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Serum-free cell culture system supplemented with lipid-rich albumin for hepatitis C virus (strain O of genotype 1b) replication

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Abstract

HuH-7 is a highly differentiated hepatoma cell line and the only cell line that supports robust RNA replication of the hepatitis C virus (HCV). HuH-7 cells cause cell death in serum-free culture condition. However, the effect is reversed by supplementation with selenium. Serum-free cell cultures are advantageous for vaccine development and experimental reproducibility. However, HCV RNA replication in HuH-7 cells in serum-free medium had not yet been achieved. Therefore, we tried to develop a system for robust HCV RNA replication in a serum-free cell culture. Although HuH-7 cells grew in serum-free medium in the presence of selenium, HuH-7 cells under these conditions did not support HCV RNA replication in long-term culture. Among the supplements tested, serum-free medium with lipid-rich albumin (LRA) was found to yield robust HCV RNA replication. HCV proteins were detected for more than 9 months in serum-free medium supplemented with LRA. This is the first report to demonstrate a long-term, serum-free cell culture that successfully maintained robust HCV RNA replication. This cell culture system is expected to be a useful tool for vaccine development, as well as for further investigation of cellular factors that are essential for HCV RNA replication.

Keywords: HCV; Serum-free cell culture; Selenium; Lipid-rich albumin; Vaccine

1. Introduction

Persistent hepatitis C virus (HCV) infection causes liver fibrosis and hepatocellular carcinoma. Approximately 170 million people worldwide are infected with HCV. The combination of pegylated interferon (IFN) with ribavirin is the current standard therapy for chronic hepatitis C and yields a sustained virological response rate of about 55% (Feld and Hoofnagle, 2005). HCV, a member of the *Flaviviridae* family, is an enveloped virus with a positive single-stranded 9.6 kilobase (kb) RNA genome, which encodes a large polyprotein precursor of about 3000 amino acids (aa) (Kato et al., 1990; Tanaka et al., 1996). This polyprotein is processed by a combination of host and viral proteases into at least 10 proteins: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Hijikata et al., 1991, 1993).

The discovery of the HCV subgenomic replicon in 1999 was a turning point for HCV RNA replication in cultured cells (Lohmann et al., 1999). Furthermore, genome-length HCV RNA replication systems were developed using N, Con1, and H strains (Blight et al., 2002; Ikeda et al., 2002; Pietschmann et al., 2002). We recently developed a genome-length HCV RNA (strain O of genotype 1b) replication reporter system (OR6) as an effective screening system (Ikeda et al., 2005). The development of infectious virus-producing cells has been a remarkable breakthrough in the fields of virology (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005).

HuH-7 is a highly differentiated hepatoma cell line that is commonly used for replication and infection studies. However, these cells have been reported by a number of groups to be heterogeneous, and the replication efficiency of this cell line differed among subclonal HuH-7 cells. Parental HuH-7 cells showed low capacity for HCV RNA replication and low susceptibility for HCV infection, but Huh7.5, Huh-Lunet, and our recently developed the RSc cells efficiently support HCV RNA replication and infection (Blight et al., 2002; Pietschmann et al., 2006; Ikeda et al., in preparation). In addition to these sub-

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clonal HuH-7 cell features, fetal bovine serum (FBS) may be another factor that affects HCV RNA replication and infection, as FBS is a pooled material containing unknown factors from different origins. Therefore, different FBS lots may affect the reproducibility of experiments conducted by different research groups. Furthermore, pathogens contained in FBS may introduce additional problems during the development of an HCV vaccine.

To resolve these issues, in this study, we investigated whether or not HCV RNA could replicate in serum-free cell culture. As HuH-7 cells produce an autocrine growth factor, hepatoma-derived growth factor, additional supplementation with growth factor seemed to be unnecessary (Nakamura et al., 1989, 1994). HuH-7 cells cause cell death in serum-free culture condition. However, when HuH-7 cells were cultured in serum-free medium supplemented with selenium, they produce a number of plasma proteins and liver-specific enzymes essential for their survival (Nakabayashi et al., 1982, 1984). Therefore, the serum-free culture of HuH-7 cells can be maintained by the addition of selenium alone. However, HCV RNA replication was not yet maintainable under these conditions.

In the present study, we found that HCV RNA replicates robustly for more than 9 months in serum-free medium supplemented with selenium and lipid-rich albumin (LRA). These results indicate the requirement of the lipid for HCV RNA replication. This cell culture system is expected to be a useful tool for the development of an HCV vaccine, and will also enhance the reproducibility of experiments, including those that evaluate anti-HCV reagents.

2. Materials and methods

2.1. Reagents

Sodium selenite (Na_2SeO_3), insulin, linoleic acid, oleic acid, IFN-α, and cyclosporine A (CsA) were purchased from Sigma–Aldrich (St. Louis, MO). Fluvastatin (FLV) and low-density lipoprotein (LDL) were purchased from Calbiochem (San Diego, CA). Lipid-rich albumin (ALBUMAX I™) was purchased from Invitrogen and is referred to as LRA in this study.

2.2. Cell cultures

The OR6 cells were cultured in Dulbecco's modified eagle's medium (DMEM; Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1% penicillin–streptomycin (referred to as 10% FBS medium in this study), and G418 (300 μg per ml; Geneticin, Invitrogen) in a 5% CO_2 atmosphere at 37 °C. The cells were supplied with fresh medium twice a week at a 5:1 split ratio. The serum-free medium was DMEM containing 100 nM sodium selenite (Na_2SeO_3, Sigma–Aldrich) with LRA. The cells were cultured on six-well plates in 10% FBS medium or the serum-free medium. The cells cultured in the serum-free medium were harvested at 1, 3, 6, 9, and 12 months, and were subjected to Western blot analysis.

2.3. Cell count

To examine cell growth in selenium-containing medium with 10% FBS, 2 mg per ml of LRA, or no supplementation, OR6 cells were seeded at a density of 1×10^5 cells per well onto six-well plates in the absence of G418. Then, the number of the cells was counted in an improved Neubauer-type hemotocytometer after trypan blue dye (Invitrogen) treatment.

2.4. Western blot analysis

Preparation of the cell lysate, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblot analysis with a polyvinylidene difluoride membrane were performed as described previously (Kato et al., 2003). The antibodies used in this study were those against Core (Institute of Immunology, Tokyo, Japan), NS3 (Novocastra Laboratories, UK) and β-actin (AC-15; Sigma–Aldrich). Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA).

2.5. Luciferase reporter assay

A luciferase reporter assay was performed as described previously (Ikeda et al., 2006). Briefly, 2×10^4 cells were plated onto 24-well plates and cultured in 10% FBS or the serum-free medium, at least in triplicate for each assay, and the cells were cultured for 24 h. Then, the cells were treated with human IFN-α, CsA, or FLV at several concentrations for 72 h. The cells were then harvested and subjected to luciferase assay using the *Renilla* luciferase assay system (Promega). The cells were washed twice with phosphate-buffered saline and were then extracted with 100 μl of *Renilla* lysis reagent. The relative luciferase unit value in 10 μl of lysates was measured by adding 50 μl of *Renilla* luciferase assay reagent according to the manufacturer's protocol. A manual Lumat LB 9501/16 luminometer (EG&G Berthold, Bad Wildbad, Germany) was used for the detection of luciferase activity.

3. Results

3.1. Efficiency of HCV RNA replication in HuH-7 cells with supplements in serum-free medium

At the early stage of the establishment of the HuH-7 cells, the serum-free cell culture was examined; the HuH-7 cells were found to replicate continuously for more than 9 months in a chemically defined medium containing selenium (Nakabayashi et al., 1982). Furthermore, the HuH-7 cells were maintained for a period of more than 3 years in improved serum-free medium containing additional supplements, i.e., oleic acid, linoleic acid, and insulin (Nakabayashi et al., 1984). We first investigated whether these serum-free conditions would support HCV RNA replication using the OR6 reporter system. The OR6 cells supported the replication of genome-length HCV-O RNA, into which the luciferase gene had been introduced (Ikeda et al., 2005). Using this OR6 system, we were able to monitor the level of HCV RNA

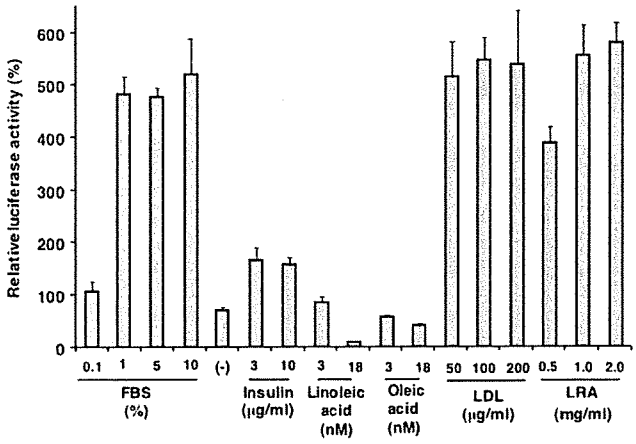

Fig. 1. HCV RNA replication in OR6 cells under different cell culture conditions. The OR6 cells were cultured in DMEM containing 100 nM sodium selenium with FBS (0.1, 1, 5, and 10%), insulin (3 and 10 μg per ml), linoleic acid (3 and 18 nM), oleic acid (3 and 18 nM), LDL (50, 100, and 200 μg per ml), or LRA (0.5, 1.0, and 2.0 mg per ml). The cells were harvested at 24 and 96 h and were subjected to luciferase assay as described in Section 2. Relative luciferase activities (%) were obtained from the value at 96 h, when the value at 24 h was assigned as 100%. The data indicate means ± standard deviations (S.D.s) from three independent experiments. (−) indicates culture in DMEM containing 100 nM sodium selenium.

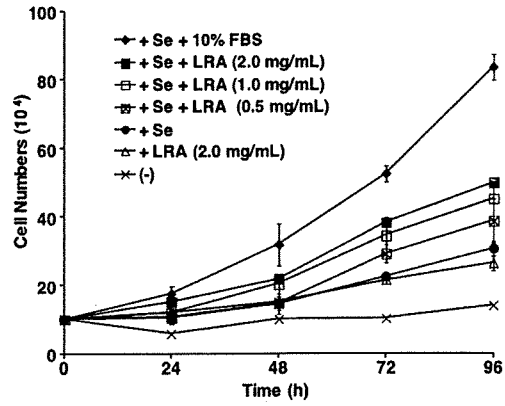

Fig. 2. Cell growth of HuH-7 cells in serum-free medium with LRA or in 10% FBS medium. The OR6 cells were plated at 1×10^5 cells per well onto six-well plates in triplicate. The cells were cultured in DMEM containing 100 nM sodium selenium with 10% FBS or 0.5, 1.0 and 2.0 mg per ml LRA or no supplement and were harvested at 24, 48, 72, and 96 h. The cells were cultured in DMEM or DMEM with LRA in the absence of selenium and were harvested at indicated time points.

replication by measuring the activity of luciferase. Luciferase activity at 96 h was five times higher than that at 24 h in 10% FBS medium (Fig. 1). However, HCV RNA replication was reduced when the OR6 cells were cultured in serum-free medium containing only selenium (Fig. 1). Serum-free medium supplemented with insulin reduced HCV RNA replication to about one-third of that observed in cultures maintained in 10% FBS medium supplemented with selenium. When used in combination with linoleic acid or oleic acid, the serum-free medium with selenium remarkably reduced HCV RNA replication (Fig. 1). However, in the serum-free medium with selenium in combination with LDL (50, 100, or 200 μg per ml) or LRA (1 or 2 mg per ml), HCV RNA replication was supported at the same level as that in 10% FBS medium, although the replication of HCV RNA was slightly low level in serum-free medium with selenium and LRA supplement at 0.5 mg per ml. These results suggest that chemically conditioned serum-free medium supplemented with selenium is not sufficient to support HCV RNA replication, but the addition of either LDL or LRA restored HCV RNA replication to almost the same level as that observed in 10% FBS medium. Thus, some of the elements essential for HCV RNA replication may be contained in LDL and LRA.

3.2. Cell growth of HuH-7 cells in selenium-containing medium supplemented with FBS or LRA

As HCV RNA replication depends on cell growth (Guo et al., 2001; Pietschmann et al., 2001), we next determined the number of cells at 24, 48, 72, and 96 h of culture. The doubling time of the OR6 cells was estimated to be approximately 29, 43, and 64 h in selenium-containing medium with 10% FBS, or 0.5, 1.0, and 2 mg per ml of LRA, or no supplementation, respectively (Fig. 2). Selenium or LRA containing medium enhanced the growth of OR6 cells and the combination of the selenium with LRA further enhanced the cell growth in a LRA dose-dependent manner (Fig. 2). Interestingly, the cell culture in serum-free medium supplemented with 2 mg per ml of LRA and selenium supported HCV RNA replication as efficiently as did that supplemented with 10% FBS and selenium (Fig. 1), although the cell growth of the culture in the medium with LRA and selenium was slower than that in the medium supplemented with 10% FBS and selenium. These results indicate that LRA may contain factors that enhance HCV RNA replication, and these LRA-derived factors appear to function in a manner that is not dependent on the cell growth factor.

3.3. Expression of HCV proteins in HuH-7 cells at 1 month of cell culture under various medium conditions

We continued to maintain the culture of OR6 cells for 1 month in different types of conditioned media. NS3 and Core HCV proteins were detected in the OR6 cell culture for 1 month in medium containing selenium with 10% or 5% FBS, but not with 1% or 0.5% FBS (Fig. 3A). The protein expression levels were higher in the cell culture with 10% FBS medium than in that with the 5% FBS medium. HCV proteins were not detected in the OR6 cells cultured in serum-free medium containing selenium alone (Fig. 3A). In contrast, HCV proteins were detected in LRA- and selenium-containing cell cultures. The levels of expression of HCV proteins were almost equal to those in the cell culture with selenium and 5% FBS (Fig. 3A). To further confirm the results, we performed luciferase assay for the OR6 cells cultured for 1 month after RNA transfection (Fig. 3B). These results indicated that HCV RNA replication was not maintained for more than 1 month in low concentrations (less than 1%) of FBS with selenium. However, the cell culture in serum-free medium with selenium and LRA at concentrations of 0.5, 1, or 2 mg per ml did efficiently support HCV RNA replication for more than

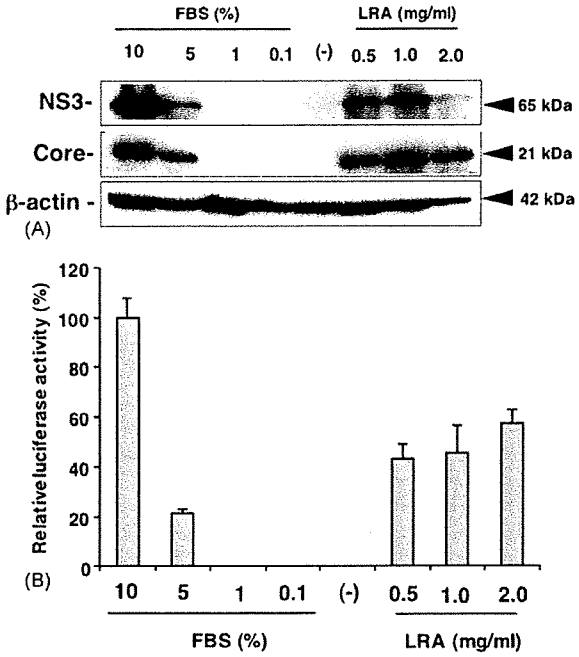

Fig. 3. Expression of HCV proteins in HuH-7 cells cultured in serum-free medium with LRA or in 10% FBS medium. OR6 cells were cultured for 30 days in DMEM containing 100 nM sodium selenium with LRA (0.5, 1.0, and 2.0 mg per ml) or FBS (0.1, 1, 5, and 10%). (−) indicates the culture in DMEM with sodium selenium. (A) The production of Core and NS3 in the OR6 cells was analyzed by immunoblotting using anti-Core and anti-NS3 antibodies. β-actin was used as a control for the amount of protein loaded per lane. (B) Relative luciferase activity was determined for the cells from 24-well plates in triplicate.

1 month. Therefore, we concluded that LRA could serve as an alternative supplement to FBS, when used in combination with selenium for HCV RNA replication.

3.4. Core expression in OR6 cells cultured in serum-free medium supplemented with LRA and selenium

Since OR6 cells cultured in serum-free medium with selenium and LRA could support HCV RNA replication at least for 1 month, we continued to culture the OR6 cells under the same conditions for 1 year. Core was detected for 9 months and for 6 months in serum-free medium containing selenium with LRA at 1.0 and 2.0 mg per ml, respectively (Fig. 4). These results suggest that serum-free cell culture supplemented with LRA supports HCV RNA replication at almost equal level to that in the culture supplemented with 10% FBS without G418 selection at least more than 9 months.

3.5. Influence of anti-HCV compounds on HCV RNA replication in OR6 cells cultured in serum-free medium supplemented with LRA and selenium

IFN is currently used as a therapeutic treatment of patients with chronic hepatitis C. Subgenomic HCV replicon-harboring cells and genome-length HCV RNA-replicating cells have been used to evaluate IFN and other candidate anti-HCV reagents, as

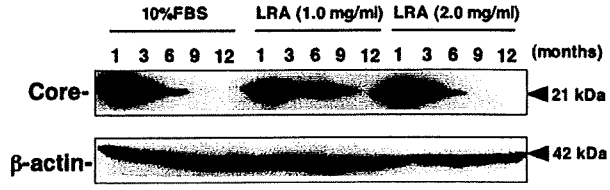

Fig. 4. Core expression in a long-term culture of OR6 cells in serum-free medium supplemented with LRA. The OR6 cells were cultured for 1 year in DMEM containing 100 nM sodium selenium with LRA at 1.0 or 2.0 mg per ml. The OR6 cells were also cultured in DMEM with 10% FBS and used as a positive control. Cells were harvested at 1, 3, 6, 9, and 12 months, and were subjected to Western blot analysis for Core using anti-Core antibody. β-actin was used as a control for the amount of protein loaded per lane.

has recently been reported in the case of CsA and the statins (Ikeda et al., 2006; Kapadia and Chisari, 2005; Watashi et al., 2003; Ye et al., 2003). However, one remaining problem with the evaluation of the anti-HCV activity of these reagents has been that FBS is derived from pooled blood materials and contains unknown cellular factors in various concentrations. These unknown factors can affect experimental results and reproducibility. Therefore, it is preferable to use a culture medium containing as few cellular factors as possible in order to ensure the reliability of the results. In this context, completely chemical conditioned medium is most ideal. Although our selenium and LRA culture system still contained animal proteins and lipids, it contained fewer unknown factors than medium containing FBS. We compared the anti-HCV activity of IFN-α, CsA, and FLV in serum-free medium with selenium and LRA, as well as in 10% FBS medium with selenium. OR6 cells were treated with these reagents for 72 h, and harvested cells were subjected to luciferase assay. When the OR6 cells were treated with IFN-α under either the 10% FBS medium condition or the selenium and LRA condition, HCV RNA replication was inhibited by IFN-α in a dose-dependent manner (range: 0.625–5 IU per ml; Fig. 5). However, the sensitivity of the cultures to IFN-α was greater in selenium and LRA medium than in 10% FBS medium (Fig. 5). When the OR6 cells were treated with CsA at low concentrations of 0.0625 and 0.125 μg per ml, sensitivity was greater in selenium and LRA medium than in 10% FBS medium, but almost identical sensitivity was observed under both medium conditions at concentrations of CsA of 0.25 and 0.5 μg per ml (Fig. 5). The most striking result was observed when the OR6 cells were treated with FLV in concentrations ranging from 1.25 to 10 μM. In contrast to the results obtained with CsA and IFN-α, the sensitivity associated with FLV was markedly reduced in the cultures treated with selenium and LRA medium, as compared to that of cultures in 10% FBS medium (Fig. 5).

4. Discussion

The development of serum-free cell culture systems will reduce the risk of contamination by infectious pathogens of animal origin in studies of vaccine development. Moreover, such systems will enhance the reproducibility of such experiments, because FBS contains unknown cellular factors that can affect the physiological state of cells. In this study, we developed a

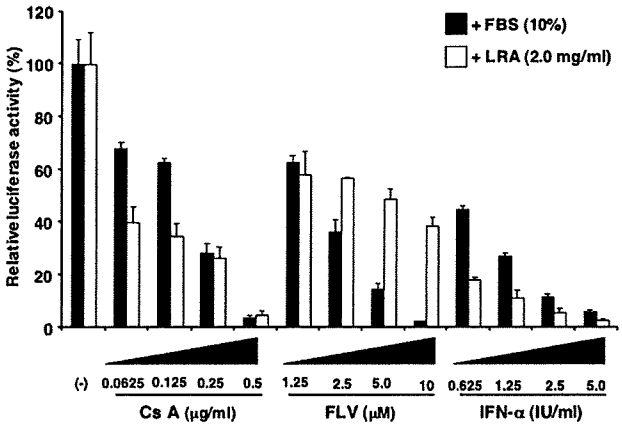

Fig. 5. Influence of anti-HCV compounds on HCV RNA replication in OR6 cells in serum-free medium supplemented with LRA or in 10% FBS medium. The OR6 cells were cultured in triplicate in 10% FBS medium with selenium (100 nM) or in serum-free medium with LRA (2 mg per ml) and selenium (100 nM). Under these cell culture conditions, the cells were treated with CsA (0.0625, 0.125, 0.25, and 0.5 μg per ml), FLV (1.25, 2.5, 5.0, and 10 μM), or IFN-α (0.625, 1.25, 2.5, and 5 IU per ml) for 72 h. Then, the cells were harvested and subjected to luciferase assay as described in Section 2. The luciferase activity of OR6 cells cultured under both medium conditions in the absence of anti-HCV compounds was assigned as 100% and is indicated by (−).

novel serum-free cell culture system supplemented with LRA that was able to support HCV RNA replication for more than 9 months. Although this cell culture system still contained animal proteins, the quantity of unknown cellular factors contained in the FBS was to a great extent reduced. The development of such a long-term cell culture is noteworthy, as it could be used for the stable mass-production of an HCV vaccine.

In a related previous study, it was reported that insulin, linoleic acid, and oleic acid enhance the growth of HuH-7 cells in serum-free cell culture (Nakabayashi et al., 1984). Therefore, we tested these supplements in HCV RNA replication experiments. Insulin was found to slightly enhance HCV RNA replication, but linoleic acid and oleic acid inhibited HCV RNA replication. In another recent study, Kapadia and co-workers reported that oleic acid enhanced HCV RNA replication in a serum-containing cell culture (Kapadia and Chisari, 2005). This discrepancy may have been due to the presence or absence of serum; moreover, some of the serum proteins may function in concert with oleic acid to support HCV RNA replication.

LDL is an exogenous source of cholesterol, and it was found to support HCV RNA replication in serum-free cell culture. When the cholesterol demand is satisfied, intrinsic mevalonate, which is a precursor of both cholesterol and non-sterol isoprenoid, is directed to non-sterol isoprenoid. Non-sterol isoprenoid is essential for the prenylation of cellular proteins that support HCV RNA replication (Ikeda et al., 2006; Kapadia and Chisari, 2005; Ye et al., 2003). For this reason, LDL may support HCV RNA replication, even in serum-free cell culture. LRA was initially developed to reduce or replace the requirement of serum supplementation by chromatographic separation from bovine plasma (Invitrogen). Here, we found that LRA supported HCV RNA replication as well as cell growth in serum-free cell culture. LRA contains free fatty acids and cholesterol associated with albumin. Therefore, cholesterol may, at least to some extent, play a role in HCV RNA replication by the mechanism described above. However, care should be taken before coming to a conclusion regarding the effects of free fatty acids on HCV RNA replication, because LRA contains a mixture of fatty acids in different states of saturation. Recent reports have demonstrated that saturated fatty acids enhance HCV RNA replication, but polyunsaturated fatty acids inhibit HCV RNA replication (Kapadia and Chisari, 2005). In addition, these diverse effects of fatty acids on HCV RNA replication in serum-containing medium cannot be simply applied to serum-free culture systems, because oleic acid has been shown to exert different effects on HCV RNA replication under serum-containing and serum-free culture conditions. To clarify the roles played by fatty acids in this context, further studies will be needed.

Here, HCV RNA replication depended on the growth of HuH-7 cells, and it has previously been shown that expression levels of HCV proteins and RNA are low in confluent cells (Guo et al., 2001; Pietschmann et al., 2001). Therefore, we examined the time course of cell growth and found that cell growth in serum-free medium with LRA was slower than that in 10% FBS medium, although the replication levels of HCV RNA were similar under both culture conditions studied. As regards HCV RNA replication and cell growth, Windisch et al. reported that HCV RNA replication in HuH-6 cells was not dependent on cell growth (Windisch et al., 2005). They demonstrated that the expression of HCV proteins was not reduced, even when the HuH-6 cells became confluent. In serum-free culture supplemented with LRA, HCV RNA replication in HuH-7 cells proceeds in a manner independent of cell growth, as was previously observed in the case of replication in HuH-6 cells.

One disadvantage associated with the use of FBS-containing cultures in virology studies is the influence exerted by unknown serum proteins, because FBS is derived from the serum pool of a bovine population. To prevent discrepancies between experiments due to differences between FBS lots, it is desirable to include only the most simple components as possible in the culture media. To this end, serum-free cultures are preferable in terms of reproducibility. Along these lines, it is expected that the use of our serum-free culture system with LRA may lead to improvements in experimental conditions for experiments in cell biology, as our culture medium contained only very simple supplements: fatty acids, cholesterol, albumin, and selenium. We tested the anti-HCV reagents CsA, FLV, and IFN-α in our serum-free culture supplemented with LRA. CsA and IFN-α were found to inhibit HCV RNA replication more efficiently in serum-free medium with LRA than in 10% FBS medium. Surprisingly, FLV inhibited HCV RNA replication less effectively in serum-free medium supplemented with LRA than it did in 10% FBS medium. One explanation for these differences may be that only FLV is a lipid metabolism-related reagent, and therefore the anti-HCV effect appeared to be antagonized by LRA. To clarify this issue, further study will be needed.

The goal of a serum-free cell culture is to develop a cell culture system containing only compounds that are of non-animal origin. Recently, a serum-free cell culture for canine

pathogenic virus production was reported using Madin Darby canine kidney cells lacking animal protein (Mochizuki, 2006). In this system, soybean peptone was used for the serum-free culture without animal protein. Canine viruses were able to grow almost as efficiently in this serum-free medium as in serum-containing medium. This plant protein-containing culture system is of the second-highest quality in terms of controlling for animal-derived pathogens in vaccine development experiments. Assessments of this animal protein-free cell culture system in terms of its usefulness for HCV RNA replication are therefore warranted.

We found HuH-7 cells supported HCV RNA replication for more than 9 months in serum-free medium supplemented with LRA at 1.0 mg per ml and selenium at 100 nM. This is the first report to describe HCV RNA replication in a long-term, serum-free culture. Recently, an infectious virus-producing cell culture system was reported using genotype 2a strain JFH1 (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). We are currently examining infectious virus production in a serum-free cell culture using the JFH1 virus. Our serum-free cell culture system may provide the useful information to the vaccine development.

In conclusion, we have established a serum-free cell culture system supplemented with LRA for the purpose of achieving HCV RNA replication. HCV proteins were detected during this series of experiments for more than 9 months. The present system has enabled an ongoing study of the production of an infectious HCV virion. Our serum-free cell culture system will yield relevant information for vaccine development, sustains only a relatively low risk of pathogenic contamination as compared to that of previous systems, and is expected to improve the reproducibility of similar experiments in the future.

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Apolipoprotein B–Dependent Hepatitis C Virus Secretion Is Inhibited by the Grapefruit Flavonoid Naringenin

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Hepatitis C virus (HCV) infects over 3% of the world population and is the leading cause of chronic liver disease worldwide. HCV has long been known to associate with circulating lipoproteins, and its interactions with the cholesterol and lipid pathways have been recently described. In this work, we demonstrate that HCV is actively secreted by infected cells through a Golgi-dependent mechanism while bound to very low density lipoprotein (vLDL). Silencing apolipoprotein B (ApoB) messenger RNA in infected cells causes a 70% reduction in the secretion of both ApoB-100 and HCV. More importantly, we demonstrate that the grapefruit flavonoid naringenin, previously shown to inhibit vLDL secretion both *in vivo* and *in vitro*, inhibits the microsomal triglyceride transfer protein activity as well as the transcription of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase and acyl-coenzyme A:cholesterol acyltransferase 2 in infected cells. Stimulation with naringenin reduces HCV secretion in infected cells by 80%. Moreover, we find that naringenin is effective at concentrations that are an order of magnitude below the toxic threshold in primary human hepatocytes and in mice. *Conclusion:* These results suggest a novel therapeutic approach for the treatment of HCV infection. (HEPATOLOGY 2008;47:1437-1445.)

Hepatitis C virus (HCV) infection is a global public health problem, affecting over 3% of the world population. HCV infection develops into a chronic condition in over 70% of the patients, ultimately leading to cirrhosis and hepatocellular carcinoma.[1] Current standards of care consist of interferon (α2A) and ribavirin, which have been found to be effective in only 50% of the cases.[1] However, this treatment is poorly tolerated by patients and is associated with significant side effects. Therefore, there is a pressing need for the development of alternative strategies for the treatment of HCV infection.

HCV has long been known to associate with β-lipoproteins [very low density lipoprotein (vLDL) and low-density lipoprotein (LDL)] circulating in patients' blood.[2] Its E1/E2 receptors have been found to bind to both LDL and high-density lipoprotein,[3] whereas HCV core protein has been shown to associate with apolipoprotein AII (ApoAII)[4] and lipid droplets in HepG2 cells.[5] In addition, HCV replication has been shown to be up-regulated by fatty acids and inhibited by statins; this suggests an interaction between HCV, cholesterol, and lipid metabolism.[6] The recent development of an efficient cell culture system in which the full lifecycle of HCV infection is captured has opened new opportunities for the study of the viral secretion.[7,8] Using this system, Gastaminza et al.[9] demonstrated that intercellular HCV particles have a higher density than their secreted counterparts, suggesting that HCV might bind low-density particles prior to viral egress. Just recently, Huang et al.[10] demon-

Abbreviations: ACAT, acyl-coenzyme A:cholesterol acyltransferase; ALT, alanine aminotransferase; ApoAII, apolipoprotein AII; ApoB, apolipoprotein B; AST, aspartate aminotransferase; cDNA, complementary DNA; DMEM, Dulbecco's modified Eagle medium; EDTA, ethylene diamine tetraacetic acid; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GFP, green fluorescent protein; HCV, hepatitis C virus; HMGR, 3-hydroxy-3-methyl-glutaryl-coenzyme reductase; i.p., intraperitoneal; LDL, low-density lipoprotein; mRNA, messenger RNA; MTP, microsomal triglyceride transfer protein; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; SCID, severe combined immunodeficient; shRNA, short hairpin RNA; Tris, trishydroxymethylaminomethane; vLDL: very low density lipoprotein.

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strated that HCV secretion is dependent on both apolipoprotein B (ApoB) expression and vLDL assembly in a chromosomally integrated complementary DNA (cDNA) model of HCV secretion.

These results strongly suggest that HCV might be "hitching a ride" along the lipoprotein lifecycle. Therefore, compounds previously shown to influence lipoprotein assembly and secretion could possibly exert a similar effect on HCV. To test this hypothesis, we used the full-length, RNA-based HCV full lifecycle model (JFH1/Huh7.5.1) previously shown to capture important aspects of viral replication, assembly, and infection. Using this model, we demonstrate that HCV is being actively secreted by infected cells in a Golgi-dependent pathway while bound to vLDL. Silencing ApoB messenger RNA (mRNA) by transfection with short hairpin RNA (shRNA) is shown to induce a 70% reduction in the secretion of ApoB, HCV core protein, and HCV RNA. More importantly, we find that the grapefruit flavonoid naringenin, previously shown to inhibit vLDL secretion both *in vivo* and *in vitro*, is able to reduce HCV secretion from infected cells by 80% ± 10%. We demonstrate that naringenin inhibits ApoB secretion by inhibiting the activity of the microsomal triglyceride transfer protein (MTP) as well as the transcription of 3-hydroxy-3-methyl-glutaryl-coenzyme reductase (HMGR) and acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2). Moreover, we find that naringenin is effective at a concentration of 200 μM, which is well below its toxic concentration for primary human hepatocytes and severe combined immunodeficient (SCID) mice.

Materials and Methods

Reagents and Antibodies. Fetal bovine serum (FBS), phosphate-buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin, and trypsin–ethylene diamine tetraacetic acid (EDTA) were obtained from Invitrogen Life Technologies (Carlsbad, CA). Lipoprotein-free FBS was purchased from Biomedical Technologies (Stoughton, MA). Insulin was obtained from Eli-Lilly (Indianapolis, IN). Oleate, naringenin, and brefeldin A were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Immunofluorescence-grade paraformaldehyde was purchased from Electron Microscope Sciences (Hatfield, PA). OptiMEM basal medium and Lipofectamine 2000 were purchased from Invitrogen Life Technologies. The SureSilencing shRNA plasmid kit for human ApoB [green fluorescent protein (GFP)] was purchased from SuperArray (Frederick, MD). An MTP fluorescent activity kit was purchased from Roar Biomedical (New York, NY). Unless otherwise noted, all other chemicals were purchased from Sigma-Aldrich Chemicals. For immunoprecipitation, Protein A-Sepharose was purchased from Invitrogen, whereas horseradish peroxidase–conjugated goat anti-mouse secondary was purchased from Santa Cruz Biotech (Santa Cruz, CA). For immunofluorescence studies, normal donkey serum and secondary F(ab')2 antibody fragments (multiple-labeling [ML] grade) were obtained from Jackson Immunoresearch (Bar Harbor, ME). Mouse anti-HCV core antigen (5 μg/mL) was purchased from US Biological (Swampscott, MA). Goat anti-ApoB (10 μg/mL) was purchased from R&D Systems, Inc. (Minneapolis, MN).

Cells and Viruses. The Huh7.5.1 human hepatoma cell line and a plasmid containing the JFH-1 genome were kindly provided by Dr. Chisari (Scripps Research Institute, La Jolla, CA) and Dr. Wakita (National Institute of Infectious Diseases, Tokyo, Japan), respectively. Huh7.5.1 cells were cultured in DMEM supplemented with 10% FBS, 200 units/mL penicillin, and 200 mg/mL streptomycin in a 5% CO_2–humidified incubator at 37°C. *In vitro* transcribed genomic JFH-1 RNA was delivered to cells by liposome-mediated transfection as described by Zhong et al.[8] Infected Huh7.5.1 cells were passaged every 3 days and used at passage <15. The presence of HCV in these cells and corresponding supernatants were determined by quantitative, reverse-transcription, polymerase chain reaction (qRT-PCR) and immunofluorescence staining. Primary human hepatocytes were purchased from BD Biosciences (San Jose, CA) and cultured on a collagen-coated 12-well plate in a C+H culture medium composed of DMEM supplemented with 10% heat-inactivated FBS, 200 U/mL penicillin/streptomycin, 7.5 μg/mL hydrocortisone, 20 ng/mL epidermal growth factor (EGF), 14 ng/mL glucagons, and 0.5 U/mL insulin. The medium was supplemented with 2% dimethyl sulfoxide for long-term culture of the primary cells.

HCV Secretion. HCV-infected Huh7.5.1 cells were plated on a 6-well plate at a density of 1×10^5 cells/cm^2 and cultured overnight in the standard medium. Prior to the beginning of the experiment, the cells were washed 3 times with PBS and cultured with DMEM containing 5% lipoprotein-free FBS. Oleate, insulin, naringenin, and brefeldin A were added at this time as described in the text. Following 24 hours of incubation, the plate was gently agitated to release mechanically bound particles, and the medium was collected, filtered to remove cellular debris, and stored at −80°C for further analysis. The attached cells were washed 3 times with PBS, harvested, pelleted, and stored at −80°C for further analysis.

Coimmunoprecipitation. The binding of Huh7.5.1-secreted JFH1 particles to ApoB was assessed with coim-

munoprecipitation. Anti-human ApoB-100 antibody (5 μg) was bound to 100 μL of Protein A-Sepharose on ice. Three milliliters of the JFH1-infected Huh7.5.1 conditioned medium (1×10^6 cells/mL) was added to the mixture, which was subsequently rotated for 4 hours at 4°C. The sample was spun down at 10,000*g* in a microcentrifuge and washed 3 times with 50 mM trishydroxymethylaminomethane (Tris)-HCl (pH 7.5) containing 5 mM EDTA. Finally, the sample was eluted in 100 μL of 10 mM Tris-HCl (pH 8.5) containing sodium dodecyl sulfate. The protein concentration in the eluted buffer was quantified as described later, and 20 μg of protein was loaded onto a 7.5% Tris-HCL resolving gel. Resolved proteins were transferred to a polyvinylidene fluoride membrane and stained against HCV core (0.5 μg/mL).

HCV Infectivity. The infectivity of the secreted HCV particles was measured as previously described.[8] Naïve Huh7.5.1 cells were grown to 80% confluence and exposed to cell culture supernatants diluted 10-fold in the culture medium. Following 1 hour of incubation at 37°C, the medium was replaced, and the cells were cultured for 3 additional days. Levels of HCV infection were determined by immunofluorescence staining for HCV core protein. The viral titer is expressed as focus forming units per milliliter of supernatant.

Human ApoB Enzyme-Linked Immunosorbent Assay (ELISA). Huh7.5.1–secreted and primary human hepatocyte–secreted ApoB was detected in the medium with the ALerCHEK, Inc. (Portland, ME), total human ApoB ELISA kit. The medium was diluted 1:10 with the specimen diluent, and the assay was carried out according to the manufacturer's directions.

HCV Core Antigen ELISA. Huh7.5.1–secreted HCV core antigen was detected in the medium with the Wako Chemicals (Cambridge, MA) ORTHO HCV antigen ELISA kit. The medium was used as is, and the assay was carried out according to the manufacturer's directions.

Total Protein Assay. The total protein content of the cells was measured with the Bio-Rad Laboratories (Hercules, CA) protein assay based on the Bardford method. Briefly, a cell pellet was lysed in 350 μL of 0.1% Triton X-100, and 5-μL samples were loaded onto a 96-well plate and incubated for 15 minutes with 250 μL of Coomassie Blue reagent at room temperature. Absorbance was measured at 595 nm and compared to a bovine serum albumin standard.

Quantitative, Real-Time, Reverse-Transcription Polymerase Chain Reaction (PCR). Virus samples collected in each experiment were filtered with a 0.45-μm filter, and a volume of 100 μL for each sample was heated at 95°C for 45 minutes. The reverse-transcription reaction step was performed on a Mastercycler epgradientS (Eppendorf) instrument using Omniscript and Sensiscript RT kits (Qiagen). Real-time PCR was performed on a Light Cycler LC-24 (Idaho Technology) using SuperScript III Platinum CellsDirect Two-Step qRT-PCR kits (Invitrogen). For the reverse-transcription step, 2 μL of a sample without RNA extraction was used. For real-time PCR, 1 μL of the reverse-transcription reactions was used. All reactions were performed according to the manufacturer's instructions with the primers detailed in Table 1.

Table 1. PCR Primers

Gene		Primer
HCV 5′ untranslated region	Forward	5′-GCAGAAAGCGTCTAGCCATGGCGT-3′
	Reverse	5′-CTCGCAAGCACCCTATCAGGCAGT-3′
MTP	Forward	5′-GAGGTTTCTCTATGCCTGTGGATTT-3′
	Reverse	5′-CCCAGGATTAACTTCTTAGCTTCCA-3′
ACAT1	Forward	5′-CAATACAATGGTGGGTGAAGAGAAG-3′
	Reverse	5′-AAAATCTTTCCTTGTTCTGGAGGTG-3′
HMGR	Forward	5′-GACCGCTTTGCTTAGATGAAAAAGA-3′
	Reverse	5′-GGACTGGAAACGGATATAAAGGTTG-3′
Actin	Forward	5′-GTCGTACCACTGGCATTGTG-3′
	Reverse	5′-CTCTCAGCTGTGGTGGTGAA-3′
ACAT2	Forward	5′-CATGCGGGAGGCTATACAAT-3′
	Reverse	5′-GTAGATGGTGCGGAAATGCT-3′

Cellular Viability. The viability of both Huh7.5.1 cells and primary human hepatocytes was studied with Thermo Fisher Scientific (Waltham, MA) Infinity aspartate aminotransferase (AST) liquid reagent. Medium samples (15 μL/well) were loaded onto a 96-well plate in triplicates and mixed with 150 μL of the AST liquid reagent. Absorbance decay was measured at the wavelength of 340 nm with 15-second intervals in a Bio-Rad Benchmark Plus spectrophotometer. Values were normalized to the total amount of AST available per culture, which was determined by total cell lysis induced by 1% Triton X-100 for 20 minutes at room temperature. Cell viability for all conditions reported in the Results section was greater than 90%.

MTP Activity Assay. MTP activity was analyzed with an MTP assay kit as previously described.[11] The assay is based on a transfer of a fluorescent signal between donor and acceptor particles due to MTP activity. Briefly, confluent Huh7.5.1 cells were stimulated with naringenin or a carrier control for 24 hours and were then washed with ice-cold PBS and scraped off the dish with a cell scraper. Samples were homogenized by sonication (3 × 5 seconds) in a buffer containing protease inhibitors. The MTP assay was performed by the incubation of 50 μg of cellular protein with 10 μL of donor and acceptor solutions in 250 μL of total buffer (15 mM Tris, pH 7.4; 40 mM NaCl; 1 mM EDTA). The in-

crease in the fluorescent signal was measured over 12 hours at 37°C at the excitation wavelength of 465 nm and emission wavelength of 538 nm.

Animal Studies. Male SCID mice (8 weeks old, 20-25 g) were obtained from Charles River Laboratories (Wilmington, MA). Animals were treated in accordance with National Institutes of Health guidelines and the Massachusetts General Hospital Subcommittee on Research Animal Care. The mice were allowed free access to laboratory chow and water *ad labium*. Naringenin was dissolved in 0.5% Tween 20 diluted in saline and given by intraperitoneal injection. Two days following the treatment, animals were sacrificed, and blood was withdrawn by cardiac puncture. AST and alanine aminotransferase (ALT) enzyme levels were assessed as described previously. Total triglycerides were measured with a kit purchased from Sigma-Aldrich Chemicals according to the manufacturer's instructions.

Silencing ApoB mRNA. HCV-infected Huh7.5.1 cells were plated in T-25 tissue culture flasks at a density of 1×10^5 cells/cm^2 and cultured overnight in the standard medium. Prior to silencing, the cells were washed 3 times with PBS, and the medium was replaced with OptiMEM basal medium. SureSilencing shRNA (GFP) plasmids against human ApoB100 as well as shRNA plasmid control (500 ng/mL) were combined with Lipofectamine 2000 in OptiMEM and incubated with the cells overnight. SureSilencing shRNA plasmids code for GFP, which was used to sort the transfected Huh7.5.1 cells with FACSAria (BD Biosciences) located at the Partners AIDS Research Center. Transfected cells (10% of the total population) were sorted directly into a 12-well plate and allowed to adhere overnight. The culture medium was conditioned by the transfected cells for 24 hours and analyzed as described previously.

Immunofluorescence Microscopy. Huh7.5.1 cells were washed 3 times with PBS and fixed in 4% electron microscopy–grade paraformaldehyde for 10 minutes at room temperature. Slides were then washed with PBS and incubated in 100 mmol/L glycine for 15 minutes to saturate reactive groups. Samples were permeabilized for 15 minutes with 0.1% Triton X-100, blocked for 30 minutes with 1% bovine serum albumin and 5% donkey serum at room temperature, and stained with primary antibodies overnight at 4°C. After additional washes with PBS, samples were stained with fluorescently tagged secondary antibodies for 45 minutes at room temperature.

Results

Huh7.5.1-Secreted HCV Is Bound to ApoB. Recent evidence suggests that HCV binds to low-density particles

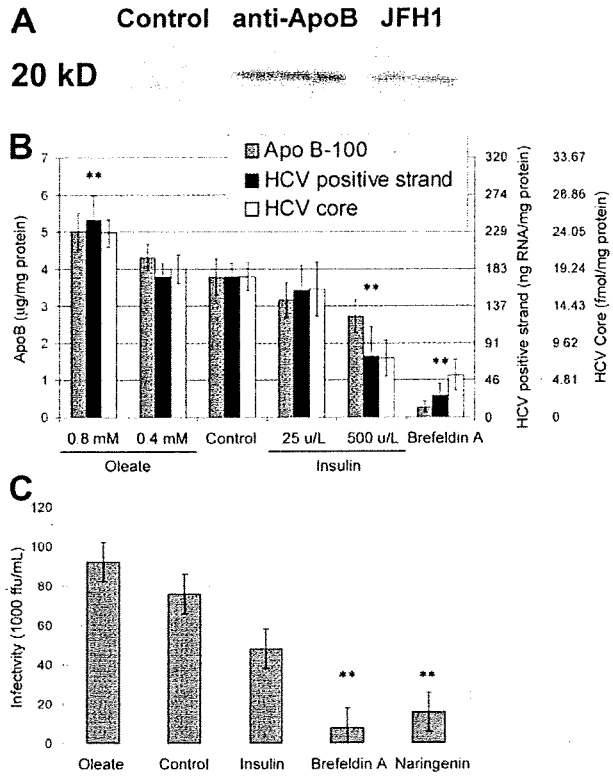

Fig. 1. (A) Immunoprecipitation of Huh7.5.1-secreted ApoB followed by anti-HCV core staining (coimmunoprecipitation). (B) Cell culture secretion of ApoB, HCV-positive strand RNA, and HCV core protein in JFH-1–infected Huh7.5.1 cells in response to oleate, insulin, and brefeldin A. The secretions of ApoB, HCV RNA, and HCV core protein are significantly up-regulated by oleate and down-regulated by insulin in a dose-dependent manner. Brefeldin A, which blocks Golgi-dependent secretion of proteins, significantly inhibits the secretion of ApoB, HCV RNA, and HCV core. Cell viability for all conditions was greater than 90%. (C) Infectivity of cell culture supernatant assessed by colony formation on naïve Huh7.5.1 cells: oleate (0.8 mM), insulin (500 U/L), brefeldin A (2.5 μg/mL), and naringenin (200 μM). $^{**}P < 0.01$.

prior to virus egress[9] and that viral secretion requires both ApoB expression and vLDL assembly to occur.[10] Therefore, HCV secreted by the JFH1/Huh7.5.1 full viral lifecycle model could potentially be secreted while bound to vLDL. To determine if Huh7.5.1-produced HCV is bound to vLDL, we immunoprecipitated the Huh7.5.1-conditioned medium against human ApoB antibodies and detected bound HCV core protein in the eluted sample. The results presented in Fig. 1A demonstrate that HCV core protein is bound to ApoB-100 in our samples. HCV core could not be detected when the sample was precipitated against irrelevant antibody (control) but was easily detected in the cell medium (JFH1).

HCV Secretion Mirrors That of vLDL. The interaction between HCV and ApoB suggests that the virus might be actively secreted by the cells while bound to vLDL. However, the interaction between these particles

might also occur outside the cell. To determine if HCV is being actively secreted by the cells while bound to vLDL, we studied viral secretion in response to oleate and insulin stimulation, which was previously shown to oppositely modulate ApoB secretion in culture.[12] Figure 1B shows ApoB, HCV core, and HCV-positive strand RNA secretion by Huh7.5.1 cells infected with the JFH-1 virus. As expected, ApoB secretion is significantly up-regulated by oleate ($P = 0.0023$, n = 5) and down-regulated by insulin ($P = 0.0073$, n = 5) in a dose-dependent manner. Similarly, HCV core protein secretion is significantly up-regulated by oleate ($P = 0.0073$, n = 3) and down-regulated by insulin ($P = 0.0223$, n = 3) in a dose-dependent manner. The secretion of HCV-positive strand RNA, measured by qRT-PCR, follows the same path. However, intracellular levels of HCV RNA remained unchanged following both treatments.

Brefeldin A is a commonly used toxin that disrupts communication between the endoplasmic reticulum and the Golgi, inhibiting the active secretion of proteins.[12,13] Not surprisingly, the addition of brefeldin A (2.5 μg/mL) blocked ApoB secretion ($P = 0.0001$, n = 5). Interestingly, brefeldin A significantly inhibits the secretion of HCV core protein ($P = 0.0021$, n = 4) and HCV-positive strand RNA ($P = 0.0006$, n = 3). To assess whether the changes in HCV core protein and RNA secretion correlate with changes of viral infectivity in the cell supernatant, we measured the ability of the secreted virus to infect naïve Huh7.5.1 cells. Figure 1C shows that the infectivity of the cell supernatant increased following oleate stimulation, decreased because of insulin, and was strongly inhibited following brefeldin A stimulation by 89% ± 10% ($P = 0.001$, n = 3). These results suggest that HCV is being actively secreted by the cells, perhaps while bound to vLDL.

HCV Core Antigen Colocalizes with ApoB. Previously, HCV core protein was shown to associate with ApoAII[4] and lipid droplets in HepG2 cells[5] overexpressing the core protein. Just recently, Huang et al.[10] demonstrated that HCV core protein colocalizes with ApoB in a chromosomally integrated cDNA model of HCV. To ascertain if HCV core protein associates with ApoB in JFH-1 virus–infected Huh7.5.1 cells, we double-stained Huh7.5.1 cells 2 days post infection by immunofluorescence for both viral and native proteins. Figure 2 demonstrates the colocalization of HCV's core and ApoB100 in infected cells. HCV core protein associates with areas in the cytoplasm that are positive to ApoB100. However, we note that although the proteins appear to be closely associated, we fail to find a one-to-one correspondence between the viral and native proteins in our model of the full viral lifecycle.

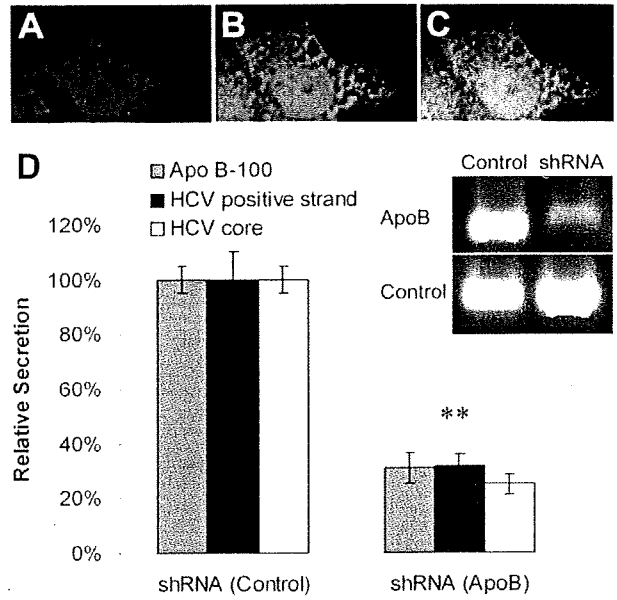

Fig. 2. Double immunofluorescence staining of JFH-1-infected Huh7.5.1 cells. (A) Staining for HCV core protein (red). (B) Staining for ApoB100 (green). (C) Superpositioning of the images demonstrates that HCV core protein associates with ApoB100 in the cytoplasm. (D) Relative secretion of ApoB, HCV-positive strand RNA, and HCV core protein in JFH-1-infected Huh7.5.1 cells following silencing of ApoB100 mRNA by SureSilencing shRNA transfection. $**P < 0.01$.

The association between ApoB100 and HCV core protein as well as previous data suggests that HCV might be "tagging along" ApoB secretion. Therefore, silencing ApoB production in the cell might decrease HCV secretion. Figure 2D demonstrates a 69% ± 6% decrease in ApoB secretion following transfection with SureSilencing shRNA ($P = 0.0001$, n = 3). Interestingly, HCV core protein secretion was significantly decreased by 75% ± 4% at the same time ($P = 0.0002$, n = 3). HCV-positive strand RNA secretion was also significantly decreased by 69% ± 4% ($P = 0.0015$, n = 3).

HCV Secretion Is Inhibited by Naringenin. Naringenin is a grapefruit flavonoid previously shown to reduce cholesterol levels both *in vivo*[14] and *in vitro*.[15] It is thought that naringenin inhibits ApoB secretion by reducing the activity and expression of MTP and ACAT.[15,16] To assess if naringenin inhibits HCV secretion in a similar manner, we cultured infected Huh7.5.1 cells in the presence of naringenin for 24 hours. Figure 3A demonstrates that naringenin inhibits the secretion of HCV core ($P = 0.0001$, n = 6) and HCV-positive strand RNA ($P = 0.0006$, n = 5) in a dose-dependent manner. At the concentration of 200 μM, naringenin inhibited HCV secretion by 80% ± 10%. Interestingly, intracellular levels of HCV-positive strand RNA (Fig. 3C) as well as intracellular HCV core protein expression (Supplemen-

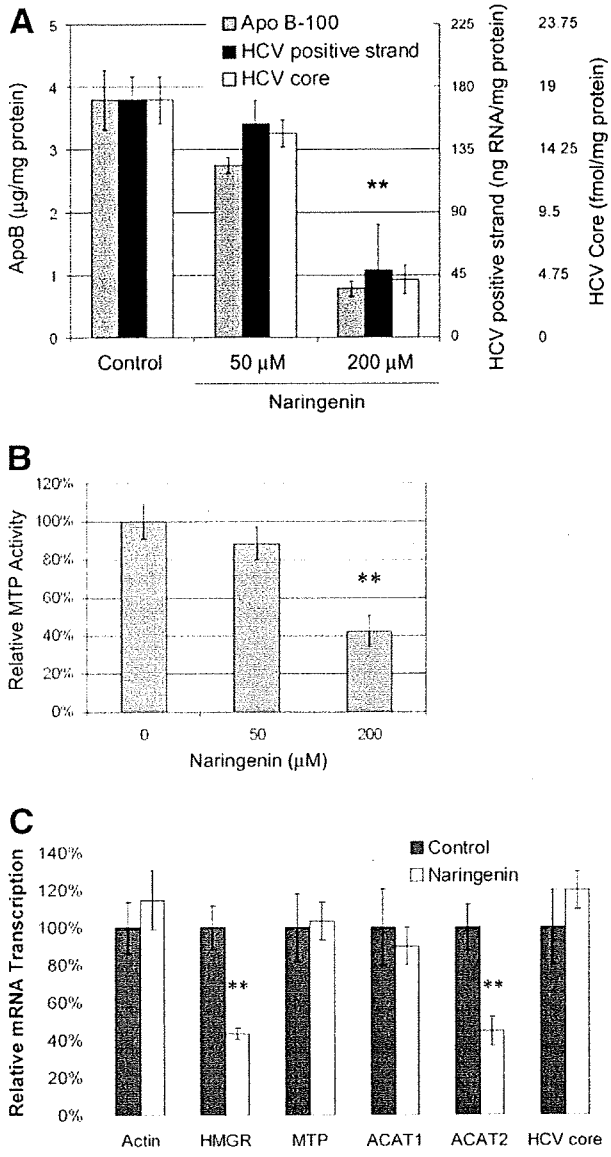

Fig. 3. (A) Inhibition of ApoB, HCV-positive strand RNA, and HCV core protein secretion by the grapefruit flavonoid naringenin. Naringenin significantly inhibits the secretion of HCV core ($P = 0.0001$, n = 6) and HCV-positive strand RNA ($P = 0.0006$, n = 5) in a dose-dependent manner. At the concentration of 200 µM, naringenin inhibited HCV secretion by 80% ± 10%. Cell viability for all conditions was greater than 90%. $^{**}P < 0.01$. (B) Naringenin inhibits the activity of MTP in a dose-dependent manner. At the concentration of 200 µM, MTP activity was reduced by 58% ± 8% ($P = 0.0012$, n = 3). (C) Naringenin induces changes in hepatic gene transcription measured by qRT-PCR. HMGR transcription was reduced by 57% ± 3% ($P = 0.010$, n = 3), whereas the transcription of ACAT2 was reduced by 55% ± 7% ($P = 0.016$, n = 3). The mRNA levels of actin, MTP, and ACAT1 remained unchanged. Intracellular RNA levels of HCV core also remained unchanged during the 24 hours of treatment. $^{**}P < 0.02$.

tary Fig. 1) remained unchanged. To assess whether the naringenin-induced inhibition of HCV core protein and RNA secretion correlated with changes of viral infectivity in the cell supernatant, we measured the ability of the secreted virus to infect naïve Huh7.5.1 cells. Figure 1C shows that the infectivity of the cell supernatant was strongly inhibited following naringenin stimulation by 79% ± 10% ($P = 0.0018$, n = 3).

Although the activity of naringenin has been described in uninfected cells,[15,17,18] it has yet to be characterized in HCV-infected cells. Figure 3B demonstrates that naringenin inhibits MTP activity in a dose-dependent manner. At the concentration of 200 µM, MTP activity was reduced by 58% ± 8% ($P = 0.0012$, n = 3). In addition, we demonstrate that naringenin induces significant changes in hepatic gene transcription measured by qRT-PCR (Fig. 3C). HMGR transcription was reduced by 57% ± 3% ($P = 0.010$, n = 3), whereas ACAT2 was reduced by 55% ± 7% ($P = 0.016$, n = 3). In contrast, the mRNA levels of actin, MTP, ACAT1, and HCV remained unchanged.

Naringenin Does Not Display Hepatic or* In Vivo *Toxicity. To assess the potential of naringenin-based treatment, we measured ApoB secretion in primary human hepatocytes following 24 hours of stimulation with naringenin. Figure 4A demonstrates a dose-dependent decrease in ApoB secretion following naringenin stimulation. At 200 µM naringenin, ApoB secretion was reduced by 60% ± 7% ($P = 0.007$, n = 3). The viability of primary human hepatocytes exposed to increasing concentrations of naringenin is shown in Fig. 4B. Human hepatocyte viability was 81% ± 3% at 200 µM naringenin and was not judged to be statistically different than that of the control (78% ± 3%). Human hepatocyte viability dropped significantly only at naringenin concentrations greater than 1000 µM.

To further assess naringenin potential, we delivered naringenin by intraperitoneal injection to 8-week-old male SCID mice at concentrations of 60, 300, and 1500 mg/kg (approximately 200, 1000, and 5000 µM). Animal survival was not affected by naringenin at these doses. To discern if liver damage occurred, we measured levels of AST and ALT in the animals' plasma 48 hours following injection. Figure 5 demonstrates that there was no elevation of ALT levels under all conditions. AST levels appeared to increase but remained under 100 U/L even at the highest dose. To assess naringenin's ability to reduce circulating vLDL levels, we measured total triglyceride levels in animal plasma. Figure 5A demonstrates a decrease in triglycerides following naringenin injection.

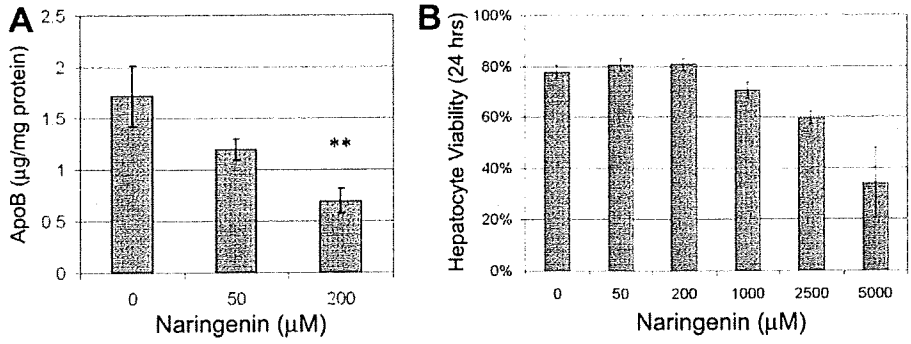

Fig. 4. (A) Naringenin stimulation inhibits ApoB secretion of primary human hepatocytes in a dose-dependent manner. At 200 μM naringenin, ApoB secretion was reduced by 60% ± 7% (P = 0.007, n = 3). (B) Viability of freshly isolated human hepatocytes exposed to increasing concentrations of naringenin for 24 hours. Human hepatocyte viability was 81% ± 3% at 200 μM naringenin and was not judged to be statistically different than the control (77% ± 3%). Human hepatocyte viability dropped significantly only at naringenin concentrations greater than 1000 μM.

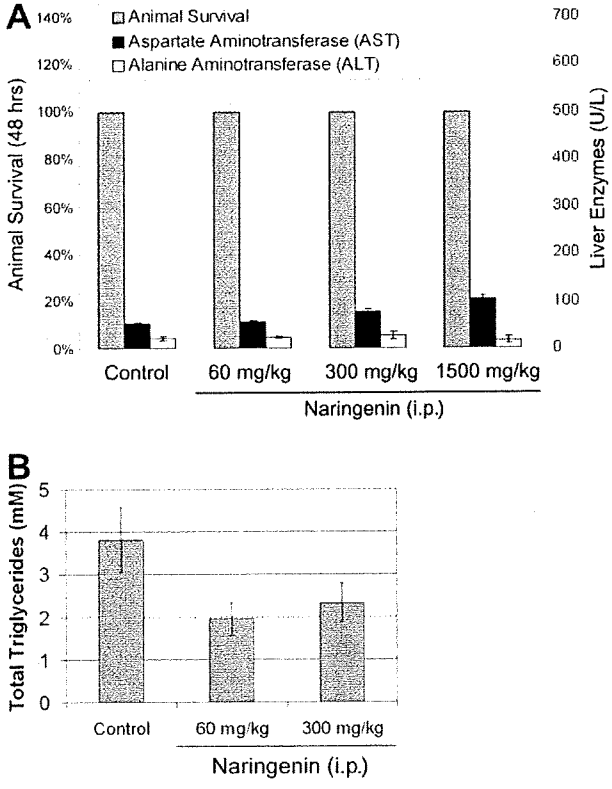

Fig. 5. Animal survival and liver enzyme release following intraperitoneal (i.p.) injection of naringenin into 8-week-old male SCID mice. Animals were injected with naringenin at 60, 300, and 1500 mg/kg of body weight. Animals were sacrificed at 48 hours, at which time liver enzymes (AST and ALT) and total triglycerides were analyzed in the animals' plasma. (A) Animal survival was monitored for several days following injection and was not affected even at the highest dose (1500 mg/kg). The ALT level appeared unchanged over all conditions, whereas AST was found to be slightly elevated at the highest dose. (B) Total triglycerides analyzed in animal plasma 24 hours following injection decreased in response to naringenin.

Discussion

HCV is a leading cause of chronic liver disease worldwide. Although the disease develops to cirrhosis in only 20% of the cases, the sheer scope of infection and lack of effective treatment make it a severe global health problem. A simulation of the US population for the years 2010-2019 predicts nearly 200,000 deaths associated with HCV infection and direct medical expenditures in excess of $10 billion. It is for these reasons that there is a pressing need for the development of alternative strategies for the treatment of HCV infection.

The interaction between HCV infection, cholesterol, and fatty acid metabolism has received significant attention, mainly because of the development of liver steatosis in chronically infected patients.[1] However, the lack of an efficient cell culture model of HCV replication and infection has significantly limited research in the field. Despite these limitations, several groups have demonstrated that HCV core protein associates with ApoAII[4] and lipid droplets in HepG2 cells[5] overexpressing the protein. The data suggest that HCV in infected patients might circulate as lipoviral particles.[19] The development of HCV replicon systems[20] has allowed for the efficient study of viral replication in culture. Using this system, Kapadia and Chisari[6] demonstrated that HCV replication is regulated by geranylgeranylation and fatty acid metabolism. Others have demonstrated that HCV nonstructural proteins, such as nonstructural protein 5A, inhibit ApoB secretion.[21]

The recent development of the JFH-1 virus[7] in combination with the Huh7.5.1 cell line[8] has allowed for the efficient infection of cells and the generation of large virus titers in culture. This model allows for the identification of intercellular infectious HCV particles with a higher density than that of their secreted counterparts,[9] suggest-

ing the binding of HCV to low-density particles in the endoplasmic reticulum. Just recently, Huang et al.[10] demonstrated that HCV assembled in ApoB and MTP enriched vesicles and that the viral secretion was dependent on both ApoB expression and vLDL assembly in a chromosomally integrated cDNA model of HCV secretion. As the association between HCV and serum β-lipoproteins (vLDL and LDL) is well known,[2] these results strongly suggest that HCV might "hitch a ride" on the lipoprotein-cholesterol lifecycle. This hypothesis is intriguing as it might explain the presence of HCV in intestinal cells, a second site of lipoprotein production.[22] In addition, it might explain HCV uptake by LDL receptor,[23,24] scavenger receptor class B type I,[25] and heparin sulfate.[26]

Our results strongly support this hypothesis. We demonstrate that HCV produced by the Huh7.5.1 cell line is bound to ApoB and that its secretion is inhibited by brefeldin A, a metabolite of the fungus *Eupenicillium brefeldianum*, which blocks the communication between the endoplasmic reticulum and the Golgi, effectively inhibiting protein secretion.[12,13] We also demonstrate that HCV secretion is up-regulated by the fatty acid oleate and down-regulated by insulin, precisely mirroring ApoB secretion by the cells.[12] Moreover, silencing ApoB100 mRNA caused a significant and parallel decrease in HCV core protein secretion. This ApoB-dependent HCV secretion pathway suggests a novel therapeutic approach for the treatment of HCV infection.

Naringin, one of the most abundant flavonoids in citrus fruits, is hydrolyzed by enterobacteria to naringenin prior to being absorbed. Naringenin has been reported to be an antioxidant,[27] MTP and ACAT inhibitor,[16] and regulator of cytochrome P4503A and 4A activity.[28,29] The ability of naringenin, or its glycosylated form, to significantly reduce plasma cholesterol levels has been demonstrated both *in vivo* and *in vitro*.[14,15] It is thought that naringenin inhibits the expression and activity of MTP, which catalyzes the transfer of lipids to the nascent ApoB molecule as it buds into the endoplasmic reticulum as a vLDL particle.[16-18] Our results demonstrate that short-term (24-hour) stimulation of infected hepatocytes with 200 μM naringenin significantly inhibits HCV secretion by 80% ± 10% and the infectivity of the titer by 79% ± 10%. At the same time, transcription of the viral RNA remains unchanged. We suggest that this is due in part to the inhibition of MTP activity by 58% ± 8% as well as the inhibition of HMGR and ACAT2 transcription. To further demonstrate naringenin as a potential therapy, we show that the compound is nontoxic to freshly isolated human hepatocytes up to concentrations greater than 1000 μM. In addition, we demonstrate that naringenin induced a 60% ± 7% decrease in ApoB secretion by primary human hepatocytes.

The concept of supplementing HCV patients' diets with naringenin is appealing. A recent clinical trial in hypercholesterolemic patients demonstrated that a low dose of naringin (400 mg/day) lowered LDL levels by 17%.[30] A similar cholesterol-lowering effect of naringenin was demonstrated in rabbits[14,31] and rats.[32] However, it is worth noting that the absorbance of naringenin through the intestinal wall is limited (less than 8%), and this suggests that short-term therapeutic doses would need to be delivered intravenously. Prior studies have suggested that the median lethal dose (50% kill) for naringenin is 2000 mg/kg for both rats and guinea pigs by intraperitoneal injection.[33] Our results show that doses up to 1500 mg/kg naringenin given by intraperitoneal injection to mice did not cause death or a marked elevation of liver enzymes, suggesting that intravenous administration of naringenin is in the realm of possibility.

The ability of the liver to regenerate in the context of the RNA-based lifecycle of HCV allows for the potential clearance of the viral infection. It is thought that clearance occurs in about 30% of HCV-infected patients. The possible reduction of HCV viral load by inhibiting viral secretion could allow uninfected cells to regenerate, potentially increasing the overall rate of viral clearance. Future studies would focus on the long-term ability of naringenin and perhaps other citrus flavonoids to reduce viral load in animal models, such as the KMT Mouse model,[34] and long-term cultures of primary human hepatocytes.

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3D cultured immortalized human hepatocytes useful to develop drugs for blood-borne HCV

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ABSTRACT

Due to the high polymorphism of natural hepatitis C virus (HCV) variants, existing recombinant HCV replication models have failed to be effective in developing effective anti-HCV agents. In the current study, we describe an *in vitro* system that supports the infection and replication of natural HCV from patient blood using an immortalized primary human hepatocyte cell line cultured in a three-dimensional (3D) culture system. Comparison of the gene expression profile of cells cultured in the 3D system to those cultured in the existing 2D system demonstrated an up-regulation of several genes activated by peroxisome proliferator-activated receptor alpha (PPARα) signaling. Furthermore, using PPARα agonists and antagonists, we also analyzed the effect of PPARα signaling on the modulation of HCV replication using this system. The 3D *in vitro* system described in this study provides significant insight into the search for novel anti-HCV strategies that are specific to various strains of HCV.

Infection with Hepatitis C virus (HCV) is a serious health problem worldwide and leads to high rates of liver cirrhosis and hepatocellular carcinoma [1]. Given that the standard HCV therapy remains insufficient for the successful treatment of many patients [2], the development of more effective and less toxic anti-HCV agents is required. *In vitro* systems like the HCV replicon-bearing cells and the infectious particle-producing JFH1 system, has contributed to the discovery of new targets for anti-HCV therapy. However, these recombinant HCV genomes only proliferate in sublines of HuH-7 cells, which do not permit infection or proliferation of blood-borne HCV. Due to the high polymorphism of natural HCV, data from recombinant HCV systems could be evaluated by studying the therapeutic response of a variety of naturally occurring HCVs. However, the current systems available for such study remain insufficient due to the low infection and replication efficiency of the natural HCV strains.

More recently, production and secretion of infectious HCV particles has been reported in two independent three-dimensional (3D) cell culture systems, termed the radial-flow bioreactor (3D/RFB) and the thermoreversible gelatin polymer (3D/TGP) systems. These results were not observed in monolayer cultures [3], suggesting that hepatocytes cultured in 3D more closely resemble liver cells *in vivo* [4] and thus support HCV proliferation. In addition, analysis of gene expression levels in 3D cultured cells revealed that the newly established immortalized human hepatocyte (HuS-E/2 cells) gene profile was altered to more closely resemble that of human liver tissue when the cells were cultured in 3D/TGP [5].

In the current study, we cultured HuS-E/2 cells in 3D/TGP and demonstrated efficient proliferation of natural HCV. Furthermore, gene expression analysis of these cells demonstrated the activation of the peroxisome proliferators-activated receptor α (PPARα) signaling pathway, suggesting an important role for this pathway in the replication of natural HCV. Thus, the *in vitro* system described appears to be a useful tool for the study of HCV infection and proliferation as well as for the development of effective anti-viral agents against various natural HCVs.

Materials and methods

Cell culture. Immortalized human hepatocytes (HuS-E/2) and LucNeo#2 replicon cells [6] were cultured as previously described [5,7]. For the 3D-TGP culture system, 1×10^5 HuS-E/2 cells were cultured in 1 ml Mebiol gel (Mebiol Inc., Kanagawa, Japan)/well in 12-well plates. Five hundred microliters of fresh medium was overlaid on the solidified gel, and was changed every 2 days. Cell

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extraction from the gel was done at the designated time points according to the manufacturer's protocol.

RNA extraction, reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR (Q-PCR). At the designated time points, total cellular RNA was extracted and 1 μg of total RNA was used as a template for RT-PCR and for the quantitative detection of HCV-RNA using real-time RT-PCR (Q-PCR) as previously described [10].

HCV infection experiment. HCV infection experiments were carried out using sera from patients infected with HCV. Infection in 2D culture was undertaken as previously described [5]. For 3D/TGP cultured cells, the gel was solidified, and 50 μl HCV-containing patient serum with a titer of 1×10^6 HCV-RNA/ml was added to the culture and mixed. The culture was continued until the cells were extracted. Following extraction from 3D-TGP, cells were centrifuged and washed three times thoroughly with PBS. RNA was then extracted from the cells as described above. HCV infection into HuS-E/2 cells was also examined in the presence of anti-E2 mouse monoclonal antibody (917) as outlined previously [8].

Treatment of cells with PPARα signaling agonists and antagonists. Fenofibrate or MK886 (Sigma–Aldrich, USA) were added to the culture medium of HuS-E/2 (2D-HuS-E/2) cells from day 0 of HCV infection; or the culture medium of LucNeo#2 replicon harboring cells. The cells were then cultured to the designated time point.

Microarray analysis. Gene expression profiles of 3D/TGP cultured HuS-E/2 cells were obtained by microarray analysis (3D-Genes Human 25, Toray, Tokyo, Japan) and compared to those of cells cultured in 2D.

Results

3D/TGP cultures enhance HCV proliferation in HuS-E/2 cells

Infection and proliferation of the HCV genotype 1b (HCV-RC5) derived from the serum of patient RC5 in HuS-E/2 cells cultured in 3D/TGP (3D/TGP-HuS-E/2 cells) was investigated and compared with that of HuS-E/2 cells cultured in 2D (2D-Hus-E/2). As outlined in Fig. 1A, the HCV-RNA levels in the 3D/TGP-HuS-E/2 cells were significantly higher at all of the time points examined following infection than in the 2D-HuS/E2 cells, suggesting that the 3D/TGP system greatly enhances the proliferation of naturally occurring HCV in HuS-E/2 cells. Similar results were also obtained for sera from additional patients (data not shown). To examine whether the infection is viral envelope-receptor mediated, the infection experiments using serum treated with anti-HCV-E2 antibody (α-E2) or with anti-tubulin (negative control) was also performed. Pre-incubation of the serum with α-E2 significantly reduced the total amount of HCV-RNA in the cells upon infection (Fig. 1B). This result suggested that the infection of natural HCV into 3D/TGP-HuS-E/2 cells was HCV-E2-dependent.

Inhibition of natural HCV replication in HuS-E/2 cells by Interferon

In order to test the effects of anti-viral agents on natural HCV replication in 3D/TGP HuS-E/2 cells, 50–100 U/ml of IFNα was added to the medium overlaying the HCV-RC5 infected 3D/TGP-HuS-E/2 cells. The two treatment concentrations resulted in the inhibition of HCV-RNA replication in 3D-HuS-E/2 cells by

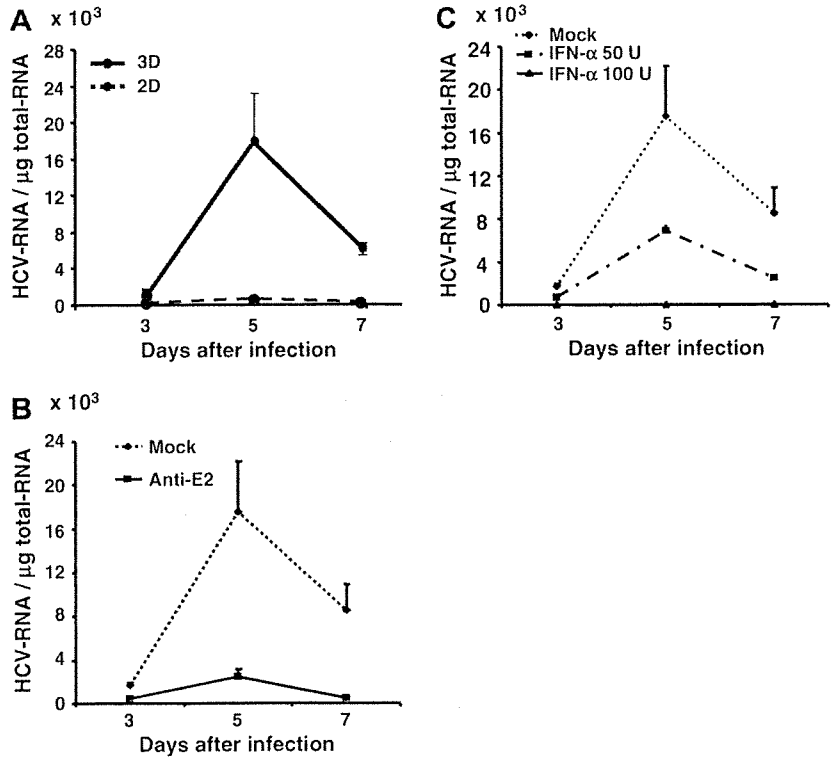

Fig. 1. HCV infection into 3D/TGP-HuS-E/2 cells. (A) 3D/TGP significantly enhanced HCV proliferation in HuS-E/2 cells. HCV patient serum was used to infect a similar number of HuS-E/2 cells cultured in 2D (hashed line) or 3D/TGP (solid line) culture for 24 h. Cells were then harvested and lysed at the indicated time points (3–7 days). The quantity of genomic HCV-RNA per 1 μg total RNA was determined by Q-PCR analysis. (B) Anti-E2 antibodies blocked HCV infection. HCV infection was performed as described in panel A in the presence of Anti-E2 specific or anti-tubulin (control) antibodies. (C) IFNα inhibits HCV replication in 3D/TGP-HuS-E/2 cells. HuS-E/2 cells were infected with HCV and fresh medium supplemented with or without (Mock), 50 U/ml, or 100 U/ml IFNα overlaid on the gel containing the cells and HCV proliferation measured as described above.

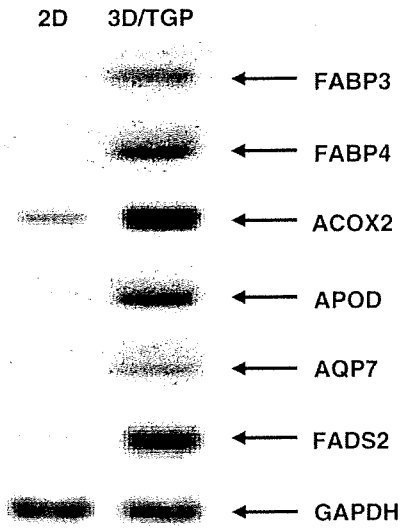

Fig. 2. RT-PCR analysis of the expression of genes identified by microarray. The PPARα regulated genes were increased in 3D/TGP-HuS-E/2 cells (3D-TGP) and their expression levels measured by RT-PCR. 2D represents RNA samples from 2D-HuS-E/2 cells. Twenty cycles of amplification were undertaken for the RT-PCR analysis. GAPDH expression served as an internal control. *Abbreviations:* FABP3, fatty acid binding proteins 3; FABP4, fatty acid binding proteins 4; ACOX2, acyl-coenzyme A oxidase 2; APOD, apolipoprotein D; AQP7, aquaporin 7; FADS2, fatty acid desaturase 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

approximately 50–60% and almost completely, respectively, when compared to the replication in cells receiving mock treatment (Fig. 1C). These results demonstrate that the IFNα treatment was effective on HCV derived from RC5 and that 3D/TGP-HuS-E/2 cells may be useful for the screening of anti-HCV drugs for the treatment of natural HCV.

Increased activation of the PPARα signaling pathway in 3D cultured HuS-E/2 cells

Given that 3D/TGP-HuS-E/2 cells demonstrated enhanced proliferation of natural HCV, the gene expression profiles of these cells was compared with that of cells cultured under normal 2D conditions using microarray analysis in order to identify the factors required for the enhanced proliferation. Among the 24,268 genes compared in this analysis, 212 genes demonstrated a greater than four folds index increase in expression in 3D/TGP than standard cultured cells. Cell signaling pathway analysis of these 212 genes showed that six genes, including fatty acid binding proteins 4 and 3 (FABP4 and 3), apolipoprotein D (APOD), aquaporin 7 (AQP7), acyl-coenzyme A oxidase 2 (ACOX2), and fatty acid desaturase 2 (FADS2), were targets of PPARα signaling [9–12]. The increased expression of these genes in the 3D/TGP-HuS-E/2 cells was further confirmed by RT-PCR analysis (Fig. 2). Given that PPARα is an essential factor for normal hepatocyte function [13], these results indicate that 3D/TGP culture enhances the hepatocyte-specific characteristics of HuS-E/2 cells.

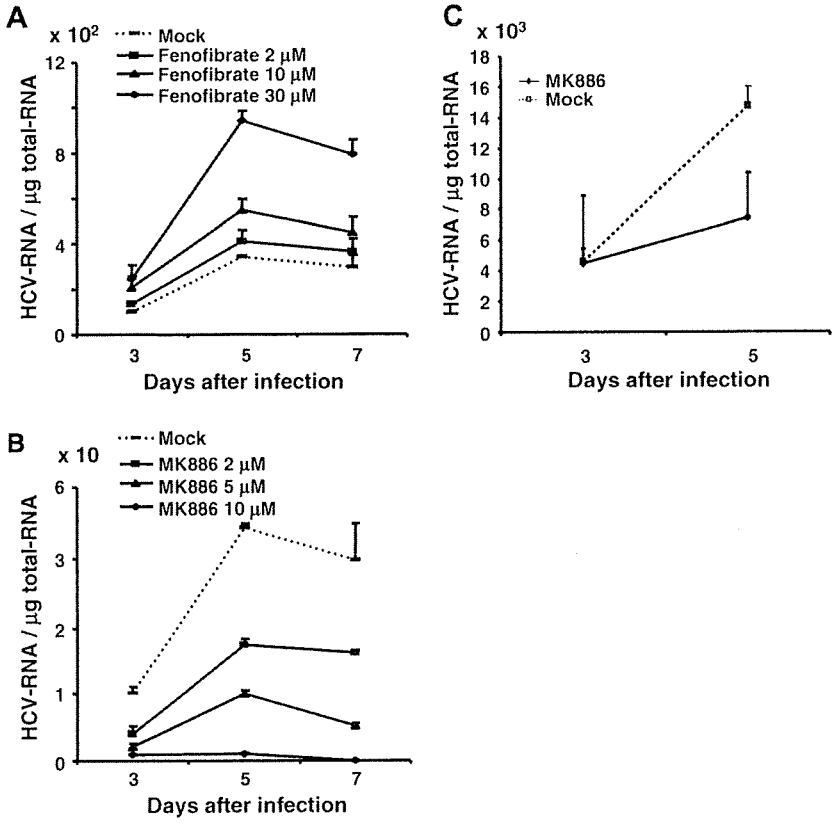

Fig. 3. The effects of PPARα agonists and antagonists on natural HCV proliferation. (A) HuS-E/2 cells were infected with HCV and fresh medium supplemented with or without (Mock) 2, 10, or 30 μM of fenofibrate overlaid on the cells. (B) Medium supplemented with or without (Mock), 2, 5, or 10 μM of MK886 was overlaid on 2D-HuS-E/2 cells infected with HCV. HCV proliferation following treatment was measured by Q-PCR. (C) Medium supplemented with or without (Mock), 10 μM of MK886 was overlaid on 3D/TGP-HuS-E/2 cells infected with HCV. HCV proliferation following treatment was measured by Q-PCR.