

## Life Style-Related Diseases of the Digestive System: Cell Culture System for the Screening of Anti-Hepatitis C Virus (HCV) Reagents: Suppression of HCV Replication by Statins and Synergistic Action With Interferon

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**Abstract.** Hepatitis C virus (HCV) infection causes chronic hepatitis and leads to liver fibrosis and hepatocellular carcinoma. Pegylated-interferon and ribavirin is the current standard therapy for chronic hepatitis C. However, the therapy is only effective in 50% of the patients. To overcome this problem, we recently developed the HCV cell culture system (OR6 system) for the screening of anti-HCV reagents. In this OR6 system, the luciferase gene was introduced into the upstream portion of the HCV genome to facilitate the monitoring of HCV RNA replication. Recently lipid metabolism is reported to be involved in HCV RNA replication. Cholesterol and sphingolipid are the major components in lipid rafts, which seem to be the scaffold for HCV RNA replication. Statins inhibit cholesterol biosynthesis and also have the pleiotropic effects by the inhibition of prenylation. We demonstrated different anti-HCV effects of statins (atorvastatin, simvastatin, fluvastatin, lovastatin, and pitavastatin) using the OR6 system. Surprisingly, in contrast to the other statins, pravastatin exhibited no anti-HCV effect. Furthermore, statins enhanced the anti-HCV effect of interferon in combination. Statins may be a promising candidate for the adjuvant in interferon therapy and may improve the efficiency of the current interferon and ribavirin therapy.

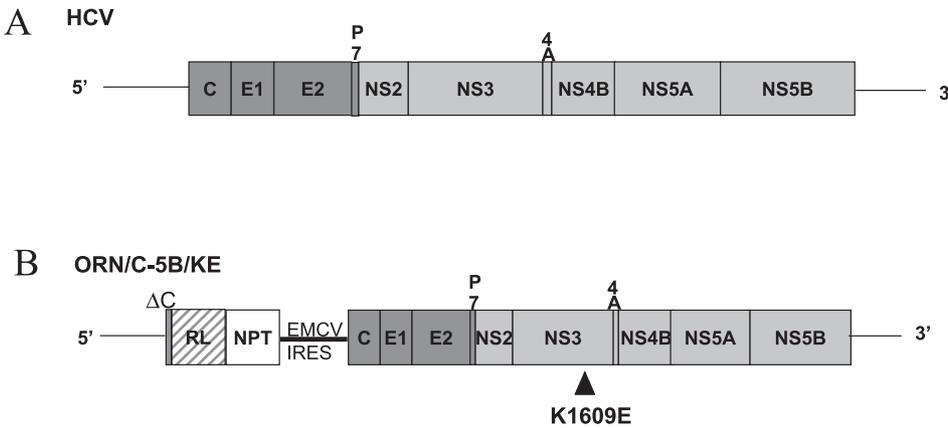
**Keywords:** life style-related disease, hepatitis C virus (HCV), statin, interferon, cell culture system

### Introduction

Approximately 170 million people worldwide are infected with the hepatitis C virus (HCV). HCV infection causes chronic hepatitis C (CH-C) and leads to liver-related death by liver cirrhosis and/or hepatocellular carcinoma. To prevent the progress of fatal liver disease after HCV infection, the elimination of the virus seems to be the most effective strategy. However, the current pegylated-interferon (PEG-IFN) and ribavirin therapy was only effective in 50% of the patients (1). Therefore, the development of more effective anti-HCV reagents is an urgent concern. When HCV replicates in hepatocytes, some of the cellular factors are essential for

HCV RNA replication. These cellular factors are the targets for antiviral as well as viral proteins such as NS3 protease or NS5B RNA-dependent RNA polymerase. Inhibition of cellular factors may cause side effects by the inhibition of their primary roles. However, one of the advantages of this strategy is that it could overcome the viral mutation leading to the resistance to the reagent against the viral proteins. Lipid metabolism is one of the candidates in the context of this strategy. To explore the best partner of IFN, we examined different six statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, using our recently developed OR6 system (2). In the OR6 system, genome-length HCV RNAs (HCV-O strain of genotype 1b) replicate efficiently and the HCV RNA level can be monitored by luciferase activities (3, 4). Statins exhibited various anti-HCV activities except for pravastatin that was not active against HCV (2). We also

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**Fig. 1.** HCV RNA with reporter gene. Schematic gene organization of genome-length HCV RNA. A: The authentic HCV RNA was composed of the N-terminal part of the structural region and C-terminal part of the nonstructural region. B: The genome-length HCV RNA with reporter gene was constructed based on the authentic HCV RNA. EMCV IRES was introduced for the translation of HCV proteins. Renilla luciferase was expressed as a fusion protein with NPT. The position of the adaptive mutation, K1609E, is indicated by a black triangle.

investigated whether or not statins could enhance the inhibitory effect of IFN on HCV RNA replication. In this review, we would like to summarize our recent findings and the literature regarding lipid metabolism as the target of anti-HCV with a focus on statins.

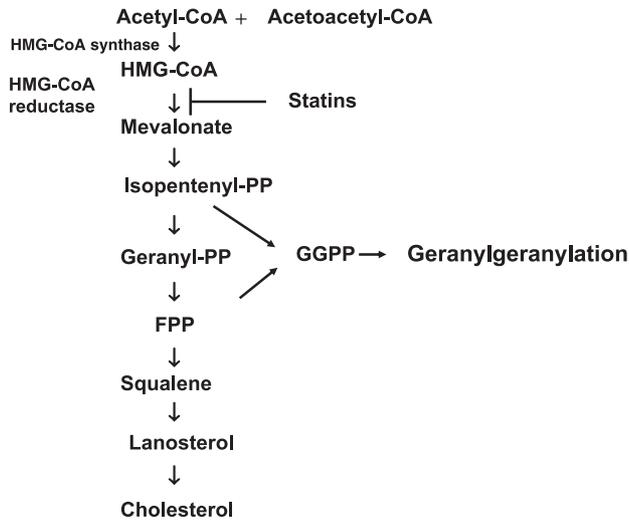
### Cell culture system for HCV RNA replication

Cell culture systems for HCV have been developed since the first breakthrough of the establishment of the subgenomic replicon by Lohmann et al. (5). The replicon system has provided the information concerning the mechanism of the replication machinery of HCV and has revealed the cellular factors essential for HCV RNA replication. After the development of the subgenomic replicon, genome-length HCV RNA replication systems using different HCV strains (H, N, Con1, and O) were developed by several groups since the subgenomic replicon did not possess the structural region in the genome (4, 6–8). For the screening of anti-HCV reagents, the replicon system has also been improved by the introduction of reporter genes (9). The introduction of the reporter gene into the HCV genome facilitated the monitoring of HCV RNA replication. For this purpose, we developed a cell culture system (OR6 system) in which genome-length HCV RNA containing renilla luciferase (RL) replicate efficiently under the selection by G418 (4). As shown in Fig. 1, RL, neomycin phosphotransferase (NPT), and encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) genes were introduced between the 5' untranslated region and Core (C) of HCV. This genome-length HCV RNA robustly replicated in the hepatoma cell line HuH-7 after the electroporation and one of the colonies designated OR6 was selected by G418 and used for the studies including determining the anti-HCV effect of statins. A recent milestone was the development of an HCV infection system using a genotype 2a HCV strain, JFH-1

(10–12). This system could reconstruct the HCV life cycle in cell culture. The future issue of the cell culture system is the development of a robust genotype 1 HCV virus production system because the efficiency of PEG-IFN and ribavirin therapy in patients with genotype 1 HCV remained lower than that in patients with genotype 2 HCV: the sustained virological responses were approximately 50% versus 80%–90%, respectively (13). More recently, pioneering studies have been reported by several groups using genotype 1 HCV strains for virus production (14, 15). However, the genotype 1 HCV virus production systems could not allow re-infection with the supernatant from the HCV-infected cells. These ongoing studies will lead to the development of a robust genotype 1 HCV infection system like genotype 2a HCV in the near future.

### HCV and lipid metabolism

Lipid metabolism is involved in the life cycle of many viruses. The resulting metabolites work as physiologically active molecules such as eicosanoids and so on, and some of them are incorporated into the lipid raft membrane. A lipid raft is distinct from other lipid membranes. It is enriched in cholesterol and sphingolipids and is detergent-resistant. Lipid rafts play an important role in virus entry, replication, and assembly. HCV also forms a replication complex on the lipid raft membrane structure (16). Therefore, the depletion of the cholesterol and sphingolipid from the lipid raft leads to the inhibition of HCV RNA replication. Aizaki et al. (17) reported that lovastatin inhibited HCV RNA replication in HCV replicon-harboring cells. Statins are inhibitors for HMG-CoA reductase in the cholesterol biosynthesis pathway (Fig. 2). Statins also possess the cholesterol-independent action (pleiotropic effect) (18). Many of these pleiotropic effects are mediated by the isoprenoid. Farnesyl pyrophosphate (FPP) and gera-



**Fig. 2.** Cholesterol biosynthesis pathway and statins. In the cholesterol biosynthesis pathway, the production of mevalonate by HMG-CoA reductase is the rate-limiting step. Statins inhibit HMG-CoA reductase, resulting in the inhibition of the production of isoprenoids as well as cholesterol. Geranyl-PP: geranylpyrophosphate and GGPP: geranylgeranylpyrophosphate.

nylgeranyl pyrophosphate (GGPP) are mevalonate-derived isoprenoids (Fig. 2). The attachment of isoprenoid to the cellular proteins is called prenylation. Prenylation regulates a variety of cellular functions, including growth, differentiation, and oncogenesis. From the aspect of the pleiotropic effect of the statins, Wang et al. (19) recently identified FBL2 as geranylgeranylated cellular protein required for HCV RNA replication. FBL2 belongs to the FBL family of proteins, all of which contain an F box and a multiple leucine-rich repeat. These two possible inhibitory mechanisms are proposed for the anti-HCV effect of statins. The low-density lipoprotein receptor (LDLR) is reported as one of the potential HCV receptors (20). However, the precise role of LDLR for HCV is still controversial (21). It will be worth trying to examine the effect of statins in the JFH-1 infection system since statins enhance the expression of LDLR.

Sphingolipid is another major component of lipid rafts and thereby is also the antiviral target for HCV. Serine palmitoyltransferase (SPT) is the enzyme responsible for the condensation of L-serine with palmitoyl-CoA to produce 3-ketodihydrospingosine in the first step of sphingolipid biosynthesis. Sakamoto et al. (22) and Umehara et al. (23) reported that myriocin, a selective inhibitor of SPT, inhibited the HCV RNA replication in replicon-harboring cells and in HCV-infected chimeric mice with humanized livers, respectively. These results further support the significance of lipid metabolism in HCV RNA replication.

Other than cholesterol and sphingolipid biosynthesis, fatty acids are reported to be metabolites that affect HCV RNA replication. Leu et al. (24) reported that polyunsaturated fatty acids (PUFAs) possessed an anti-HCV effect using HCV-replicon harboring cells. Arachidonic acid, docosahexaenoic acid, linoleic acid, and eicosapentaenoic acid belonging to PUFAs possessed anti-HCV activity. On the other hand, saturated fatty acids enhanced HCV RNA replication. The precise mechanisms of fatty acids regarding HCV RNA replication have remained unclear. Very recently, we examined the effect of ordinary nutrients on HCV RNA replication using the OR6 system (25). Interestingly, we found that vitamin E negated the anti-HCV effect of linoleic acid (25). Given that linoleic acid and vitamin E are an oxidant and antioxidant, respectively, oxidative stress may be involved in HCV RNA replication. Further study in this field will provide clues for developing anti-HCV reagents.

### Different anti-HCV effects of statins

Statins are one of the most worldwide used reagents for the treatment of hypercholesterolemia and they are beneficial in the prevention of coronary heart disease. In the cholesterol biosynthesis pathway, the production of mevalonate by HMG-CoA reductase is the rate-limiting step. Statins inhibit mevalonate synthesis by inhibiting HMG-CoA reductase, resulting in decreased production of isoprenoids as well as cholesterol. The activities of some cellular proteins are regulated by the attachment of isoprenoids (prenylation). For example, statins inhibited the function of small G proteins, Ras and Rho. Ras and Rho are major substrates for prenylation with FPP and GGPP, respectively. So far, among the statins, lovastatin is the only one with a well-characterized inhibitory effect against HCV RNA replication in cell culture (17, 26, 27). Recently, FBL2 was identified as one of the geranylgeranylated cellular proteins required for HCV RNA replication (19). Geranylgeranylated FBL2 binds to NS5A of HCV and the resulting complex is required for HCV RNA replication (19).

The anti-HCV effect of the statins other than lovastatin remains to be clarified. Therefore, we used the OR6 system to test anti-HCV effect of five statins: lovastatin, simvastatin, atorvastatin, fluvastatin, and pravastatin (2). More recently, we also added pitavastatin to this list, so that finally six statins were tested for their effects on HCV RNA replication. None of the statins exhibited cytotoxicity at the concentrations tested. The 50% effective concentrations (EC<sub>50</sub>) of statins are summarized in Table 1. The anti-HCV effects of simvastatin, atorvastatin, fluvastatin, and pitavastatin

**Table 1.** EC<sub>50</sub> of statins on HCV RNA replication

Statins	EC <sub>50</sub> ( $\mu$ M)
Lovastatin	2.16
Simvastatin	1.57
Atorvastatin	1.39
Fluvastatin	0.90
Pitavastatin	0.45

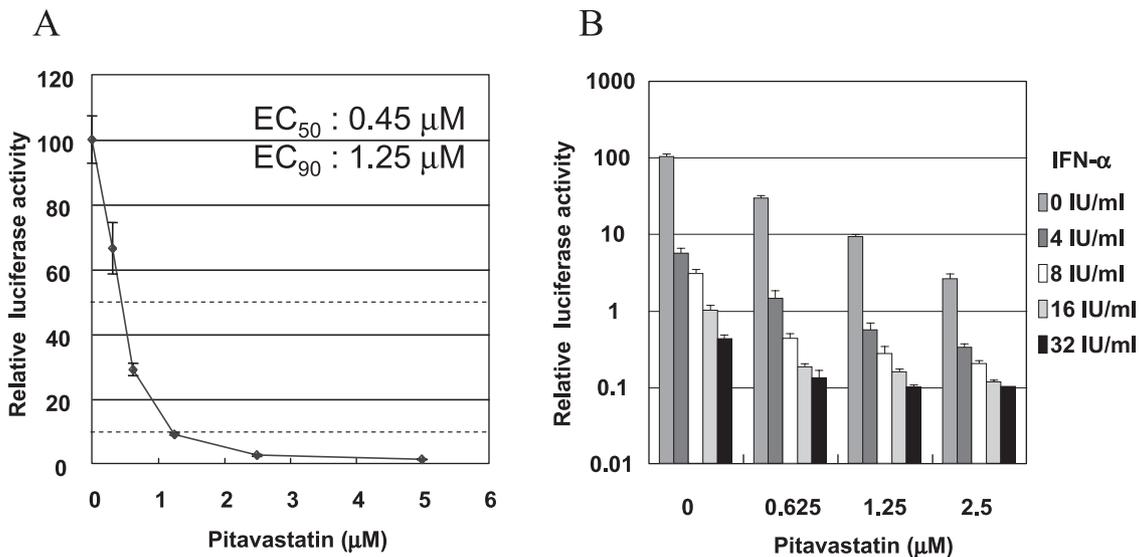
were stronger than that previously reported for lovastatin. The EC<sub>50</sub> of lovastatin, simvastatin, atorvastatin, fluvastatin, and pitavastatin were 2.16, 1.57, 1.39, 0.90, and 0.45  $\mu$ M, respectively. Pitavastatin possessed the strongest anti-HCV activity among the statins tested and its EC<sub>90</sub> was calculated as 1.25  $\mu$ M (Fig. 3A). In contrast, pravastatin exhibited no anti-HCV effect. Pravastatin is the only hydrophilic statin among the statins tested and does not cross the cellular membrane passively. It has been reported that a human liver-specific organic anion transporter, LST-1, mediates the uptake of pravastatin in human hepatocytes (28). Therefore, we examined the expression levels of LST-1 in OR6 cells. OR6 cells expressed the mRNA of LST-1 at levels equivalent to that in normal human liver (2). We ruled out the possibility that pravastatin didn't actually work as the inhibitor for HMG-CoA reductase in the cells. We confirmed that pravastatin induced HMG-CoA reductase by a positive feedback mechanism in response to the

decrease of cholesterol by the inhibition of HMG-CoA reductase by pravastatin (2). These results suggest that there may be another mechanism underlying the depletion of GGPP and cholesterol by statins. One of the clues for resolving this puzzle is that pravastatin has a different effect on P450 induction compared with the other statins (29). However, further study will be needed to clarify this issue.

### Statins in combination with IFN

The combination therapy of PEG-IFN and ribavirin is a current standard therapy for patients with CH-C. Ribavirin by itself possessed no anti-HCV effect for the patients. However, ribavirin alone exhibited an anti-HCV effect in the OR6 cell culture system when it was used at a concentration higher than that in the serum of patients undergoing ribavirin treatment. The EC<sub>50</sub> of ribavirin is calculated as 76  $\mu$ M in the OR6 system and this is approximately 5–7 times higher concentration than that in serum from the patients with ribavirin treatment (3). Furthermore, the synergistic effect of ribavirin at the low concentration with IFN was also confirmed in different cell culture systems, including the OR6 system (3, 30, 31). These results suggest that ribavirin works as a kind of the adjuvant for IFN at the low concentration.

To test the effect of statins in combination with IFN- $\alpha$  on HCV RNA replication, we treated the OR6 cells with



**Fig. 3.** Anti-HCV effect of pitavastatin in combination with IFN- $\alpha$ . A: OR6 cells were treated with pitavastatin at concentrations of 0, 0.625, 1.25, 2.5, and 5  $\mu$ M for 72 h. The EC<sub>50</sub> and EC<sub>90</sub> were calculated from the result. Shown here is the relative luciferase activity (%) calculated when the luciferase activity of untreated cells was assigned as 100%. B: The effect of pitavastatin in combination with IFN- $\alpha$ . OR6 cells were treated with pitavastatin (0, 0.625, 1.25, and 2.5  $\mu$ M) and IFN- $\alpha$  (0, 4, 8, 16, and 32 IU/ml) for 72 h. The relative luciferase activity was calculated as shown above.

pitavastatin (0, 0.625, 1.25, and 2.0  $\mu\text{M}$ ) and IFN- $\alpha$  (0, 4, 8, 16, and 32 IU/ml) (Fig. 3B). Pitavastatin enhanced the anti-HCV effect of IFN- $\alpha$  in a dose-dependent manner for a fixed concentration of IFN- $\alpha$ , 0, 4, 8, 16, or 32 IU/ml (Fig. 3B). Furthermore, we observed the decrease of luciferase activity to almost the background level in the OR6 reporter assay when OR6 cells were co-treated with 32 IU/ml of IFN- $\alpha$  and pitavastatin at the concentration of 1.25 or 2.5  $\mu\text{M}$  (Fig. 3B). The concentrations of the statins tested in the cell culture were higher than that in the sera from patients with statin administration. However, the statins may enhance the anti-HCV effect of IFN for patients with CH-C at a lower concentration than the  $\text{EC}_{50}$  in cell culture. Recently O'Leary et al. (32) reported that the monotherapy of atorvastatin does not exhibit anti-HCV activity in a pilot clinical trial. Although the monotherapy of statin seems to be insufficient for patients with CH-C, statin may be a candidate for the adjuvant of IFN therapy like ribavirin.

## Conclusions

The OR6 system was developed for the precise and quantitative assay of HCV RNA replication in cell culture. The statins were compared for their anti-HCV effects using the OR6 system and were found to possess different effects on HCV RNA replication. Lovastatin, simvastatin, atorvastatin, fluvastatin, and pitavastatin had different anti-HCV profiles in cell culture. However, pravastatin had no anti-HCV effect, although it worked as inhibitor for HMG-CoA reductase. Pitavastatin exhibited the strongest anti-HCV effect ( $\text{EC}_{50}$ : 0.45  $\mu\text{M}$ ) among the statins tested and enhanced the effect of IFN- $\alpha$ . It may be difficult to achieve the cell culture based  $\text{EC}_{50}$  of statins in patients with CH-C. However, statins at lower concentration than the  $\text{EC}_{50}$  in cell culture may enhance the anti-HCV effect of IFN- $\alpha$  in patients with CH-C. Therefore, statins may be suitable as an adjuvant of IFN- $\alpha$  like ribavirin rather than for monotherapy. Lipid metabolism including cholesterol, sphingolipid, and fatty acid biosynthesis seems to be an attractive field for the development of antiviral reagents for HCV.

## References

- Hayashi N, Takehara T. Antiviral therapy for chronic hepatitis C: past, present, and future. *J Gastroenterol.* 2006;41:17–27.
- Ikeda M, Abe K, Yamada M, Dansako H, Naka K, Kato N. Different anti-HCV profiles of statins and their potential for combination therapy with interferon. *Hepatology.* 2006;44:117–125.
- Naka K, Ikeda M, Abe K, Dansako H, Kato N. Mizoribine inhibits hepatitis C virus RNA replication: effect of combination with interferon-alpha. *Biochem Biophys Res Commun.* 2005;330:871–879.
- Ikeda M, Abe K, Dansako H, Nakamura T, Naka K, Kato N. Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. *Biochem Biophys Res Commun.* 2005;329:1350–1359.
- Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science.* 1999;285:110–113.
- Pietschmann T, Lohmann V, Kaul A, Krieger N, Rinck G, Rutter G, et al. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. *J Virol.* 2002;76:4008–4021.
- Blight KJ, McKeating JA, Marcotrigiano J, Rice CM. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. *J Virol.* 2003;77:3181–3190.
- Ikeda M, Yi M, Li K, Lemon SM. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. *J Virol.* 2002;76:2997–3006.
- Yokota T, Sakamoto N, Enomoto N, Tanabe Y, Miyagishi M, Maekawa S, et al. Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep.* 2003;4:602–608.
- Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med.* 2005;11:791–796.
- Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. *Science.* 2005;309:623–626.
- Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, et al. Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci U S A.* 2005;102:9294–9299.
- Kasahara A, Hayashi N, Hiramatsu N, Oshita M, Hagiwara H, Katayama K, et al. Ability of prolonged interferon treatment to suppress relapse after cessation of therapy in patients with chronic hepatitis C: a multicenter randomized controlled trial. *Hepatology.* 1995;21:291–297.
- Yi M, Villanueva RA, Thomas DL, Wakita T, Lemon SM. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc Natl Acad Sci U S A.* 2006;103:2310–2315.
- Heller T, Saito S, Auerbach J, Williams T, Moreen TR, Jazwinski A, et al. An in vitro model of hepatitis C virion production. *Proc Natl Acad Sci U S A.* 2005;102:2579–2583.
- Shi ST, Lee KJ, Aizaki H, Hwang SB, Lai MM. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. *J Virol.* 2003;77:4160–4168.
- Aizaki H, Lee KJ, Sung VM, Ishiko H, Lai MM. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. *Virology.* 2004;324:450–461.
- Liao JK, Laufs U. Pleiotropic effects of statins. *Annu Rev Pharmacol Toxicol.* 2005;45:89–118.
- Wang C, Gale M Jr, Keller BC, Huang H, Brown MS, Goldstein JL, et al. Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. *Mol Cell.* 2005;18:425–434.

- 20 Molina S, Castet V, Fournier-Wirth C, Pichard-Garcia L, Avner R, Harats D, et al. The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. *J Hepatol.* 2007;46:411–419.
- 21 von Hahn T, McKeating JA. In vitro veritas? The challenges of studying hepatitis C virus infectivity in a test tube. *J Hepatol.* 2007;46:355–358.
- 22 Sakamoto H, Okamoto K, Aoki M, Kato H, Katsume A, Ohta A, et al. Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. *Nat Chem Biol.* 2005;1:333–337.
- 23 Umehara T, Sudoh M, Yasui F, Matsuda C, Hayashi Y, Chayama K. Serine palmitoyltransferase inhibitor suppresses HCV replication in a mouse model. *Biochem Biophys Res Commun.* 2006;346:67–73.
- 24 Leu GZ, Lin TY, Hsu JT. Anti-HCV activities of selective polyunsaturated fatty acids. *Biochem Biophys Res Commun.* 2004;318:275–280.
- 25 Yano M, Ikeda M, Abe K, Dansako H, Ohkoshi S, Aoyagi Y, et al. Comprehensive analysis of the effects of ordinary nutrients on hepatitis C virus RNA replication in cell culture. *Antimicrob Agents Chemother.* 2007;51:2016–2027.
- 26 Kapadia SB, Chisari FV. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. *Proc Natl Acad Sci U S A.* 2005;102:2561–2566.
- 27 Ye J, Wang C, Sumpter R Jr, Brown MS, Goldstein JL, Gale M Jr. Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. *Proc Natl Acad Sci U S A.* 2003;100:15865–15870.
- 28 Nakai D, Nakagomi R, Furuta Y, Tokui T, Abe T, Ikeda T, et al. Human liver-specific organic anion transporter, LST-1, mediates uptake of pravastatin by human hepatocytes. *J Pharmacol Exp Ther.* 2001;297:861–867.
- 29 Kocarek TA, Dahn MS, Cai H, Strom SC, Mercer-Haines NA. Regulation of CYP2B6 and CYP3A expression by hydroxymethylglutaryl coenzyme A inhibitors in primary cultured human hepatocytes. *Drug Metab Dispos.* 2002;30:1400–1405.
- 30 Tanabe Y, Sakamoto N, Enomoto N, Kurosaki M, Ueda E, Maekawa S, et al. Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-alpha. *J Infect Dis.* 2004;189:1129–1139.
- 31 Kim SS, Peng LF, Lin W, Choe WH, Sakamoto N, Schreiber SL, et al. A cell-based, high-throughput screen for small molecule regulators of hepatitis C virus replication. *Gastroenterology.* 2007;132:311–320.
- 32 O’Leary JG, Chan JL, McMahon CM, Chung RT. Atorvastatin does not exhibit antiviral activity against HCV at conventional doses: a pilot clinical trial. *Hepatology.* 2007;45:895–898.