of HCV. In 1986, Hoofnagle et al. reported that IFN-α treatment normalized the serum alanine aminotransferase (ALT) levels in patients with non-A, non-B hepatitis [41]. Since the initial discovery of its anti-HCV activity, IFN-α has become the major reagent for CH–C treatment [4]. The replication of HCV RNA itself seems to stimulate IFN production signaling, and our recent results have suggested that core and/or NS5B induce IFN-stimulated genes [42–44]. However, viral NS3-4A protease inhibits the IFN production, although it does not completely shut it off. Therefore, exogenous IFN administration is needed for patients with CH–C. The SVR is affected by multiple factors, such as genotype, viral load and duration of therapy. IFN-α monotherapy was begun in the early 1990s, but an SVR was achieved in only about 10% of patients. In the early 2000s, IFN-α and ribavirin combination therapy was developed and the SVR was improved to about 30-40%. Furthermore, IFN itself has been modified by the attachment of PEG, thereby enhancing its stability in the blood. The SVR of the current standard therapy by PEG-IFN and ribavirin is as high as 50% [4]. In the current PEG-IFN and ribavirin combination therapy, the genotype of HCV is one of the major determinants of the SVR. HCV genotypes are classified into 6 groups, and genotype 1 is currently considered a problem due to its IFN resistance [45]. For example, in genotype 1 HCV, 12 months of treatment resulted in an SVR in 50% of patients, while in genotype 2, 6 months of treatment achieved an SVR of 80-90% [46]. The precise mechanisms of the IFN resistance remain unclear. However, the recently developed IFN-resistant HCV replicon-harboring cells will be useful for studies examining ways to improve the SVR [47–49]. Therefore, the focus in the treatment of patients with CH–C has shifted to increasing the SVR in genotype 1 HCV.

## 3. Cell culture-based HCV RNA-replication system

Before the development of an HCV replicon system, screening of anti-HCV reagents was rather difficult. The HCV replicon system developed by Lohmann et al. [5] was the first milestone in HCV study using a cell culture system. The replicon system has provided a wealth of information concerning the replication machinery of HCV. We can make strategies for the Achilles' heel of HCV based on the information regarding HCV RNA replication. The HCV replicon has been improved to be a suitable system for the screening of anti-HCV reagents by the introduction of reporter genes such as luciferase [50]. However, this system does not contain a structural region. Therefore, selectable genome-length HCV RNA-replicating cell culture systems have been developed [51–54]. The second milestone was the infectious virus production system established by the three groups using a genotype 2a HCV strain, JFH1 [6–8]. This system has extended the range of the HCV study to the viral entry and release. Therefore, the life cycle of HCV in the cells has been reconstructed *in vitro*. Since the development of the HCV replicon and infectious HCV production systems, many cellular proteins have been identified as essential host molecules for HCV RNA replication.

### 3.1. *From HCV replicon to infectious HCV production*

The HCV replicon reported by Lohmann et al. contained neomycin phosphotransferase (Neo) and encephalomyocarditis virus (EMCV) internal ribosome entry sites (IRES) instead of the HCV structural regions (Fig. 1) [5]. This HCV replicon consists of 2 cistrons. In the first cistron, Neo is translated by HCV-IRES and in the second cistron NS3-NS5B is translated by EMCV-IRES introduced in the region upstream of the NS region (Fig. 1). After the development of the HCV replicon system [52,53,55–58], genome-length HCV RNA replication systems using different HCV strains (H, N, Con1, and O) were developed by several groups [51–54]. In these genome-length HCV RNA replication systems, a complete open reading frame (ORF) of HCV was introduced into the second cistron instead of the NS region (Fig. 1).

For the mass screening for anti-HCV reagents, evaluation of the levels of HCV RNA or HCV proteins requires time and complicated procedures. To facilitate the monitoring of the replication level of HCV RNA, the reporter gene (Renilla luciferase) was fused to the Neo gene. In this system, anti-HCV activity was evaluated by the value of the reporter instead of the

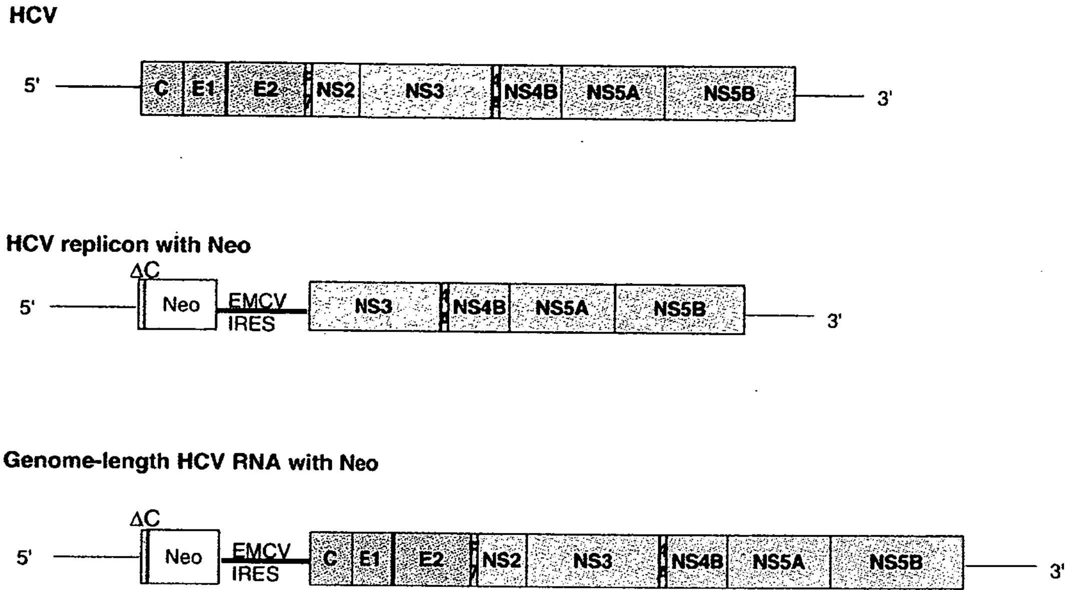


Fig. 1. Gene organizations of HCV and selectable HCVs. HCV ORF, untranslated regions, EMCV IRES, and Neo are depicted as shaded boxes, thin lines, thick lines, and open boxes, respectively. ΔC indicates the 12 N-terminal amino acid residues of the core as a part of IRES.

quantification of HCV RNA or HCV proteins. As shown in Fig. 2A, ORN/C-5B/KE contains the fused Renilla luciferase and Neo genes in the first cistron [51]. One of the cloned cell lines, OR6, was established by the G418 selection after introduction of ORN/C-5B/KE RNA into HuH-7 cells. HCV RNA and HCV proteins were stably expressed in the OR6 cells, and the Renilla luciferase activity was correlated well with the level of HCV RNA [51]. Therefore, the antiviral effect of the reagents on HCV RNA replication could be monitored by the activity of Renilla luciferase. The OR6 assay system

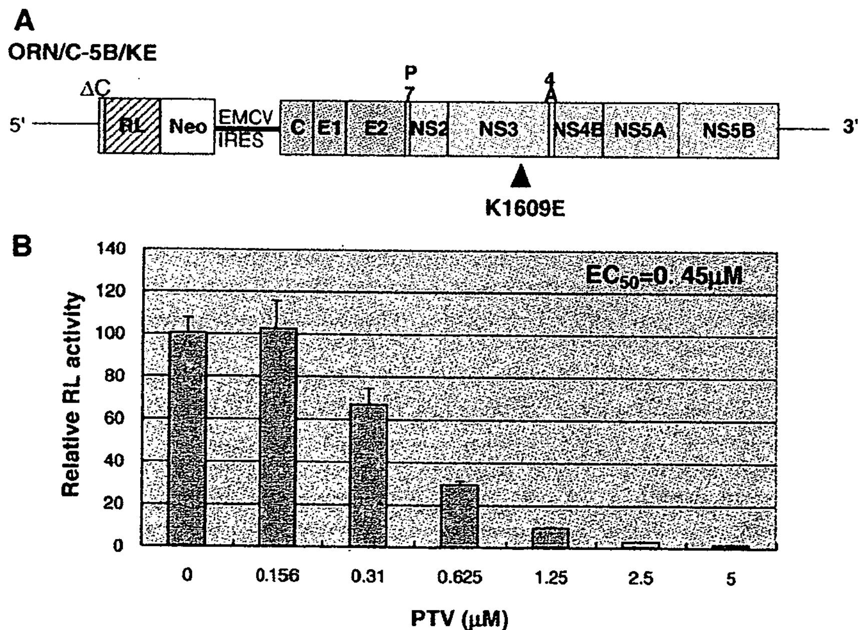


Fig. 2. Inhibitory effect of statin on HCV RNA replication in OR6 cells. (A) Schematic gene organization of genome-length HCV RNA (ORN/C-5B/KE) derived from genotype 1b, strain O. The Renilla luciferase gene, which is symbolized as RL, is depicted as a striped box and is expressed as a fusion protein with Neo. The adaptive mutation from lysine (K) to glutamine (E) at amino acids position 1609 was previously reported [51] and introduced into the genome-length HCV RNA. (B) Inhibition of HCV RNA by PTV. OR6 cells were cloned cell line selected by G418 [51]. OR6 cells were treated with PTV at a concentration of 0, 0.156, 0.31, 0.625, 1.25, 2.5, or 5 μM. After 72 hours of treatment the RL activities were determined. Shown here is the relative RL activity (%) calculated when the RL activity of untreated cells was assigned as 100%. The data indicate the means ± standard deviation from three independent experiments. The $EC_{50}$ of PTV was determined as 0.45 μM.

facilitates the mass screening for anti-HCV reagents. HCV RNA replicating in OR6 cells contained an adaptive mutation, K1609E, in the NS3 region. Adaptive mutations have been reported to enhance the replication level of HCV RNA in cell culture [59–61]. In the case of HCV-O, two adaptive mutations were required for robust replication of the genome-length HCV RNA replication [60]. For example, authentic HCV-O RNA with the adaptive mutations of E1202G and K1609E can robustly replicate in HuH-7 cells for 9 months or more (Ikeda et al., unpublished data).

In 2005, three groups reported infectious HCV production systems using the JFH1 strain in cell culture [6–8]. These reports showed that the life cycle of HCV could be reconstructed in HuH-7 cells, and thus became landmarks in the search for an ideal HCV cell culture system. The unique features of these systems were the origin of this strain and the cell lines. JFH1 was a genotype 2a strain derived from a patient with fulminant hepatitis and did not require any adaptive mutations for robust replication, unlike other HCV strains. The unique feature of this system was that it employed HuH-7 cells such as Huh-7.5 or Huh-lunet cells, since the parental HuH-7 cells could not support robust production of infectious HCV [6–8,62]. Recently, the genotype 1a H77-S strain was reported to produce infectious HCV in cell culture, although the production level of infectious H77-S was lower compared with that by JFH1 [63]. Interestingly, five adaptive mutations were introduced into the H77-S genome in order to enhance the efficiency of infectious virus production. The presence of these adaptive mutations is the most striking and controversial characteristic regarding the production of infectious HCV described above. Further study will be needed to understand the role of adaptive mutations on infectious virus production.

### 3.2. HCV life cycle

The establishment of an infectious HCV production system gradually led to clarification of the life cycle of HCV. Information regarding the HCV RNA replication has been accumulated since the development of the HCV replicon system, and the infectious HCV production system [6–8] has further provided information about the step of virus entry and release. The life cycle of HCV includes the (1) receptor binding and cell entry, (2) cytoplasmic release and uncoating, (3) IRES-mediated translation, (4) processing, (5) RNA replication, (6) packaging and assembly, (7) virion maturation, and (8) virion release. Although some of the mechanisms are still unclear, each of these steps is a target for antivirals. Among the proteins involved in these steps, the protease in step (4) and polymerase in step (5) have been especially well characterized. Specific inhibitors for these proteins have been developed and some of them are now in clinical trials for patients with CH–C [21,64].

### 3.3. Cellular proteins required for HCV RNA replication

Cellular proteins are required for HCV RNA replication and may determine the cell tropism of HCV. As HCV is a parasite, it utilizes the cellular proteins for its replication machinery. Therefore, cellular proteins essential for HCV RNA replication are the targets for antivirals. Using cell culture systems, several cellular proteins have been identified as effective molecules for HCV RNA replication (Table 1). La and PTB were representative molecules reported as essential host factors for HCV RNA replication [40]. Recently, an immunosuppressant, CsA, has been reported to inhibit HCV RNA replication by blocking the binding of CyPB to NS5B [28]. HSP90 and the FK-506-binding protein 8 (FKBP8) form a complex with NS5A and geldanamycin, an inhibitor of HSP90, suppressed HCV RNA replication by blocking the formation of these complex [38]. The advantage of the inhibitors targeting cellular factor is that these reagents do not affect the viral escape achieved through mutations. The high mutation rate caused by RdRp frequently produced escape mutants toward the antiviral reagents for HCV proteins. A disadvantage of the inhibitors targeting cellular factors may be that they induce side effects by inhibiting the primary roles of the cellular factors.

## 4. Host metabolism as anti-HCV targets

The cellular factors are the targets of the antivirals independent of the viral escape via the genetic mutations caused by RdRp. The cellular factors were synthesized in their metabolic pathways and modified by the enzymes. These enzymes are also targets in the antiviral strategy (Table 1). Furthermore, some of the reagents have already been used in the clinical treatment of the respective diseases. One of the advantages of using existing reagents is that their characterizations—including safety and side effects—have already been performed. Therefore, screening of the existing reagents for anti-HCV will be a new field of antivirals. The development of a cell culture system for HCV led to the revelation that HCV incorporates many cellular factors into the replication machinery of the virus. Now we have both the information of the HCV life cycle and the cell culture assay system—the input and output—that we need to develop a pool of antiviral reagents. Below, we will discuss the particular host cell metabolic pathways that are currently being targeted by anti-HCV reagents including more recently found pitavastatin (PTV) (Fig. 2B).

### 4.1. Cholesterol-biosynthesis pathway and geranylgeranylation

In the cholesterol-biosynthesis pathway, the region downstream of mevalonate branches into separate pathways for cholesterol and isoprenoid synthesis (Fig. 3). The attachment of the isoprenoid is called prenylation of the protein. Prenylation regulates a variety of cellular functions, such as growth, differentiation, and oncogenesis. Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are mevalonate-derived isoprenoids and are attached to the target proteins by farnesyltransferase (FTase) and geranylgeranyl transferase type I (GGTase-I), respectively. FTase and GGTase-I recognize protein substrates with a C-terminal tetrapeptide recognition motif called the CaaX box: in the case of GGTase-I, C is cysteine, a is an aliphatic amino acid, and X is leucine, isoleucine, valine, or phenylalanine. Production of mevalonate by 3-hydroxy-3-methylglutaryl coenzyme A (HMG–CoA) reductase is the rate-

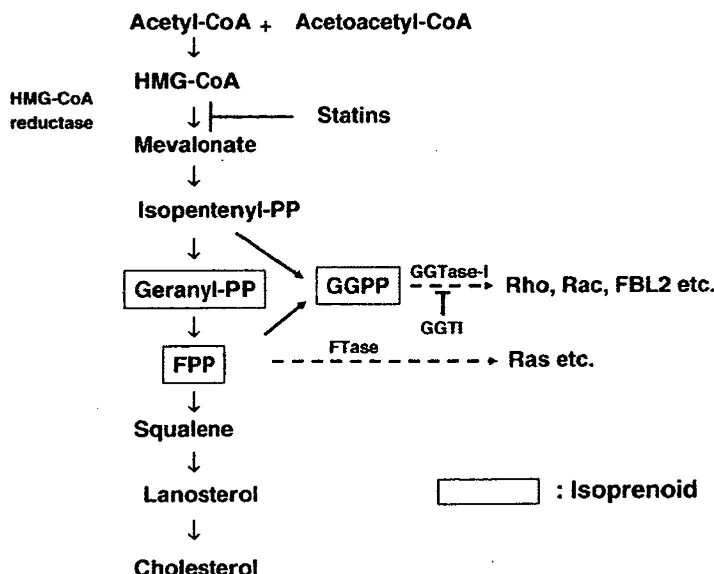


Fig. 3. Cholesterol-biosynthesis pathway. The inhibition of HMG–CoA reductase by statins leads to the suppression of mevalonate and of the production of its downstream metabolites. Decreased prenylation on the GTP-binding proteins had a significant effect on the signal transduction.

limiting step in the cholesterol biosynthesis. Statins are potent HMG–CoA reductase inhibitors and are beneficial in the prevention of coronary heart disease. Statins also inhibit the prenylation of the proteins.

Lipid metabolism is essential for the life cycle of many viruses. The cholesterol-rich lipid raft plays an important role in virus entry, replication, and assembly. HCV also forms a replication complex on the lipid raft membrane structure [65]. HCV RNA replication occurs in the lipid raft and the cholesterol supply is crucial to maintain the structure of the lipid raft [65]. Aizaki et al. [66] reported that lovastatin (LOV), one of the HMG–CoA reductase inhibitors, inhibited HCV RNA replication in HCV replicon-harboring cells.

Statins also possess the cholesterol-independent action (pleiotropic effect) [67]. Many of these pleiotropic effects are mediated by the isoprenoid. For example, inhibition of small GTP-binding proteins, Ras and Rho, whose proper membrane localization and function are dependent on prenylation, may play a significant role in the pleiotropic effect of statins. Ras and Rho are major substrates for prenylation with FPP and GGPP, respectively. GDP-bound Ras and Rho are localized in the cytoplasm. When FPP or GGPP is bound to the inactive Ras or Rho, they are translocated to the cell membrane and converted to GTP-bound active forms. Recently, Wang et al. [68] identified FBL2 as one of the geranylgeranylated cellular proteins required for HCV RNA replication. FBL2 belongs to the FBL family of proteins, all of which contains an F box and a multiple leucine-rich repeat, with the F box binding to a multicomponent ubiquitin ligase complex. Geranylgeranylated FBL2 binds to NS5A, and the resulting complex seems to be required for HCV RNA replication. In HCV replicon-harboring cells, knockdown of FBL2 by siRNA has been shown to reduce HCV RNA by 65% [68]. Depletion of the GGPP by statins may inhibit the geranylgeranylation of cellular proteins such as FBL2 and cause the anti-HCV effect in the cells.

Statins are among the most widely used reagents to lower cholesterol. One of the statins used clinically, LOV, has been well characterized and shown anti-HCV activity in cell culture. [66,69,70]. However, the anti-HCV activities of other statins remain to be clarified. Recently the anti-HCV activities of several statins were characterized using an OR6 assay system [71]. The anti-HCV activities were tested for five statins: atorvastatin (ATV), fluvastatin (FLV), pravastatin (PRV), simvastatin (SMV), and LOV. FLV exhibited the strongest anti-HCV activity (50% effective concentration to inhibit HCV RNA replication ($EC_{50}$): 0. 9 μM), while ATV and SMV showed moderate inhibitory effects ($EC_{50}$: 1.39 and 1.57 μM, respectively). However, LOV, which has been reported to inhibit HCV replication, was shown to possess the weakest anti-HCV activity ($EC_{50}$: 2.16 μM). More recently, we found that PTV possessed stronger anti-HCV activity than FLV (Fig. 2B). The $EC_{50}$ of PTV was calculated as 0.45 μM. The anti-HCV activities of statins were reversed by supplying mevalonate or geranylgeraniol. However, surprisingly, PRV exhibited no anti-HCV activity, although it worked as an inhibitor for HMG–CoA reductase. Although PRV is a water-soluble reagent (the others are lipophilic), PRV induced the expression of HMG–CoA reductase by a positive feedback mechanism. There may be another mechanism underlying the depletion of GGPP by the statins. Interestingly, it has been reported that only PRV has a different effect on the induction of P450 compared with the other statins [72].

Ribavirin is the only reagent currently used with IFN-α to treat patients with CH–C [73]. In the previous study on anti-HCV activity using the OR6 assay system, the $EC_{50}$ of ribavirin was 76 μM [74]. This concentration is much higher than the clinically achievable ribavirin concentration (10–14 μM) reported previously [75,76]. Since FLV exhibited strong anti-HCV activity, FLV was examined for its anti-HCV activity in combination with IFN-

α in OR6 cells [71]. Co-treatment of IFN-α and FLV exhibited synergistic inhibitory effects on HCV RNA replication. For example, when administered in combination with IFN-α (2 IU/ml) and FLV (5 μM), the level of HCV RNA replication was remarkably reduced to approximately 3%, compared with the effects of treatment with IFN-α alone. The combination therapy of FLV may be effective for the treatment of patients with CH–C.

It is not appropriate to further reduce the cholesterol level of CH–C patients who already have a normal cholesterol level. For these patients, statin-related anti-HCV reagents possessing no cholesterol-lowering activity would be good candidates for future clinical use. The specific inhibition of GGPP synthesis and prenylation will be worth testing, and GGTase-I inhibitor (GGTI) is one of the candidates for this purpose. Furthermore, specific inhibition of the proteins modified by GGTase-I may be more effective. FBL2 may be one of the target proteins, because its formation of a complex with NS5A is required for HCV RNA replication. Therefore, the reagents blocking the association of FBL2 with NS5A will be able to inhibit the HCV RNA replication with fewer side effects. Prenyltransferase recognizes a broad range of protein substrates with a CaaX motif. Reid et al. [77] reported a list of hypothetical prenyltransferase substrates within the human genome. Other than FBL2, the host molecules involved in HCV RNA replication may be exist in this list.

Antiviral activity of statins has also been reported in other viruses. In the respiratory syncytial virus (RSV), LOV exhibited antiviral activity via the inhibition of RhoA [78]. RhoA is activated by geranylgeranylation, and activated RhoA interacts with the F glycoprotein of RSV. FLV inhibited cytomegalovirus (CMV) replication by abolishing CMV-induced NF-κB activity, which is involved in a pathway that is crucial for CMV replication [79]. In human immunodeficiency virus (HIV), LOV and SIV reduced HIV replication via suppression of the binding between the integrin intercellular adhesion molecule 1(ICAM1) and lymphocyte function associated antigen-1 (LFA-1) [80]. Statins were recently shown to bind to LFA-1, and ICAM1-bearing viruses were reduced by statins in a dose-dependent manner. It is noteworthy that the inhibition of LFA-1 binding to ICAM-1 by statins is independent of the inhibition of HMG CoA reductase. Statins inhibited the cholesterol-biosynthesis pathway and branched prenylation pathways by depletion of mevalonate. The latter caused pleiotropic effects in growth, differentiation, and antivirals. However, an unknown function of statins may exist—for example, the binding of LFA-1 is likely independent of the cholesterol-lowering and the inhibition of prenylation. Furthermore, the finding that PRV has a different effect on the induction of P450 than the other statins has not been clearly explained by the characterization of these mechanisms of statins. A better understanding of this finding may lead to the discovery of statin-related anti-HCV reagents that do not have exhibit any cholesterol-lowering activity or inhibition of prenylation.

### 4.2. Sphingolipid synthesis pathway

Lipid rafts are detergent resistant membranes (DRM) and are enriched in cholesterol and sphingolipids. The active replication complex of HCV is present in lipid rafts [65]. Therefore, sphingolipid metabolism is also an antiviral target for HCV. Serine palmitoyltransferase (SPT) is the enzyme responsible for the condensation of L-serine with palmitoyl–CoA to produce 3-ketodihydrosphingosine in the first step of sphingolipid biosynthesis (Fig. 4). Myriocin, a selective inhibitor of SPT, inhibited the replication of HCV replicon [81,82].

Sakamoto et al. [81] reported that the compound NA255, which is structurally similar to myriocin, also inhibited the replication of the HCV replicon. NA255 has been identified as the secondary fungal metabolite derived from *Fusarium* sp. NA255 suppressed HCV replicon in a dose-dependent manner, and its $EC_{50}$ was 2 nM. They further examined the involvement of the sphingolipid synthetic pathway in HCV RNA replication. Fumonisin B1, an inhibitor of dihydroceramide synthase, also suppressed the replication of HCV replicon. In mammalian cells, ceramide is synthesized in the endoplasmic reticulum (ER) and translocates to the Golgi compartment for conversion to sphingomyelin. HPA-12, an inhibitor of ceramide trafficking from the ER to the Golgi apparatus, also inhibited the replication of HCV replicon. Glycosphingolipids (GSLs) are also a component of lipid rafts, and PPMP, an inhibitor of GSL biosynthesis, also suppressed the replication of HCV replicon. Furthermore, they demonstrated that after treatment with NA255, the NS5B ratio in the DRM was markedly decreased. Interestingly, however, the DRM fraction of NS3 and NS5A were not affected. Inhibition of sphingolipid biosynthesis by NA255 disrupted the association of lipid rafts with NS5B, but not with NS3 or NS5A. They identified a helix-turn-helix motif (Glu230-Gly263) in NS5B as a sphingolipid-binding domain (SBD), which was similar in structure to the SBD of the V3 loop of HIV-1.

Umehara et al. [82] reported that myriocin suppressed HCV RNA replication *in vivo*, using HCV-infected chimeric mice with humanized livers. Myriocin reduced the HCV RNA levels in both serum and liver to 1/10–1/100 of the levels prior to the 8 day treatment. They also demonstrated that the combined treatment of myriocin with PEG-IFN reduced the HCV RNA level to less than 1/1000 of the control levels. These results

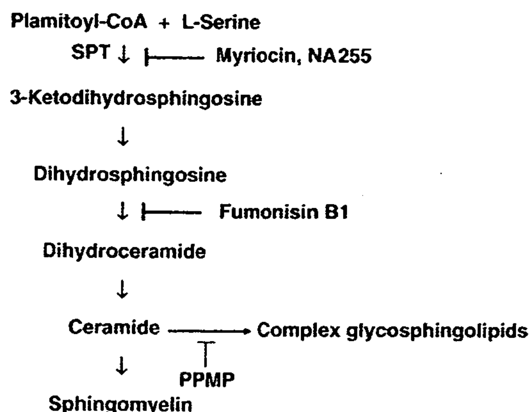


Fig. 4. Sphingolipid-biosynthesis pathway. The spingolipid-biosynthesis pathway. Myriocin and NA255 inhibited the SPT and caused the depletion of spingomyelin and glycosphingolipids.

suggest that the sphingolipid biosynthetic pathway is also a suitable target for the development of HCV therapies.

### 4.3. GTP-biosynthesis pathway

At the beginning of GTP-biosynthesis pathway, inosine monophosphate dehydrogenase (IMPDH) is the enzyme responsible for the conversion of inosine 5′ monophosphate (IMP) into xanthosine 5′ monophosphate (XMP) (Fig. 5). Ribavirin, mizoribine, mycophenolic acid (MPA), and VX-497 are IMPDH inhibitors and inhibit HCV RNA replication.

Ribavirin enhanced the SVR of PEG-IFN therapy from 29% to 56% compared to the PEG-IFN monotherapy [83]. However, the antiviral mechanisms of ribavirin remain to be clarified. Four possible mechanisms have been proposed [73,84]: (1) direct inhibition of RNA replication; (2) inhibition of IMPDH; (3) immunomodulation; (4) mutagenesis. Ribavirin is phosphorylated to mono-, di-, and triphosphate (RMP, RDP, and RTP, respectively). (1) RTP, an analog of GTP, is incorporated into replicating RNA by RdRp and caused termination of the RNA synthesis. (2) RMP competitively inhibits the host enzyme IMPDH, which is essential for the synthesis of GTP, and causes a depletion of the GTP pool. (3) Rivavirin has been suggested to cause immunomodulatory effects, such as the shift of Th2 to Th1 in immune response, and to induce an HCV-specific T cell response. (4) Ribavirin acts as an RNA mutagen and causes error catastrophe. In poliovirus replication, 100 μM of ribavirin increased the mutation rate from about 1.5 mutations/genome (wild type) to about 1.9 mutations/genome and resulted in a decrease of infectivity of 70% [85]. The mutation rate increased in a ribavirin dose-dependent manner: 6.9 mutations/genome and 15.5 mutations/genome at 400 μM and 1000 μM, respectively [85].

In the clinical study of CH–C, the enhancement of SVR has been observed only in combination therapy of ribavirin with IFN, but not in ribavirin monotherapy. It may be difficult to test the effect of ribavirin monotherapy, since the clinically achievable concentration of ribavirin without severe side effects such as anemia is too low (10–14 μM). However, in the cell culture model [74,86], a higher concentration of ribavirin suppressed HCV RNA replication ($EC_{50}$: 76 μM) [74].

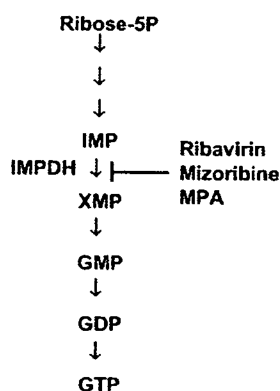


Fig. 5. GTP-biosynthesis pathway. The de novo GTP-biosynthesis pathway. Ribavirin, mizoribine, and MPA suppressed the XMP synthesis by the inhibition of IMPDH.

Mizoribine is an imidazole nucleoside that is isolated from culture medium of the mold *Eupenicillium brefeldianum* M-2166 and is structurally similar to ribavirin. Mizoribine was authorized by the Japanese Government as an immunosuppressive drug for renal transplantation; thereafter, lupus nephritis, rheumatoid arthritis, and nephritic syndrome were also added to the list of diseases for which this agent is indicated [87,88]. Based on the similarity of mizoribine to ribavirin, the anti-HCV activity of mizoribine has been tested using an OR6 assay system. The anti-HCV activity of mizoribine ($EC_{50}$: 99 μM) was similar to that of ribavirin [74]. Furthermore, a low dose (at least 5 μM) of mizoribine was able to enhance the antiviral activity of IFN [74]. Mizoribine was reported to exhibit antiviral activity on influenza virus types A and B [87] and recently on bovine viral diarrhea virus [89] and severe acute respiratory syndrome (SARS)-associated coronavirus [90]. The precise antiviral mechanism of mizoribine remains unclear. However, any of the four hypothesized mechanisms of ribavirin mentioned above may be possible. Since mizoribine has not been associated with severe side effects, it will be an alternative reagent for combination therapy with IFN.

Like mizoribine, MPA is used as an immunosuppressant and is known to inhibit IMPDH. It has been reported to show *in vitro* antiviral activity against dengue virus [91,92], hepatitis B virus (HBV) [93], avian reovirus [94], yellow fever virus [95], and West Nile virus [96]. The anti-HCV activity of MPA was reported by Henry et al. [97]. At clinically relevant concentrations (1.0–6.0 μg/ml), MPA inhibited HCV RNA replication to approximately 75% in a study using HCV replicon-harboring cells. Furthermore, combination treatment of MPA with CsA or IFN showed synergistic inhibition of HCV RNA replication. We also recently confirmed that the combination of CsA and mizoribine had a synergistic effect on the inhibition of HCV RNA replication (Yano et al., unpublished data). These data suggest that immunosuppressive drugs possessing anti-HCV activity, such as CsA, MPA, and mizoribine, may prevent not only the rejection of the graft but also the recurrence of HCV infection after liver transplantation, and that a combination of these drugs may be of additional benefit for such patients.

VX-497 is a reversible uncompetitive IMPDH inhibitor that is structurally unrelated to other known IMPDH inhibitors. Markland et al. [98] reported the broad-spectrum antiviral activity of VX-497.VX-497 exhibited 10- to 100-fold more potency than ribavirin against HBV, human CMV, RSV, herpes simplex virus type 1, parainfluenza 4 virus, EMCV, and Venezuelan equine encephalomyelitis virus in cell culture [98]. Zhou et al. [99] reported that VX-497 alone had only marginal effect on HCV replicon, although combination treatment with ribavirin and VX-497 enhanced anti-HCV activity. They also reported that in their HCV replicon assay system, MPA showed only a marginal anti-HCV effect [99]. This result is different from the anti-HCV effect of MPA reported by Henry et al. [97]. Further study will be needed to clarify these controversial results.

### 4.4. N-glycosylation pathway

HCV morphogenesis is a target of antivirals in the life cycle of the virus. The HCV envelope glycoproteins E1 and E2 are highly N-glycosylated [100]. The consensus sequence for N-glycosylation is Asn-X-Ser/Thr, where X is any amino acid except for Pro, and E1 and E2 contain 5-6 and 11 glycosylation sites, respectively. From the previous study using bovine viral diarrhea virus, inhibition of α-glucosidase is expected to prevent the proper folding and assembly of HCV. Therefore, the N-glycosylation pathway may be a novel molecular target for antivirals. Chapel et al. [101] reported an anti-HCV effect of the α-glucosidase inhibitor in the binding step using HCV virus-like particles (VLPs) derived from baculovirus. The glucose analogue deoxynojirimycin derivatives, which are α-glucosidase inhibitors, caused the retention of unprocessed, hyperglycosylated N-linked glycans on HCV glycoproteins and led to the reduction in binding of VLP to the cells [101]. These results will be examined using a recently developed infectious HCV production cell culture system. α-glucosidase inhibitor may be one of the candidates for an effective combination therapy.

### 4.5. STAT1 methylation

It is crucial that the SVR for patients with CH–C receiving the current standard therapy of PEG-IFN plus ribavirin is improved from the current value of about 50%. The anti-HCV effect of IFN-α is caused through the Jak-STAT signaling pathway. Duong et al. [102] proposed that hypomethylation of STAT1 by HCV protein caused the resistance to IFN therapy. Unmethylated STAT1 is less active because it can be bound and inactivated by its inhibitor, the protein inhibitor of activated STAT1 (PIAS1). Protein arginine methyltransferase 1 (PRMT1) is the enzyme responsible for the methylation of STAT1. HCV proteins induced the expression of the catalytic subunit of protein phosphatase 2A (PP2Ac), and overexpression of PP2Ac induced STAT1 hypomethylation via the inhibition of PRMT1. Finally, PIAS1 interacted with and inhibited hypomethylated STAT1 and resulted in the suppression of IFN signaling [102].

S-adenosyl-L-methionine (AdoMet) is a methyl group donor for STAT1 methylation by PRMT1. AdoMet is used for the treatment of alcoholic liver disease and is available in many countries as a nonprescription drug. Betaine has been known to raise the intracellular concentration of AdoMet and plays the central role in the recycling of AdoMet. When PP2Ac was overexpressed in HuH-7 and UHVH 57.3 cells, IFN-α signaling was suppressed [102]. However, the co-treatment of AdoMet and betaine restored the IFN-α signaling. These results suggest that the addition of AdoMet and betaine to the current standard therapy with PEG-IFN and ribavirin may enhance the SVR for patients with CH–C.

### 4.6. Fatty acid-biosynthesis pathway

Lipid metabolism is one of the most important pathways for HCV RNA replication. Other than cholesterol and sphingolipid synthesis, fatty acids are reported to be metabolites involved in HCV RNA replication [70,103]. However, the precise mechanisms of fatty acids on HCV RNA replication have remained unclear.

Leu et al. [103] reported that polyunsaturated fatty acids (PUFAs) inhibited HCV replicon replication. Arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) belong to PUFAs (Fig. 6) and possessed anti-HCV activity. The $EC_{50}$ of AA was 4 μM. However, at 100 μM, α-linolenic acid, γ-linolenic acid (GLA), and linoleic acid reduced HCV RNA levels slightly, and saturated fatty acids, including oleic acid, myristic acid, palmitic acid, and steric acid, slightly enhanced HCV RNA levels. Similar results were also reported by Kapadia et al. [70] using a genome-length HCV RNA-replicating cell line.

AA produces lipid mediators such as prostaglandins (PGs), thromboxanes (Txs), leukotriens (LTs), and lipoxins (LXs) (Fig. 6). However, the antiviral activity of these eicosanoids remains unclear. In their clinical study, Hyman et al. [104]

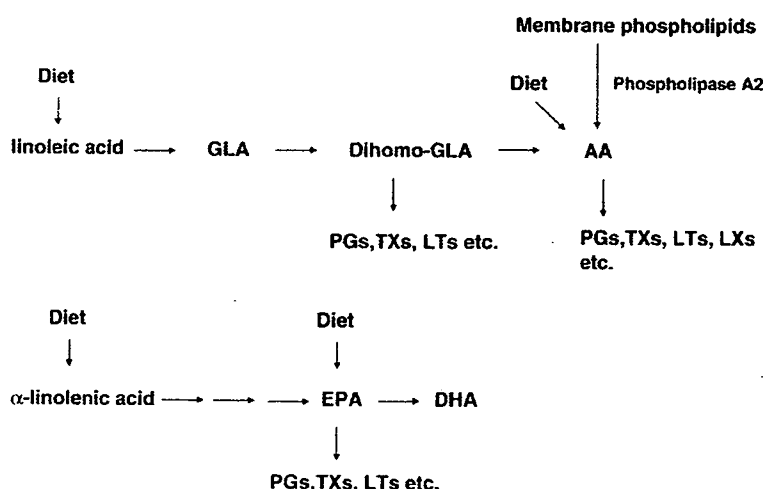


Fig. 6. Fatty acid-biosynthesis pathway. The PUFA metabolism from diet or membrane phospholipids.

reported that oral prostaglandin E2 therapy resulted in no beneficial effect on patients with CH–C. Investigation of the anti-HCV effects of the metabolites of PUFAs will lead to a new field of antivirals based on the host metabolism.

## 5. Conclusions

Ever since HCV was discovered to be the causative agent of non-A, non-B hepatitis virus, IFN has played the central role in treating the disease. Currently IFN has been modified by PEG and accompanied by the powerful partner, ribavirin, which boosts the anti-HCV activity of IFN. During the development of IFN therapy for patients with CH–C, the lack of a robust method of HCV RNA replication in cell culture has hampered research into the HCV life cycle and the discovery of potent new anti-HCV reagents. It is difficult to attack the Achilles' heel of HCV without information on the replication machinery of the virus. However, the development of a subgenomic replicon system by Lohmann et al. [5] partially revealed the HCV life cycle. The information about HCV RNA replication in the virus life cycle provided clues to the development of antivirals both from the standpoint of the virus and the host. A representative example is the discovery that NS3-4A inhibits innate immunity [105]. HCV runs through the cellular first defenses of the IFN-production system. NS3-4A, a serine protease, cleaved the unexpected cellular target Cardif and disrupted RIG-I signaling [106]. HCV replicon contributed to the discovery of the viral serine protease inhibitor. Surprisingly, a serine protease inhibitor, SCH6, inhibited HCV RNA replication not only by the inhibition of NS3-4A activity but also by the inhibition of the RIG-I signaling [105]. This serine protease inhibitor possesses dual functions, inhibiting both viral (NS3-4A) and cellular (Cardif) proteins involved in IFN production.

Viral and cellular molecules are the targets of antivirals. HCV RdRp caused a high mutation rate and the mutations accumulated in virus genome [107]. The high mutation rate enhances the viral evolution. As for the reagents targeting viral proteins, such as NS3-4A or NS5B, resistance to the therapy happens by the frequent mutations caused by RdRp. In fact, in the clinical trial of the NS3-4A protease inhibitor, VX-950, HCV RNA rapidly decreased within 3 days after treatment [20]. However, HCV RNA increased again at around 14 days after treatment [20]. HCV mutants may not be the problem in the anti-HCV reagent against cellular proteins, although the inhibition of the primary functions of the cellular proteins may cause side effects. In this review, host metabolic pathways are overviewed. One of the advantages of targeting host metabolism as antivirals is that multiple enzymes involved in the metabolism could become candidates for antivirals. In the strategy targeting host metabolism, we should be careful in regard to the side effects caused by inhibition of the primary function of the metabolite. To minimize these undesirable effects, pinpoint inhibition of the enzyme should be done.

Lipid metabolism is one of the important targets for antivirals among cellular factors. Very recently, we examined the effect of ordinary nutrients on HCV RNA replication [108]. Using an OR6 assay system, we found that linoleic acid possessed an anti-HCV effect and its combination with CsA exerted synergistic inhibitory effect on HCV RNA replication [108]. However, the anti-HCV mechanism of PUFAs remains unclear. An improved understanding of the anti-HCV effect of PUFAs will extend the field of host metabolism as a target of antivirals in the future.

One recent striking advance is the development of a method for infectious HCV production in cell culture. This system provides information regarding the complete life cycle of HCV and will extend our understanding of the antivirals to virus entry, assembly and release. The discovery of anti-HCV reagents targeting host metabolism in the HCV life cycle will improve the SVR in combination with IFN. Or, the development of new anti-HCV reagents could lead to the retirement of IFN in the near future.

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# DDX3 DEAD-Box RNA Helicase Is Required for Hepatitis C Virus RNA Replication[▽]

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**DDX3, a DEAD-box RNA helicase, binds to the hepatitis C virus (HCV) core protein. However, the role(s) of DDX3 in HCV replication is still not understood. Here we demonstrate that the accumulation of both genome-length HCV RNA (HCV-O, genotype 1b) and its replicon RNA were significantly suppressed in HuH-7-derived cells expressing short hairpin RNA targeted to DDX3 by lentivirus vector transduction. As well, RNA replication of JFH1 (genotype 2a) and release of the core into the culture supernatants were suppressed in DDX3 knockdown cells after inoculation of the cell culture-generated HCVcc. Thus, DDX3 is required for HCV RNA replication.**

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DEAD-box RNA helicases are involved in various RNA metabolic processes, including transcription, translation, RNA splicing, RNA transport, and RNA degradation (9, 20). DDX1 and DDX3, DEAD-box RNA helicases, have been implicated in the replication of human immunodeficiency virus type 1 (HIV-1). Both DDX1 and DDX3 interact with HIV-1 Rev and enhance Rev-dependent HIV-1 RNA nuclear export (10, 24).

On the other hand, DDX3 binds to the hepatitis C virus (HCV) core protein (17, 19, 25), and DDX3 expression is deregulated in HCV-associated hepatocellular carcinoma (HCC) (7, 8). However, the biological function of DDX3 in HCV replication is still not understood. To address this issue, we first used lentivirus vector-mediated RNA interference to stably knock down DDX3 in three HuH-7-derived cell lines: O cells, harboring a replicative genome-length HCV RNA (HCV-O, genotype 1b) (13); sO cells, harboring its subgenomic replicon of HCV RNA (14); or RSc cured cells, which cell culture-generated HCV (HCVcc) (JFH1, genotype 2a) (23) could infect and effectively replicate in (M. Ikeda et al., unpublished data). Oligonucleotides with the following sense and antisense sequences were used for the cloning of short hairpin RNA (shRNA)-encoding sequences against DDX3 in the lentivirus vector: for DDX3i#3, 5′-GATCCCCGGAGGAAATTATAACTCCCTTCAAGAGAGGGAGTTATAATTTCCTCCTTTTTGGAAA-3′ (sense) and 5′-AGCTTTTCCAAAAAGGAGGAAATTATAACTCCCTCTCTTGAAGGGAGTTATAATTTCCTCCGGG-3′ (antisense); for DDX3i#7, 5′-GATCCCCGGTCACCCTGCCAAACAAGTTCAAGAGACTTGTTTGGCAGGGTGACCTTTTTGGAAA-3′ (sense) and 5′-AGCTTTTCCAAAAAGGTCACCCTGCCAAACAAGTCTCTTGAACTTGTTTGGCAGGGTGACCGGG-3′ (antisense). These oligonucleotides were annealed and subcloned into the BglII-HindIII site, downstream from an RNA polymerase III promoter of pSUPER (6). To construct pLV-DDX3i#3 and pLV-DDX3i#7, the BamHI-SalI fragments of the corresponding pSUPER plasmids were subcloned into the BamHI-SalI site of pRDI292 (5), an HIV-1-derived self-inactivating lentivirus vector containing a puromycin resistance marker allowing for the selection of transduced cells. The vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1-based vector system has been described previously (18). We used the second-generation packaging construct pCMV-ΔR8.91 (26) and the VSV-G-envelope plasmid pMDG2. The lentivirus vector particles were produced by transient transfection of 293FT cells with FuGene 6 (Roche).

Western blot analysis of the lysates demonstrated the only trace of DDX3 protein in DDX3 knockdown O cells (DDX3i#3) (Fig. 1A). In this context, the HCV core expression level was significantly decreased in the DDX3 knockdown O cells (Fig. 1A). To further confirm this finding, we examined the level of HCV RNA in these cells. We found that accumulation of genome-length HCV-O RNA was notably suppressed in DDX3 knockdown O cells (Fig. 1B). Furthermore, the efficiency of colony formation in DDX3 knockdown Oc cells (created by eliminating genome-length HCV RNA from O cells by interferon treatment) transfected with the genome-length HCV-O RNA with an adapted mutation at amino acid (aa) position 1609 in the NS3 helicase region (K1609E) (13) was also notably reduced compared with that in control cells (Fig. 1C). In contrast, highly efficient knockdown of an unrelated host factor, poly(ADP-ribose) polymerase 1 (PARP-1) (4), had no observable effects on HCV RNA replication, the efficiency of colony formation, or the core expression level (data not shown), suggesting that our finding was not due to a nonspecific event. Interestingly, accumulation of the subgenomic replicon RNA (HCV-sO) was also suppressed in DDX3 knockdown sO cells (Fig. 1D). Moreover, we examined the potential role of DDX3 in an HCV infection and produc-

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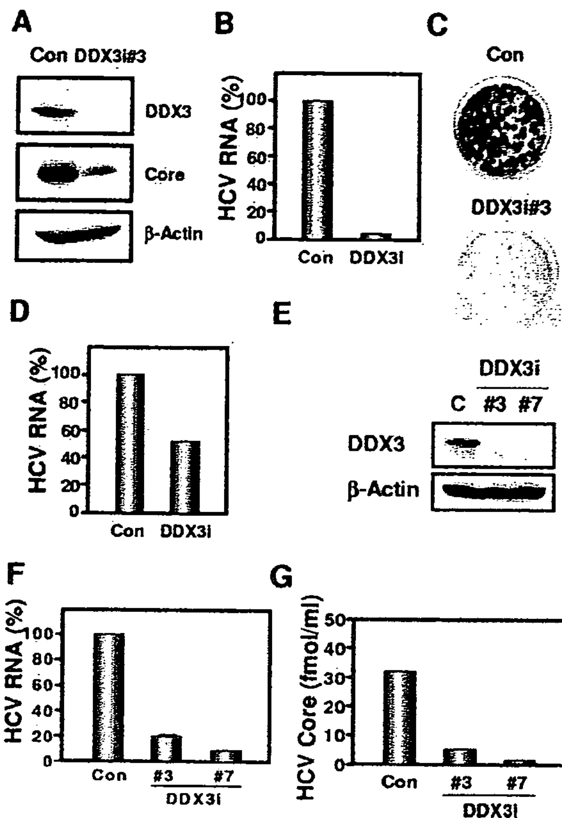


FIG. 1. Requirement of DDX3 for HCV replication. (A to D) Effect of DDX3 knockdown on HCV RNA replication. (A) Inhibition of DDX3 expression by shRNA-producing lentivirus vector. The results of Western blot analysis of cellular lysates with anti-DDX3 (ProSci), anti-HCV core (CP-9; Institute of Immunology), or an anti-β-actin antibody (Sigma) in O cells expressing shRNA against DDX3 (DDX3i#3) as well as in O cells transduced with a control lentivirus vector (Con) are shown. (B) The level of genome-length HCV RNA was monitored by real-time LightCycler PCR (Roche). Experiments were done in duplicate, and bars represent the mean percentages of HCV RNA. (C) Efficiency of colony formation in DDX3 knockdown cells. In vitro-transcribed ON/C-5B K1609E RNA (2 μg) was transfected into the DDX3 knockdown Oc cells (DDX3i#3) or the Oc cells transduced with a control lentivirus vector (Con). G418-resistant colonies were stained with Coomassie brilliant blue at 3 weeks after electroporation of RNA. Experiments were done in duplicate, and representative results are shown. (D) The level of subgenomic replicon RNA was monitored by real-time LightCycler PCR. Experiments were done in duplicate, and bars represent the mean percentages of HCV RNA. (E to G) Effect of DDX3 knockdown on HCV infection. (E) Inhibition of DDX3 expression by shRNA-producing lentivirus vector. The results of Western blot analysis of cellular lysates with anti-DDX3 or an anti-β-actin antibody for RSc cells expressing the shRNA DDX3i#3 or DDX3i#7 and for RSc cells transduced with a control lentivirus vector (Con) are shown. (F) The level of genome-length HCV (JFH1) RNA was monitored by real-time LightCycler PCR after inoculation of the cell culture-generated HCVcc. Results from three independent experiments are shown. (G) The levels of the HCV core in the culture supernatants were determined by an enzyme-linked immunosorbent assay (Mitsubishi Kagaku Bio-Clinical Laboratories). Experiments were done in duplicate, and bars represent the mean HCV core protein levels.

tion system (23). We found 80 to 90% reductions in the accumulation of JFH1 RNA and 82 to 94% reductions in the release of the core into the culture supernatants in DDX3 knockdown HuH-7-derived RSc cured cells at 4 days after inoculation of HCVcc (Fig. 1E to G). Thus, DDX3 seems to be required for HCV RNA replication.

Previously, DDX3 was identified as an HCV core-interacting protein by yeast two-hybrid screening. This interaction required the N-terminal domain of the core (aa 1 to 59) and the C-terminal domain of DDX3 (aa 553 to 622) (17, 19, 25). To determine whether the core can interact with DDX3 regardless of the HCV genotype, we used the HCV-O core (genotype 1b) and the JFH1 core (genotype 2a) (Table 1). We first examined their subcellular localization by confocal laser scanning microscopy as previously described (3). Consistent with previous reports (17, 19, 25), both the HCV-O core and JFH1 core mostly colocalized with DDX3 in the perinuclear region (Fig. 2A). Then we immunoprecipitated lysates from 293FT cells in which hemagglutinin (HA)-tagged DDX3 and HCV-O core, JFH1 core, or their 40-aa N-truncated mutants were overexpressed with an anti-HA antibody. Cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 0.5% NP-40, 10 mM NaF, 0.1 mM $Na_3VO_4$, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Lysates were precleared with 30 μl of protein G-Sepharose (GE Healthcare Bio-Sciences). Precleared supernatants were incubated with 1 μg of anti-HA antibody (3F10; Roche) at 4°C for 1 h. Following absorption of the precipitates

TABLE 1. Primers used for construction of the HCV core-expressing plasmids[a]

| Plasmid name | Direction | Primer sequence |
|---|---|---|
| pCXbsr/core(HCV-O) | Forward | 5′-GGAATTCCCACCATGAG CACGAATCCTAAACCTC-3 |
| | Reverse | 5′-ATAAGAATGCGGCCGCC TATCAAGCGGAAGCTGG GATGGT-3′ |
| pcDNA3/core(HCV-O) | Forward | 5′-CGGGATCCAAGATGAGC ACGAATCCTAAACCTCAA AGA-3′ |
| pcDNA3/FLAG-core(HCV-O) | Reverse | 5′-CCGCTCGAGTCAAGCGG AAGCTGGGATGGTCAAA CA-3′ |
| pcDNA3/Δcore(HCV-O) | Forward | 5′-CGGGATCCAAGATGGGC CCCAGGTTGGGTGTGCG C-3′ |
| pcDNA3/FLAG-Δcore(HCV-O) | Reverse | 5′-CCGCTCGAGTCAAGCGG AAGCTGGGATGGTCAAA CA-3′ |
| pcDNA3/core(JFH1) | Forward | 5′-CGGGATCCAAGATGAGC ACAAATCCTAAACCTCAA AGA-3′ |
| pcDNA3/FLAG-core(JFH1) | Reverse | 5′-CCGCTCGAGTCAAGCAG AGACCGGAACGGTGATG CA-3′ |
| pcDNA3/Δcore(JFH1) | Forward | 5′-CGGGATCCAAGATGGGC CCCAGGTTGGGTGTGCG C-3′ |
| pcDNA3/FLAG-Δcore(JFH1) | Reverse | 5′-CCGCTCGAGTCAAGCAG AGACCGGAACGGTGATG CA-3′ |

[a] To construct pCXbsr/core(HCV-O), a DNA fragment encoding the core was amplified by PCR from pON/C-5B (13) with the indicated primers. The PCR product was digested with EcoRI-NotI and subcloned into the same site of pCX4bsr (1). To construct pcDNA3/core(HCV-O), pcDNA3/FLAG-core(HCV-O), pcDNA3/Δcore(HCV-O), and pcDNA3/FLAG-Δcore(HCV-O), DNA fragments encoding the core were amplified by PCR from pON/C-5B (13) with the indicated primer sets. To construct pcDNA3/core(JFH1), pcDNA3/FLAG-core-(JFH1), pcDNA3/Δcore(JFH1), and pcDNA3/FLAG-Δcore(JFH1), DNA fragments encoding the core were amplified by PCR from pJFH1 (23) with the indicated primer sets. The PCR products were digested with BamHI and XhoI and then subcloned into the same site of pcDNA3 (Invitrogen) or pcDNA3-FLAG (2).

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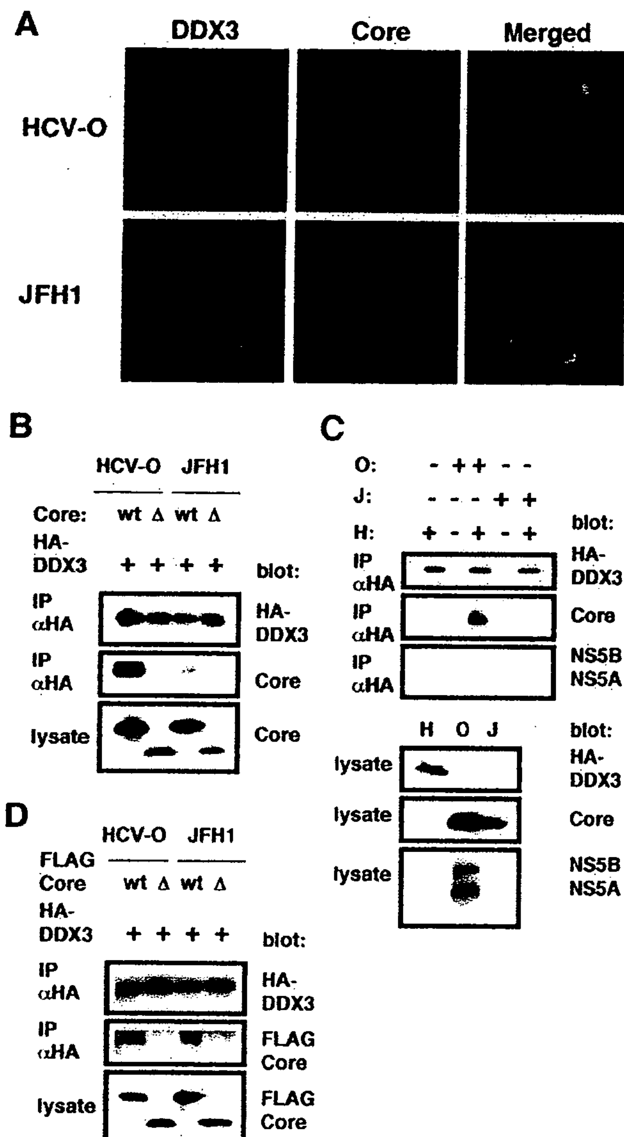


FIG. 2. Interaction of the HCV core with DDX3. (A) The HCV core colocalizes with DDX3. 293FT cells cotransfected with 100 ng of pCXbsr/core(HCV-O) or pcDNA3/core(JFH1) and 200 ng of pHA-DDX3 were examined by confocal laser scanning microscopy. Cells were stained with anti-HCV core (CP-9 and CP-11 mixture) and anti-DDX3 antibodies and were then visualized with fluorescein isothiocyanate (DDX3) or Cy3 (core). Images were visualized using confocal laser scanning microscopy (LSM510; Carl Zeiss). The right panels exhibit the two-color overlay images (Merged). Colocalization is shown in yellow. (B) The core binds to DDX3. 293FT cells were cotransfected with 4 μg of pHA-DDX3 and 4 μg of pCXbsr/core(HCV-O) (wt), pcDNA3/Δcore(HCV-O) (Δ), pcDNA3/core(JFH1) (wt), or pcDNA3/Δcore(JFH1) (Δ). The cell lysates were immunoprecipitated with an anti-HA antibody (3F10; Roche), followed by immunoblot analysis using either anti-HA (HA-7; Sigma) or anti-HCV core antibody (CP-9 and CP-11 mixture). (C) 293FT cells transfected with 4 μg of pHA-DDX3 (H), O cells (O), or RSc cells 3 days after inoculation of HCVcc (JFH1) (J) were lysed and immunoprecipitated (IP) with 1 μg of anti-HA antibody (3F10), followed by immunoblotting with anti-HA (HA-7), anti-core (CP-9 and CP-11 mixture), or anti-HCV NS5A (no. 8926) and anti-HCV NS5B. (D) 293FT cells transfected with 4 μg of pHA-DDX3 and 4 μg of pcDNA3/FLAG-core(HCV-O) (wt), pcDNA3/FLAG-Δcore(HCV-O) (Δ), pcDNA3/FLAG-core(JFH1) (wt), or pcDNA3/FLAG-Δcore(JFH1) (Δ) were lysed and immunoprecipitated with 1 μg of an anti-HA antibody (3F10), followed by immunoblotting with an anti-HA (HA-7) or anti-core (CP-9 and CP-11 mixture) antibody.

on 30 μl of protein G-Sepharose resin for 1 h, the resin was washed four times with 700 μl lysis buffer. Proteins were eluted by boiling the resin for 5 min in 1× Laemmli sample buffer. The proteins were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblot analysis using either anti-HA (HA-7; Sigma) or anti-HCV core antibody (CP-9 and CP-11 mixture). We observed that the HCV-O core but not its N-truncated mutant could strongly bind to HA-tagged DDX3 (Fig. 2B). In contrast, the binding activity of the JFH1 core to HA-tagged DDX3 seemed to be fairly weak (Fig. 2B). As well, immunoprecipitation of lysates of 293FT cells expressing HA-tagged DDX3, O cells, or JFH1-infected RSc cells, or mixtures of these lysates, with an anti-HA antibody revealed that HCV-O core but not JFH1 core could bind strongly to DDX3 (Fig. 2C). The anti-HCV core antibody we used could detect both HCV-O core and JFH1 core (Fig. 2), while both anti-HCV NS5A and anti-NS5B antibodies failed to detect JFH1 NS5A and NS5B (Fig. 2C). At least, we failed to detect an interaction between DDX3 and either HCV-O NS5A or NS5B under experimental conditions that permitted the core to interact with DDX3 by immunoprecipitation (Fig. 2C). In contrast, the FLAG-tagged JFH1 core could bind to HA-tagged DDX3 just as efficiently as the FLAG-tagged HCV-O core could (Fig. 2D). Thus, the binding affinity or stability of the complex formed between the JFH1 core and DDX3 might be weaker than that between the HCV-O core and DDX3.

Since DDX3 is required for HIV-1 and HCV replication, we hypothesized that the HCV core might affect the function of HIV-1 Rev when both proteins were coexpressed. To test this hypothesis, we used the Rev-dependent luciferase-based reporter plasmid pDM628, harboring a single intron that includes both the Rev-responsive element (RRE) and the luciferase coding sequences (Fig. 3A) (10). In this system, Rev binds to RRE on the unspliced reporter mRNA, allowing its export from the nucleus for luciferase reporter gene expression, while the intron containing the luciferase gene is excised during RNA splicing when cells are transiently transfected with pDM628 alone. As previously reported (10), the luciferase activity in 293FT cells transfected with this reporter plasmid was stimulated by Rev, which induced a fourfold increase in the reporter signal (Fig. 3B). Luciferase activity was increased eightfold by the combination of Rev and DDX3, whereas neither the HCV-O core nor the JFH1 core had any effect on this Rev function (Fig. 3B). Since the Rev-binding domain (the N-terminal domain) and the core-binding domain (the C-terminal domain) do not overlap in DDX3, the HCV core might not compete with HIV-1 Rev for binding with DDX3. However, the development of a novel DDX3 inhibitor might provide a powerful antiviral agent against both HIV-1 and HCV (15).

Taking these results together, this study has shown for the first time that DDX3 is required for HCV RNA replication.

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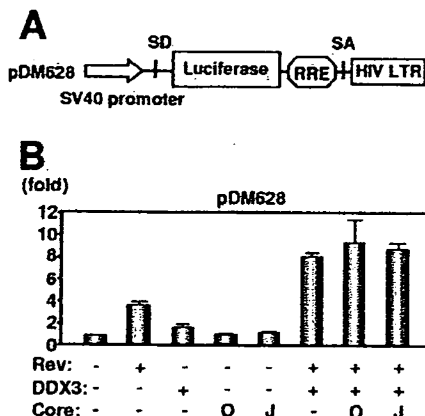


FIG. 3. HCV core does not affect the DDX3-mediated synergistic activation of Rev function. (A) Schematic representation of HIV-1 Rev-dependent luciferase-based reporter plasmid pDM628 harboring a splicing donor (SD), splicing acceptor (SA), and RRE. (B) 293FT cells were cotransfected with 100 ng of pDM628, 200 ng of pcRev, 200 ng of pHA-DDX3, and/or 100 ng of either pcDNA3/core(HCV-O) (O) or pcDNA3/core(JFH1) (J). A luciferase assay was performed 24 h later. All transfections utilized equal total amounts of plasmid DNA owing to the addition of the empty vector pcDNA3 to the transfection mixture. The relative stimulation of luciferase activity (*n*-fold) is shown. The results shown are means from three independent experiments.

Since helicases are motor enzymes that use energy derived from nucleoside triphosphate hydrolysis to unwind double-stranded nucleic acids, the DDX3–core complex might unwind the HCV double-stranded RNA and separate the RNA strands or might contribute to the function of HCV NS3 helicase. Since the replication of subgenomic replicon RNA was also partially suppressed in DDX3 knockdown cells (Fig. 1D), DDX3 might be associated with an HCV nonstructural protein(s) or HCV RNA itself. Indeed, Tingting et al. recently reported that DDX1 bound to both the HCV 3′ untranslated region (3′ UTR) and the HCV 5′ UTR and that short interfering RNA-mediated knockdown of DDX1 caused a marked reduction in the replication of subgenomic replicon RNA (22). Furthermore, Goh et al. demonstrated that DDX5/p68 associated with HCV NS5B and that depletion of endogenous DDX5 correlated with a reduction in the transcription of negative-strand HCV RNA (11). However, we failed to observe an interaction between DDX3 and NS5A or NS5B by immunoprecipitation under our experimental conditions in which the core could interact with DDX3 (Fig. 2C). Importantly, our DDX3 knockdown study demonstrated a more significant reduction in the accumulation of genome-length HCV RNA (95% reduction) than in the accumulation of subgenomic replicon RNA (52% reduction) (Fig. 1B and D). To date, it has been demonstrated that the 5′ UTR, the 3′ UTR, and the NS3-to-NS5B coding region are sufficient for HCV RNA replication (16); however, the core might be partly involved in the replication of genome-length HCV RNA. Importantly, DDX1 and DDX3 were specifically detected in the lipid droplets of core-expressing Hep39 cells by proteomic analysis (21), suggesting that DDX3 might be associated with HCV assembly or might incorporate into the HCV virion through interaction with the core to act as an RNA chaperone.

Recent studies have suggested a potential role of DDX3 and DDX5 in the pathogenesis of HCV-related liver diseases. DDX3 expression is deregulated in HCV-associated HCC (7, 8), and Huang et al. identified single-nucleotide polymorphisms in the DDX5 gene that were significantly associated with an increased risk of advanced fibrosis in patients with chronic hepatitis C (12). Interestingly, DDX3 might be a candidate tumor suppressor. DDX3 inhibits colony formation in various cell lines, including HuH-7, and up-regulates the $p21^{waf1/cip1}$ promoter (8). If DDX3 could in fact suppress tumor growth, then the core might overcome DDX3-mediated cell growth arrest and down-regulate $p21^{waf1/cip1}$ through interaction with DDX3, and it might also be involved in HCC development.

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# Hepatitis C virus NS5B delays cell cycle progression by inducing interferon-β via Toll-like receptor 3 signaling pathway without replicating viral genomes

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**Abstract**

To clarify the pathogenesis of hepatitis C virus (HCV), we have studied the effects of HCV proteins using human hepatocytes. Here, we found that HCV NS5B, an RNA-dependent RNA polymerase, delayed cell cycle progression through the S phase in PH5CH8 immortalized human hepatocyte cells. Since treatment with anti-interferon (IFN)-β neutralizing antibody restored the cell cycle delay, IFN-β was deemed responsible for the cell cycle delay in NS5B-expressing PH5CH8 cells. The induction of IFN-β and the cell cycle delay were overridden by the down-regulation of Toll-like receptor 3 (TLR3) through RNA interference in NS5B-expressing PH5CH8 cells. Moreover, the NS5B full form was required for the cell cycle delay, the induction of IFN-β, and the activation of the IFN-β signaling pathway. Our findings revealed that NS5B induced IFN-β through the TLR3 signaling pathway in immortalized human hepatocytes even without replicating viral genomes.
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*Keywords:* Hepatitis C virus; NS5B; Interferon-β; TLR3; Hepatocyte cells

## Introduction

Since more than 170 million individuals are estimated to be infected with hepatitis C virus (HCV) worldwide, this disease is a global health problem (Thomas, 2000). HCV belongs to the family Flaviviridae, whose positive-stranded RNA genome encodes a large polyprotein precursor of approximately 3000 amino acid residues. This polyprotein is processed by a combination of the host and viral proteases into at least ten proteins in the following order: $NH_2$-core-envelope 1-envelope 2-p7-nonstructural protein 2 (NS2)-NS3-NS4A-NS4B-NS5A-NS5B-COOH (Kato, 2001; Kato et al., 1990). These viral proteins are not only involved in viral replication but also may affect a variety of cellular functions (Bartenschlager and Lohmann, 2000; Kato, 2001). Although persistent infection with HCV is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) (Colombo, 1996; Kato, 2001), the molecular mechanisms leading to liver cell dysplasia and HCC remain elusive.

It has been thought that unregulated cell cycle progression may be a cause of malignant transformation of normal cells. On the other hand, inhibition of cell cycle progression through the S phase may cause replication error during DNA replication, which induces genomic instability and malignant transformation. Therefore, it is important to clarify the effect of HCV proteins on cell cycle progression in order to understand the molecular mechanism underlying the pathogenesis of HCV, including the development of HCC. A number of previous reports suggested that four HCV proteins—the core, NS3, NS4B, and NS5A—are involved in modulating cell cycle progression (Arima et al., 2001; Kato, 2001; Ray and Ray, 2001; Reed and Rice, 2000). For instance, the core protein promotes cell proliferation through the Ras/Raf signaling pathway and the anti-apoptotic function (Mar-

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