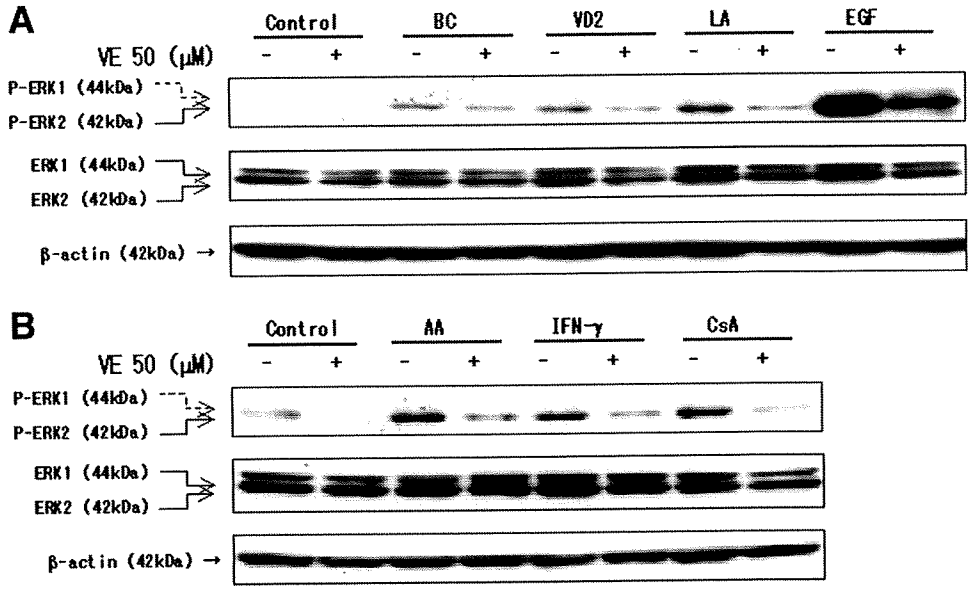

Fig. 6. VE attenuated ERK1/2 phosphorylation by the anti-HCV nutrients and reagents. OR6 cells were precultured as described in Figs. 4A and B, and then pretreated with ethanol (−) or 50 μM VE (+) for 1 hour. The cells were then treated with control medium, 20 μM BC, 10 μM VD2, 100 μM LA, or 50 ng/mL EGF (A) and control medium, 100 μM AA, 2 IU/mL IFN-γ, and 2 μg/mL CsA (B), respectively, in either the absence (ethanol) (−) or presence (+) of 50 μM VE for 15 minutes. After the treatment, cell lysates underwent western blot analysis as described in Fig. 5.

et al.,[24] we also confirmed that U0126 inhibited the anti-HCV activity of IFN-γ in OR6 cells stably replicating genome-length HCV RNA. Although they did not identify the direct activation of the MEK–ERK1/2 signaling pathway by IFN-γ, we demonstrated that IFN-γ could stimulate this cascade in HCV RNA replication cells. Moreover, this stimulation was not only inhibited by U0126 but also by antioxidant VE. This result indicates the involvement of oxidative stress in the anti-HCV activity of IFN-γ as well as the MEK–ERK1/2 signaling pathway. IFNs induce the transcription of IFN-stimulated genes through the JAK–STAT pathway, but the induction of IFN-stimulated genes by IFN-γ has been far more complex than that by IFN type I.[30] A study using a macrophage cell line revealed that IFN-γ activated ERK1/2, followed by the expression of IFN-γ–stimulated genes downstream of the JAK–STAT signaling pathway.[31] Another study reported that the defensive activity of IFN-γ against hepatitis B virus in hepatoblastoma cells was mediated through the induction of oxidative stress.[32] Furthermore, ROS itself has been reported to suppress HCV RNA replication in human hepatoma cells.[33] These reports support our proposal regarding anti-HCV activity of oxidative stress that the generation of intracellular ROS inhibits HCV RNA replication through activation of the MEK–ERK1/2 signaling pathway. Waris and Siddiqui[34] reported that calcium-dependent ROS generation induced cyclooxygenase-2 and prostaglandin E(2) via the activation of nuclear factor kappa B, leading to the suppression of HCV RNA replication. Choi et al.[35] also demonstrated that elevated calcium suppressed HCV RNA replication. The activation of nuclear factor kappa B by ROS was mediated through the MEK–ERK1/2 signaling pathway. Therefore, we suggest that the oxidative reagents and nutrients in this study also may induce anti-HCV status by calcium-dependent ROS generation.

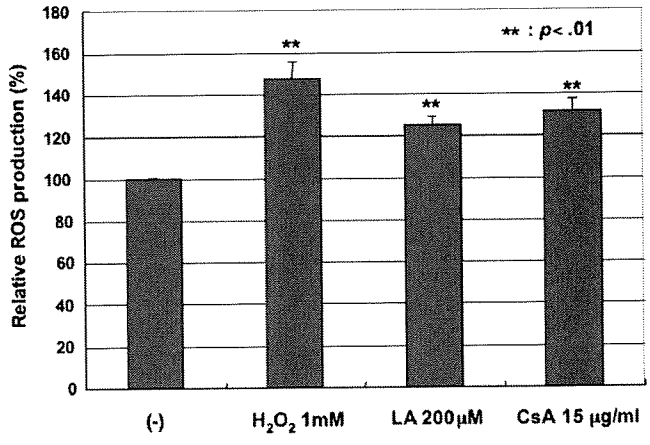

Fig. 7. ROS production by H_2O_2, LA, and CsA. OR6 cells were untreated or treated with H_2O_2 (1 mM), LA (200 μM), and CsA (15 μg/mL) and then incubated with dihydrodichlorocarboxyfluorescein diacetate. Fluorescence was measured with a fluorescence plate reader. $^{**}P <$ 0.01 versus untreated cells.

In the course of our study of the anti-HCV activities of these three nutrients, we found that treatment with U0126 more strongly inhibited their anti-HCV activities than treatment with PD98059. U0126 has been shown to possess approximately 100-fold-higher MEK1/2-specific inhibitory activity than PD98059.[36] This different potential between the two inhibitors was considered to cause a gap in their effects on anti-HCV activities. We further found that, much like EGF, all three nutrients enhanced the phosphorylation of ERK1/2 and MEK1/2, which was reduced by treatment with U0126 or VE. In addition, the

present study was the first to observe that BC, which has been shown to produce ROS,[37] activates the MEK–ERK1/2 signaling pathway, an action that VD2[38] and LA[39] have already been shown to exhibit in leukemia cell and dendritic cell lines, respectively. Furthermore, we found the involvement of the MEK–ERK1/2 signaling pathway in the anti-HCV mechanism of the three nutrients as well as various PUFAs, which were reported to be mediated through lipid peroxidation.[29] These results suggest that the anti-HCV nutrients BC, VD2, and PUFAs, including LA, as well as IFN-γ may suppress HCV RNA replication via activation of the MEK–ERK1/2 signaling pathway in response to ROS production.

We also investigated the involvement of the MEK–ERK1/2 signaling pathway in the suppressive mechanism of anti-HCV reagents other than IFN-γ. In our previous study, the anti-HCV activity of CsA, but not FLV, was prevented by VE.[13] Consequently, these results implied that CsA, but not statins, could be potent activators of the MEK–ERK1/2 signaling pathway as oxidants, leading to down-regulation of HCV RNA replication. CsA has been demonstrated to bind to cyclophilins and suppress HCV RNA replication by abolishing their interaction with NS5B polymerase.[40] This CsA binding to cyclophilins, especially cyclophilin A (CyPA), has been shown to result in the generation of ROS through inhibition of the peptidylprolyl-cis-trans-isomerase–like activity of CyPA.[41] Moreover, CyPA was reported to be secreted in response to oxidative stress,[42] and to bind to a cell surface receptor, CD147, followed by ERK1/2 activation.[43] These reports and our results suggest that CsA, acting as an oxidant, may trigger activation of the MEK–ERK1/2 signaling pathway, both directly by producing ROS by way of interaction with CyPA in the early phase, and indirectly by secreting CyPA in the late phase. Both activations could lead to an inhibition of HCV RNA replication. Thus, CyPA may play a critical role as an intermediator in the oxidative anti-HCV activity of CsA. In the latest study, CyPA was identified as the most essential cellular cofactor of HCV RNA replication among cyclophilins.[44] Further studies will be needed to clarify whether CyPA is required for the oxidative suppressive mechanism of anti-HCV nutrients/reagents other than CsA.

Although we expected that strong activation of the MEK–ERK1/2 signaling pathway would suppress HCV RNA replication, EGF exhibited only slight anti-HCV activity in OR6 cells. The promotion of cell growth by EGF might prevent its primary inhibitory effect on HCV RNA replication. A portion of the ERK1/2 phosphorylation by EGF was also reduced by treatment with VE (Fig. 6A), suggesting that EGF might stimulate the MEK–ERK1/2 signaling pathway, in part, as an oxidant, and that this oxidative activity of EGF could exhibit its slight anti-HCV activity.

In this study, using MEK1/2 specific inhibitors, we revealed that the MEK–ERK1/2 signaling pathway is involved in the oxidative antiviral mechanism of the anti-HCV nutrients BC, VD2, and PUFAs and the anti-HCV reagents IFN-γ and CsA. Our results suggest that this oxidative induction of the MEK–ERK1/2 signaling pathway could be a novel therapeutic strategy for the eradication of HCV infection. Although oxidants themselves cause liver damage, they may work as anti-HCV factors during therapy in patients with chronic hepatitis C.

In conclusion, this study suggests that the anti-HCV activity of oxidative stress is closely linked to the activation of the MEK–ERK1/2 signaling pathway.

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Efficient replication systems for hepatitis C virus using a new human hepatoma cell line

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ABSTRACT

Persistent hepatitis C virus (HCV) infection causes chronic liver diseases and is a serious global health problem. Cell culture-based persistent HCV RNA replication systems and infectious HCV production systems are widely used in HCV research. However, persistent HCV production systems have been developed only for HuH-7 hepatoma cells. Here we found a new human hepatoma cell line, Li23, that enables persistent HCV production and anti-HCV reagent assay. Li23's cDNA expression profile differed from HuH-7's, although the two cells had similar liver-specific expression profiles. We used HCV RNA with a specific combination of adaptive mutations to develop an HCV replicon system and genome-length HCV RNA replicating systems including a reporter assay system. Finally, Li23-derived cells persistently produced infectious virus of an HCV strain. Li23-derived cells are potentially useful for understanding the HCV life cycle and for finding antiviral targets.

1. Introduction

Hepatitis C virus (HCV) infection frequently causes active liver diseases such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Choo et al., 1989; Saito et al., 1990; Thomas, 2000). Although the combination of pegylated-interferon (PEG-IFN) and ribavirin is the standard therapy worldwide, only half of the patients receiving this treatment exhibit a sustained virological response (Chevaliez and Pawlotsky, 2007; Hadziyannis et al., 2004). Since more than 170 million people are infected with HCV worldwide, the virus remains a serious global health problem (Thomas, 2000). HCV is an enveloped positive single-stranded RNA virus of the *Flaviviridae* family. The HCV RNA genome encodes a large polyprotein precursor of approximately 3000 amino acids, which is cleaved into 10 proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Hijikata et al., 1991, 1993; Kato et al., 1990). Although many issues have been addressed since HCV was first identified, the lack of a virus culture system has long been a serious handicap in the fight against HCV infection (Kato and Shimotohno, 2000). The development of an HCV replicon system enabling HCV subgenomic RNA replication in HuH-7 human hepatoma cells has allowed the study of the mechanisms underlying HCV replication (Lohmann et al., 1999). After the first replicon of genotype 1b was developed, HCV replicons derived from several HCV strains appeared, and tissue, genotype, and host ranges were expanded (Ali et al., 2004; Date et al., 2004; Ikeda et al., 2002; Kato et al., 2003a,b; Kishine et al., 2002; Zhu et al., 2003). However, most of RNA replication systems using the culture cells other than HuH-7 cells have been fairly low-level. Furthermore, genome-length HCV RNA replication systems and drug assay systems have been developed (Blight et al., 2002; Ikeda et al., 2002, 2005; Mori et al., 2008; Pietschmann et al., 2002). To date, however, robust genome-length HCV RNA replication and anti-HCV reagent assays have been developed for only one human cell line, HuH-7 (Bartenschlager and Sparacio, 2007; Lindenbach and Rice, 2005). In 2005, an efficient virus production system using the JFH1 genotype 2a strain was developed using HuH-7-derived cell lines (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). In this system also, HuH-7 is still the only cell line that enables persistent HCV production without additional host factors such as CD81 (Gottwein and Bukh, 2008), although transient virus production in human hepatoma cell line LH86 was recently reported (Zhu et al., 2007). Furthermore, it is uncertain whether or not the recent advances obtained from the HuH-7 cell system reflect the general features of the HCV life cycle. Here, we found a new human hepatoma cell line, Li23, that enables robust genome-length

Abbreviations: HCV, hepatitis C virus; PEG-IFN, pegylated-interferon; E1, envelope 1; NS2, non-structural 2; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; p.i., post-infection; dsRNA, double-stranded RNA; EC_{50}, 50% effective concentration.

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HCV RNA replication. Using Li23-derived cell lines, we developed a novel drug assay system and a persistent HCV production system.

2. Materials and methods

2.1. Cell line

The Li23 cell line, established in 1987, consists of human hepatoma cells from a Japanese male (age 56) and was kindly provided by Drs. Y. Ishikawa and S. Hirohashi (National Cancer Center Research Institute, Tokyo). The Li23 cell line is free of both the hepatitis B virus antigen and HCV (Kato et al., 1995).

2.2. Cell culture

The six HuH-7-derived cell lines: sO cells, harboring the subgenomic replicon RNA of HCV-O (genotype 1b) (Kato et al., 2003a); O cells, harboring a replicative genome-length HCV-O RNA (Ikeda et al., 2005); Oc cured cells, which were created by eliminating HCV RNA from O cells by IFN treatment (Ikeda et al., 2005); OAc cured cells, which were created by eliminating HCV RNA from genome-length HCV-O RNA replicating OA cells (Abe et al., 2007); OR6 cells, harboring the genome-length HCV-O RNA with luciferase as a reporter (Ikeda et al., 2005); RSc cured cells that cell culture-generated HCV-JFH1 (HCVcc) (JFH1 strain of genotype 2a) (Wakita et al., 2005) could infect and efficiently replicate (Ariumi et al., 2007, 2008; Kuroki et al., 2009), or their parental HuH-7 cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The sO, O, and OR6 cells were maintained in the presence of G418 (0.3 mg/ml, Geneticin; Invitrogen). Li23 cells were maintained in modified culture medium for the PH5CH8 human immortalized hepatocyte cell line (Ikeda et al., 1998). The medium for Li23 cells consists of F12 medium and DMEM (1:1 in volume) supplemented with 1% FBS, epidermal growth factor (50 ng/ml), insulin (10 μg/ml), hydrocortison (0.36 μg/ml), transferrin (5 μg/ml), linoleic acid (5 μg/ml), selenium (20 ng/ml), prolactin (10 ng/ml), gentamycin (10 μg/ml), kanamycin monosulfate (0.2 mg/ml), and fungizone (0.5 μg/ml). Li23-derived cells harboring the HCV replicon or genome-length HCV RNA were cultured in the above medium supplemented with G418 (0.3 mg/ml). The cured Li23-derived cells were maintained in the above medium without G418. The HeLa and HEK293 cells were cultured in DMEM supplemented with 10% FBS.

2.3. Plasmid construction

To introduce the mutations into plasmid pON/3-5B, pON/C-5B, or pORN/C-5B (Ikeda et al., 2005) (GenBank accession no. AB191333; Supplemental Fig. S1), a PCR-based site-directed mutagenesis method was used as previously described (Abe et al., 2007; Mori et al., 2008).

2.4. RNA synthesis

Plasmid DNAs were linearized with XbaI and used for RNA synthesis with the T7 MEGAscript kit (Ambion) as previously described (Kato et al., 2003a). Synthesized RNA was purified by lithium chloride precipitation and dissolved in nuclease-free water.

2.5. RNA transfection and selection of G418-resistant cells

RNA was transfected to Li23 or Li23-derived cells as previously described (Lohmann et al., 1999). Cells were selected in complete medium with G418 (0.3 mg/ml) and sodium bicarbonate solution (0.15%) for 3 weeks as previously described (Kato et al., 2003a). For the staining of G418-resistant colonies, Coomassie brilliant blue (0.06% in 50% methanol–10% acetic acid) was used as previously described (Ikeda et al., 2005).

2.6. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting analysis with a PVDV membrane were performed as previously described (Kato et al., 2003a). The antibodies used for the O strain in this study were those against core (CP11; Institute of Immunology), E1 and E2 (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), NS3 (Novocastra Laboratories), NS4A and NS5A (a generous gift from Dr. A. Takamizawa, Research Foundation for Microbial Diseases, Osaka University), and NS5B (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science). The antibodies used for the JFH1 strain were those against core (CP11; Institute of Immunology) and NS5B (Murakami et al., 2008). β-Actin antibody (Sigma) was used as the control for the amount of protein loaded per lane. Immunocomplexes were detected by the Renaissance enhanced chemiluminescence assay (PerkinElmer Life Sciences).

2.7. Northern blot analysis

Total RNA from the cultured cells was prepared using an RNeasy extraction kit (Qiagen). Three micrograms of total RNA was used for the analysis. HCV-specific RNA and β-actin mRNA were detected according to a method described previously (Ikeda et al., 2005; Kato et al., 2003a). The synthetic RNA transcribed from pON/3-5B, pON/C-5B, or pORN/C-5B (10^7 and 10^8 genome equivalents spiked into cellular total RNA) was used to compare HCV RNA levels.

2.8. Quantification of HCV RNA

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis for HCV RNA was performed using a real-time LightCycler PCR as described previously (Ikeda et al., 2005). We used the following forward and reverse primer sets for the real-time LightCycler PCR: HCV-O, 5′-AGAGCCATAGTGGTCTGCGG-3′ (forward), 5′-CTTTCGCGACCCAACACTAC-3′ (reverse); and HCV-JFH1, 5′-AGAGCCATAGTGGTCTGCGG-3′ (forward), 5′-CTTTCGCAACCCAACGCTAC-3′ (reverse). Experiments were done in triplicate.

2.9. Preparation of cured cells

To prepare cured cells, the cells harboring the HCV replicon or genome-length HCV RNA were treated with IFN-γ as described previously (Abe et al., 2007). Briefly, the cells were treated with IFN-γ (1000 IU/ml) in the absence of G418. The treatment was continued for 3 weeks with the addition of IFN-γ at 4-day intervals. The cured cells obtained from O, OA, sOL, OL8, OL11, OL14, ORL8, and ORL11 cells were named Oc, OAc, sOLc, OL8c, OL11c, OL14c, ORL8c, and ORL11c, respectively. RT-PCR confirmed the absence of HCV RNA in these cured cells.

2.10. Immunofluorescence and confocal microscopic analyses

Four days after the cells were seeded on the collagen-coated coverslip, they were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) and then permeabilized in 0.1% Triton X-100 in PBS at room temperature. After blocking with 1% bovine serum albumin, the cells were incubated with the primary antibodies and then with the secondary antibody. The primary antibodies used to detect the core and dsRNA were anti-core (CP11; Institute of Immunology) and anti-double-stranded (ds) RNA (K1; English and

Scientific Consulting), respectively. The primary antibodies used to detect NS5B of O strain and JFH1 strain were anti-NS5B (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science) and anti-NS5B (Murakami et al., 2008), respectively. The secondary antibody was Cy2-conjugated anti-mouse secondary antibody or FITC-conjugated anti-rabbit secondary antibody (for NS5B of JFH1) (Jackson ImmunoResearch). The nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma). The coverslips were mounted on glass slides by PermaFluor Aqueous Mountant (ThermoFisher) and then the cells were photographed under a confocal laser scanning microscope (LSM510; Carl Zeiss).

2.11. cDNA microarray analysis

HuH-7, Oc, OAc, Li23, OL8, OL11, OL8c, and OL11c cells (1×10^6 each) were plated onto 10-cm diameter dishes and cultured for 2 days in the absence of G418. Total RNAs from these cells were prepared using the RNeasy extraction kit (Qiagen). cDNA microarray analysis was performed by Dragon Genomics Center of Takara Bio. (Otsu, Japan) through an authorized Affymetrix service provider. cDNA was synthesized by the GeneChip T7-Oligo(dT) Promoter Primer Kit (Affymetrix) and TaKaRa cDNA Synthesis Kit (Takara Bio) from 3 μg total RNA. Biotinylated complementary RNA (cRNA) was synthesized by the IVT Labeling Kit (Affymetrix). Following fragmentation, 10 μg of cRNA was hybridized for 16 h at 45 °C on the GeneChip Human Genome U133 Plus 2.0 Array. GeneChips were washed and stained in the Affymetrix Fluidics Station 450, and then were scanned using GeneChip Scanner 3000 7G. Single array analysis was calculated by Microarray Suite version 5.0 (MAS5.0) with the Affymetrix setting. Differentially expressed genes were selected by comparing HuH-7-derived cells and Li23-derived cells.

2.12. RT-PCR

RT-PCR was performed to detect cellular mRNA as described previously (Dansako et al., 2003). Briefly, total RNA (2 μg) was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen) using an oligo dT primer (Invitrogen) according to the manufacturer's protocol. One-tenth of synthesized cDNA was used for PCR. The primers and PCR cycles used in this study are listed in Supplemental Table 1.

2.13. Quantification of HCV core protein

The levels of the core protein in the culture supernatants were determined by enzyme-linked immunosorbent assay (Mitsubishi Kagaku Bio-Clinical Laboratories).

2.14. Renilla luciferase assay for anti-HCV reagents

To monitor the effects of anti-HCV reagents, the cells were plated onto 24-well plates (2×10^4 cells per well) and cultured with the medium for Li23-derived cells in the absence of fungizone and G418 for 24 h. The cells were then treated with anti-HCV reagent at several concentrations for 72 h (sometimes 24 or 48 h), or the cells were treated with a combination of IFN-α and another anti-HCV reagent at several concentrations for 72 h. After treatment, the cells were subjected to luciferase assay using the renilla luciferase assay system according to the manufacturer's protocol (Promega). A manual Lumat LB 9501/16 luminometer (EG&G Berthold) was used to detect luciferase activity. The experiments were performed in at least triplicate.

2.15. Cell viability

To examine the cytotoxic effects of anti-HCV reagents on the cells, the cells were plated onto 24-well plates (2×10^4 cells per well) and cultured for 24 h. They were then treated with or without anti-HCV reagents for 72 h in the absence of G418. The viable cells were then counted in an improved Neubauer-type hemotocytometer after Trypan blue dye (Invitrogen) treatment. The experiments were performed in triplicate.

2.16. Infection of cells with secreted HCV

The inoculum for HCV infection was the culture medium of RSc cells (Ariumi et al., 2007, 2008; Kuroki et al., 2009) at 145 days after transfection with JFH1 RNA *in vitro* synthesized from pJFH1 (Wakita et al., 2005). This inoculum was passed through a 0.2-μm filter after low-speed centrifugation before use for infection. We seeded cells 24 h before infection at a density of 2×10^4 cells per well in a 24-well plate. We infected cells with 100 μl (equivalent to $10^{4.3}$ $TCID_{50}$) of inoculum for 2 h, washed them, added complete medium and cultured them for a maximum of 30 days with adequate passage of the cells. In some cases, at 7 or 8 days p.i., supernatant was used as an inoculum for the next HCV infection. The cells at 7 or 14 days p.i. were used to detect HCV proteins by Western blot analysis, to quantify HCV RNA by quantitative RT-PCR or to analyze the immunofluorescence of HCV proteins or dsRNA.

3. Results

3.1. Efficient replication system with HCV replicon or genome-length HCV RNA using human hepatoma Li23 cells

We previously established several genome-length HCV RNA (O strain of genotype 1b) replicating cell lines and found that a specific combination of adaptive mutations – either Q1112R, P1115L, and S2200R (QR,PL,SR) or Q1112R, K1609E, and S2200R (QR,KE,SR) – drastically enhanced the level of genome-length HCV RNA replication (Abe et al., 2007; Ikeda et al., 2005). This finding led us to hypothesize that such combinations of adaptive mutations may overcome the barrier that has made HuH-7 the only cell line thus far to allow the robust replication of genome-length HCV RNA. To test this hypothesis, HCV replicon RNA (ON/3-5B) possessing QR,PL,SR or QR,KE,SR (Supplemental Fig. 1) was transfected into various kinds of human cell lines (HuH-6, Li21, Li23, Li24, PH5CH, OUMS29, IHH10.3, IHH12 etc.), and the G418 selection was performed as described previously (Kato et al., 2003a). Although we failed to obtain the G418-resistant colonies in the most cell lines, fortunately, we found that the Li23 human hepatoma cell line gave only G418-resistant colonies (Fig. 1A). Approximately 200 and 700 colonies obtained from ON/3-5B/QR,PL,SR and ON/3-5B/QR,KE,SR-transfected cells, respectively, were pooled. Western blot analysis revealed that the expression levels of HCV proteins NS5A and NS5B were much higher in ON/3-5B/QR,KE,SR-derived colonies than in ON/3-5B/QR,PL,SR-derived colonies (Fig. 1B). We used the former for further analysis and referred to them as sOL cells. We demonstrated that the replicon in sOL cells showed a high level of sensitivity to anti-HCV reagents, similar to the level shown by the replicon (ON/3-5B/SR) in sO cells (Kato et al., 2003a) (Fig. 1C).

To obtain a source of cells with which to develop a genome-length HCV RNA replication system, we prepared cured cells (sOLc) from sOL cells by IFN-γ treatment, because cured cells are known to extremely enhance HCV RNA replication levels (Ikeda et al., 2005; Kato et al., 2003a). A genome-length HCV RNA (ON/C-5B/QR,KE,SR; Supplemental Fig. 1) was transfected into sOLc cells. Following G418 selection, many colonies were obtained (Fig. 2A). Fourteen

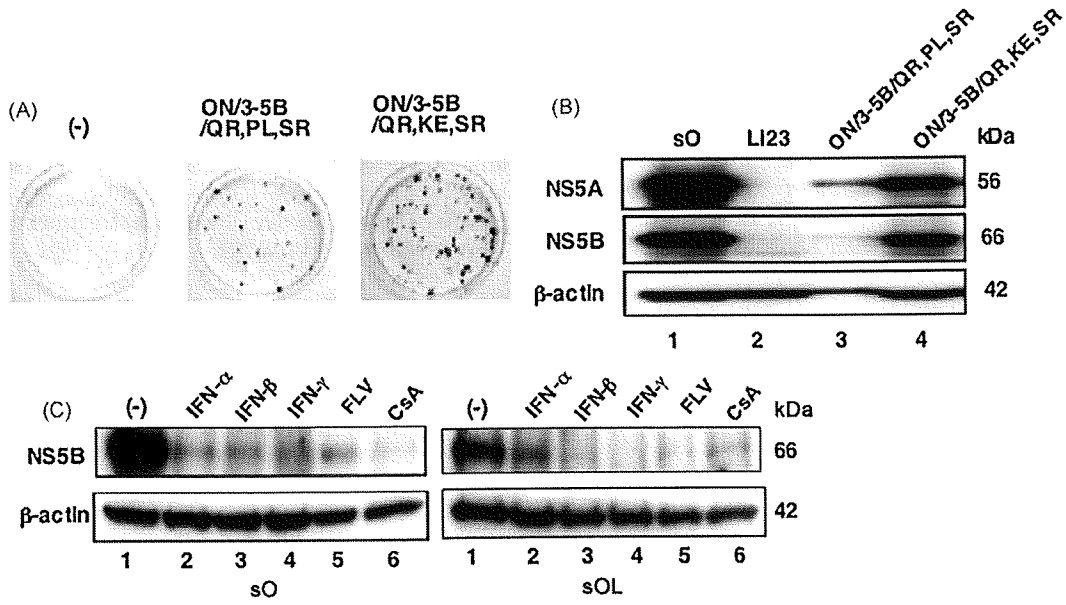

Fig. 1. Li23-derived cells harboring HCV replicon. (A) G418-resistant colonies from Li23 cells transfected with replicon RNA. ON/3-5B RNA with three additional mutations (ON/3-5B/QR,PL,SR or ON/3-5B/QR,KE,SR) was transfected into Li23 cells. The panels show G418-resistant colonies (57 colonies/μg RNA for ON/3-5B/QR,PL,SR and 132 colonies/μg RNA for ON/3-5B/QR,KE,SR) that were stained with Coomassie brilliant blue at 3 weeks after RNA transfection. (B) Western blot analysis of Li23-derived G418-resistant cells for HCV proteins NS5A and NS5B. Lane 1, sO (HuH-7-derived cell line harboring HCV replicon, ON/3-5B/SR); lane 2, Li23 as a negative control; lane 3, polyclonal G418-resistant cells obtained by transfection with ON/3-5B/QR,PL,SR RNA; lane 4, polyclonal G418-resistant cells (sOL) by transfection with ON/3-5B/QR,KE,SR RNA. (C) Sensitivity of sOL replicon to anti-HCV reagents. sOL cells were treated with IFN-α (lane 2, 20 IU/ml), IFN-β (lane 3, 20 IU/ml), IFN-γ (lane 4, 20 IU/ml), fluvastatin (FLV) (lane 5, 5 μM), or cyclosporine A (CsA) (lane 6, 0.5 μg/ml) for 5 days. Lane 1 shows no treatment. For comparison, sO cells were treated as well as sOL cells. NS5B was detected by Western blot analysis.

colonies (referred to as OL1–OL14) and a mixture of approximately 200 other colonies (referred to as OL) were successfully proliferated as cell lines. Using quantitative RT-PCR, we selected OL8, OL11, and OL14 because of their high levels (>9×10^6 copies/μg total RNA) of HCV RNA, although the titer of HCV RNA from genome-length HCV RNA replicating HuH-7-derived O cells (Ikeda et al., 2005) was approximately 4.5×10^7 copies/μg total RNA (Supplemental Fig. 2). We also demonstrated that the HCV sequence was not

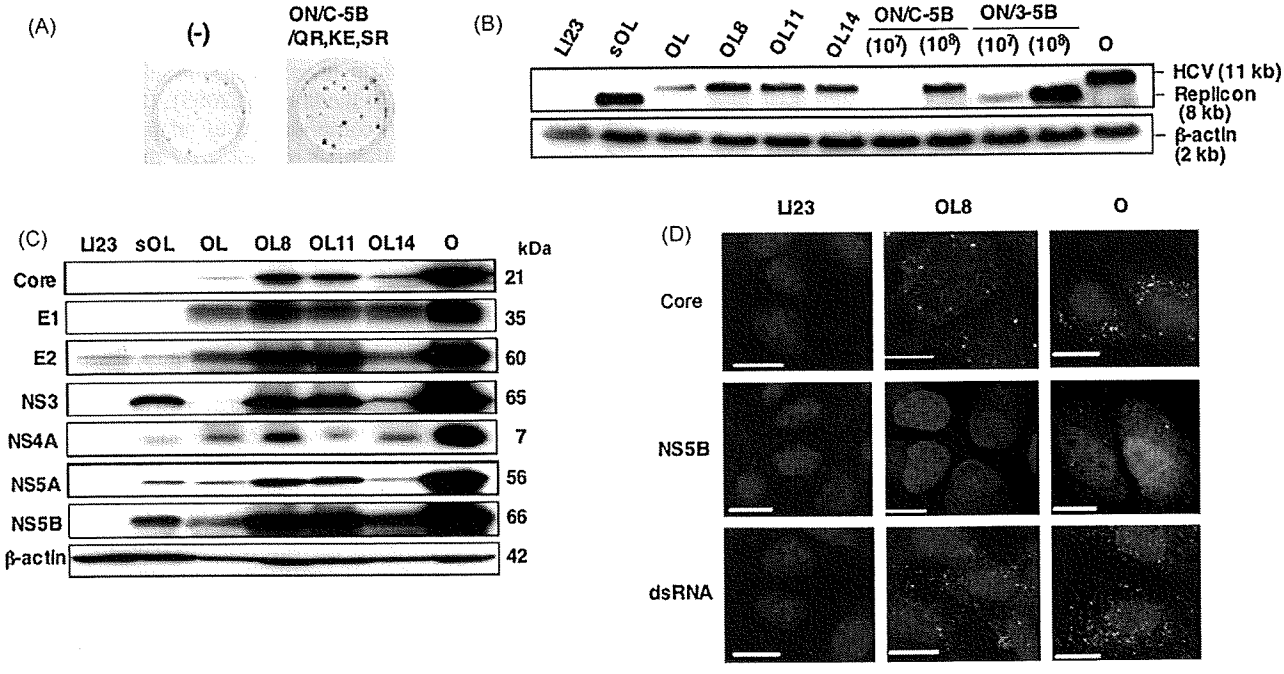

Fig. 2. Establishment of Li23-derived cell lines harboring replicative genome-length HCV RNA. (A) G418-resistant colonies from sOLc cells transfected with genome-length HCV RNA (ON/C-5B/QR,KE,SR). The panels show G418-resistant colonies (100 colonies/μg RNA) that were stained with Coomassie brilliant blue at 3 weeks after RNA transfection. (B) Northern blot analysis of total RNA prepared from sOL cells and genome-length HCV RNA-replicating cells (OL, OL8, OL11, and OL14). Synthetic RNA, given number of ON/C-5B or ON/3-5B RNA. HuH-7-derived O cells harboring replicative genome-length HCV RNA (ON/C-5B/KE,SR) and Li23 cells served as positive and negative controls, respectively. (C) Western blot analysis of sOL and genome-length HCV RNA-replicating cells (OL, OL8, OL11, and OL14) for HCV proteins, core, E1, E2, NS3, NS4A, NS5A, and NS5B. O cells and Li23 cells served as positive and negative controls, respectively. (D) Immunofluorescence analysis of OL8 cells. The cells were processed and stained with anti-core, anti-NS5B, or anti-dsRNA antibodies and Cy2-conjugated secondary antibody. The O cells and Li23 cells served as positive and negative controls, respectively. Bar, 20 μm.

integrated into the genomic DNA in OL, OL8, OL11, OL14, or sOL cells (data not shown). Northern and Western blot analyses also showed that the levels of HCV RNA and proteins in OL8 and OL11 cells were somewhat lower than those in O cells (Fig. 2B and C). Immunofluorescence analysis of the intracellular localization of HCV proteins and dsRNA, which is an intermediate of RNA replication, showed that the staining levels of HCV proteins and dsRNA located in the cytoplasm of OL8 cells, were also comparable to those in O cells (Fig. 2D). Both OL8 and O cells had two types of core protein staining patterns (detergent-resistant dots or patches and detergent-sensitive ring-like structures), as described previously (Matto et al., 2004) in HuH-7 cells harboring the genome-length HCV RNA (Con1 strain of genotype 1b) (Fig. 2D). These results suggest that robust replication of genome-length HCV RNA occurs in OL8 and OL11 cells. We performed sequence analysis of HCV RNAs derived from OL8, OL11, and OL14 cells, but no additional mutations were detected commonly among the three independent clones sequenced (data not shown). This suggested that no mutations other than Q1112R, K1609E, and S2200R are needed for genome-length HCV RNA replication in Li23-derived cells.

3.2. Genes differentially expressed between Li23- and HuH-7-derived cells

RT-PCR analysis revealed that Li23 and HuH-7 cells had similar liver-specific gene expression profiles (Fig. 3A). However, there is no information regarding the Li23-specific gene expression profile. To address this, we performed cDNA microarray analysis using total RNAs prepared from Li23, OL8, OL11, cured OL8 (OL8c), and OL11c cells in addition to HuH-7, Oc (Ikeda et al., 2005), and OAc (Abe et al., 2007). As the first step in this analysis, we selected 206 and 326 genes whose expression levels were upregulated and downregulated at ratios of more than 2^5 and less than 2^{-5} in Li23 vs. HuH-7, respectively. Then, from among those selected in the first step, we performed an additional selection of genes whose expression levels were commonly upregulated or downregulated among Li23-derived cells when compared with HuH-7-derived cells, and each of several already-known genes were identified (data not shown). Fig. 3B shows the results of RT-PCR regarding the representative genes belonging to such a category in the expression levels between Li23- and HuH-7-derived cells. The most characteristic feature of Li23-derived cells was the high expression levels of cancer antigens (NY-ESO-1, MAGEA, etc.) compared with no expression in HuH-7-derived cells (Fig. 3B). We demonstrated that such drastic differences were not attributable to differences in culture media (Supplemental Fig. 3). These results exclude the possibility that OL8 and OL11 cells are derived from contamination of HuH-7-derived cells. On the other hand, this microarray analysis revealed that HuH-7- and Li23-derived cells showed similar expression levels of CD81, scavenger receptor class B type I (SR-BI), Claudin-1, and Occludin, which have been identified as the host factors for HCV entry (Burlone and Budkowska, 2009). RT-PCR analysis confirmed them (Fig. 3C).

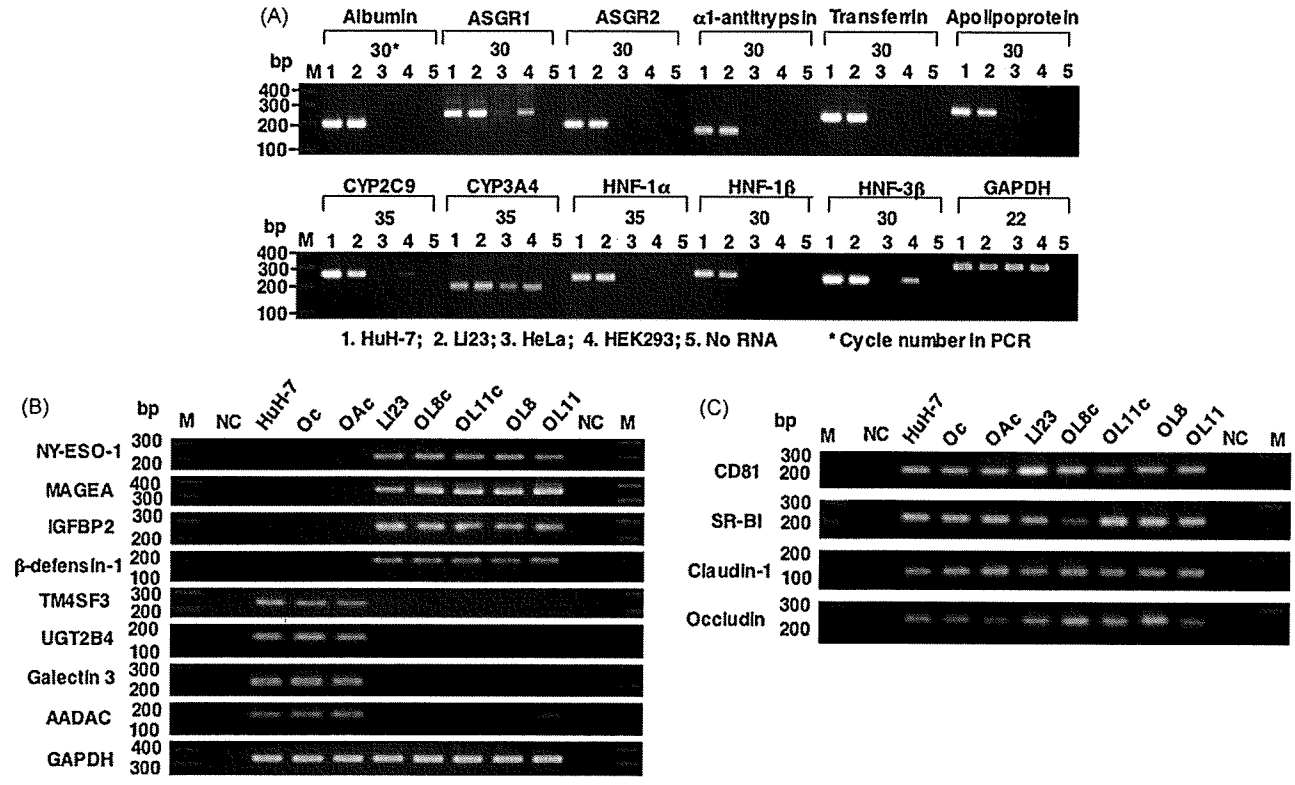

Fig. 3. Representative genes differentially expressed among Li23- and HuH-7-derived cells. (A) Li23 and HuH-7 cells showed similar liver-specific gene expression profiles. Total RNAs prepared from HuH-7, Li23, HeLa, and HEK293 cells were subjected to RT-PCR to detect liver-specific mRNAs using the primer sets listed in Supplementary Table 1. Presented data are the results of the following mRNA species: albumin, asialoglycoprotein receptor 1 (ASGR1), ASGR2, α1-antitrypsin, transferrin, apolipoprotein, cytochrome P450 2C9 (CYP2C9), CYP3A4, hepatocyte nuclear factor 1α (HNF-1α), HNF-1β, and HNF-3β. (B) Representative genes that were differentially expressed between HuH-7-derived cell lines and Li23-derived cell lines. Total RNAs prepared from HuH-7-derived cells (HuH-7, Oc, and OAc) and Li23-derived cells (Li23, OL8c, OL11c, OL8, and OL11) were subjected to RT-PCR using the primer sets listed in Supplementary Table S1. Lane M, 100 bp DNA ladder; NC, no RNA. The data presented are the results of the following mRNA species: cancer testis antigen (NY-ESO-1), melanoma-specific antigen family A (MAGEA), insulin-like growth factor binding protein 2 (IGFBP2), β-defensin-1, transmembrane 4 superfamily member 3 (TM4SF3), UDP glycosyltransferase 2 family polypeptide B4 (UGT2B4), galectin 3, and arylacetamide deacetylase (AADAC). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. (C) Expression levels of CD81, SR-BI, Claudin-1, and Occludin between HuH-7- and Li23-derived cells. RNA preparation and RT-PCR were performed as described in (B).

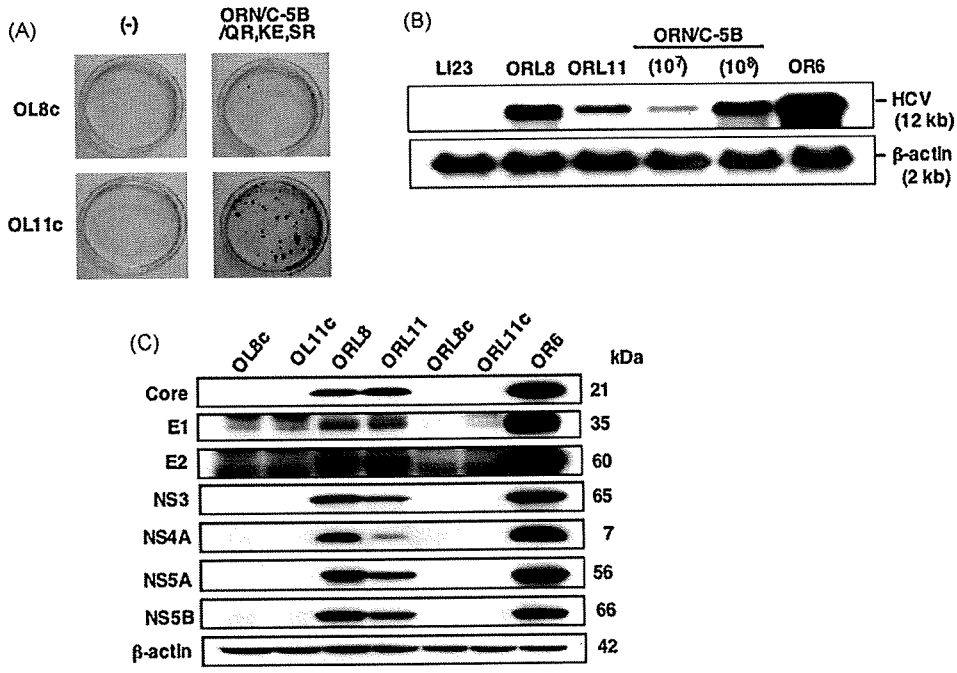

Fig. 4. Establishment of OL8- and OL11-derived cell lines harboring replicative genome-length HCV RNA encoding renilla luciferase. (A) G418-resistant colonies from OL8c or OL11c cells transfected with genome-length HCV RNA (ORN/C-5B/QR,KE,SR) encoding renilla luciferase gene. The panels show G418-resistant colonies that were stained as described in Fig. 1A. (B) Northern blot analysis of total RNA prepared from genome-length HCV RNA replicating ORL8 and ORL11 cells. Synthetic RNA, given number of synthetic ORN/C-5B RNA; Li23, negative control. HuH-7-derived OR6 cells replicating genome-length HCV RNA encoding renilla luciferase gene (ORN/C-5B/KE,SR) served as positive control. (C) Western blot analysis of ORL8 and ORL11 cells for HCV proteins, core, E1, E2, NS3, NS4A, NS5A, and NS5B. OL8c, OL11c, ORL8c, and ORL11c, negative controls; OR6, positive control.

3.3. *Development of new luciferase reporter assay systems that facilitate the quantitative monitoring of HCV RNA replication*

Since the reporter assay system using HuH-7-derived OR6 cells, which robustly replicates genome-length HCV RNA encoding renilla luciferase, is potentially useful for the quantitative evaluation of anti-HCV activity (Ikeda et al., 2005, 2006; Ikeda and Kato, 2007), we have tried to develop an Li23-derived assay system corresponding to the OR6 assay system. A genome-length HCV RNA encoding renilla luciferase (ORN/C-5B/QR,KE,SR) (Supplemental Fig. 1) was transfected into OL8c or OL11c cells. Following G418 selection, several OL8c colonies and several hundred OL11c colonies were obtained from the cells transfected with ORN/C-5B/QR,KE,SR (Fig. 4A). Regarding ORN/C-5B/QR,KE,SR, 9 OL8c-derived clones and 16 OL11c-derived clones were successfully proliferated as cell lines. Each clone possessing the highest

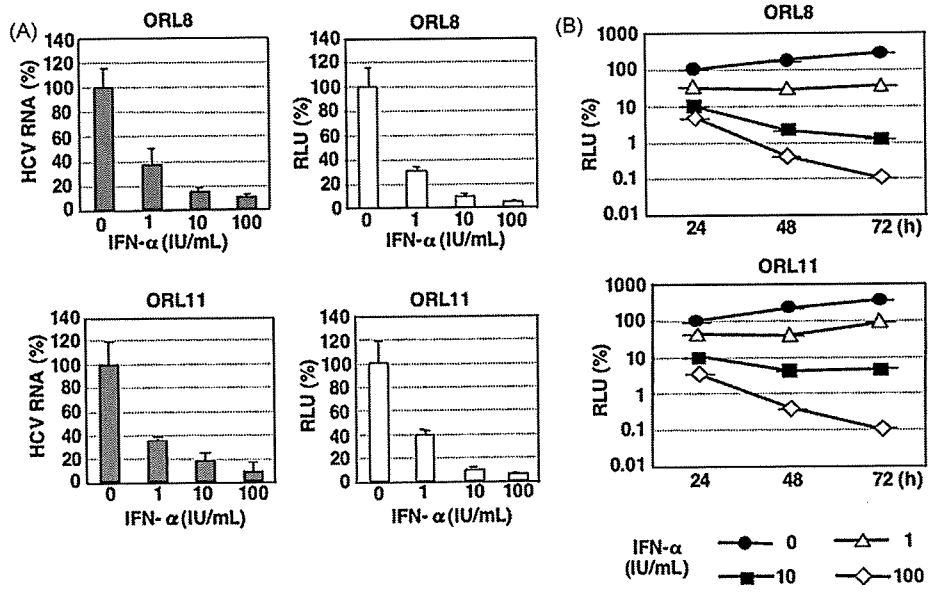

Fig. 5. ORL8 and ORL11 reporter assay system to monitor genome-length HCV RNA replication. (A) Renilla luciferase activity is correlated with HCV RNA level. The ORL8 (upper panels) and ORL11 (lower panels) cells were treated with IFN-α (0, 1, 10, and 100 IU/ml) for 24 h, and then luciferase reporter assay (right panels) and quantitative RT-PCR (left panels) were performed. The relative luciferase activity (RLU) (%) or HCV RNA (%) calculated at each point, when the level of luciferase activity or HCV RNA in non-treated cells was assigned to be 100%, is presented here. (B) IFN-α sensitivity of HCV RNA replication in ORL8 and ORL11 cells. The ORL8 (upper panel) and ORL11 (lower panel) cells were treated with IFN-α (0, 1, 10, and 100 IU/ml); the luciferase assay was performed at 24, 48, and 72 h after the treatment. The RLU (%) calculated at each point, when the luciferase activity of non-treated cells at 24 h was assigned to be 100%, is presented here. The experiments were performed in at least triplicate.

titer of HCV RNA was selected by quantitative RT-PCR and was thereafter referred to as ORL8 and ORL11 (data not shown). We demonstrated that the HCV RNA sequence was not integrated into the genomic DNA in ORL8 or ORL11 cells (data not shown). Northern and Western blot analyses showed that ORL8 and ORL11 cells expressed sufficient levels of HCV RNA and proteins for the quantitative monitoring of HCV RNA replication, although these levels were somewhat lower than those in OR6 cells (Fig. 4B and C). We performed sequence analysis of HCV RNAs derived from ORL8 and ORL11 cells, but no additional mutations were detected commonly among the three independent clones sequenced (data not shown). We demonstrated good correlations between the levels of luciferase activity and HCV RNA in ORL8 and ORL11 cells (Fig. 5A), as we previously demonstrated in OR6 cells treated with IFN-α for 24 h (Ikeda et al., 2005). Time course assays (24, 48, and 72 h) on IFN-α treatment demonstrated that the luciferase activity decreased in a dose- and time-dependent manner, and revealed that the luciferase activity had decreased to less than 0.1% at 72 h after treatment with 100 IU/ml IFN-α (Fig. 5B).

3.4. ORL8 and ORL11 assay systems are frequently more sensitive than the OR6 assay system

Using ORL8 and ORL11 assay systems, we evaluated the anti-HCV activities of representative reagents identified by HuH-7-derived assay systems (Ikeda and Kato, 2007; Moriishi and Matsuura, 2007). For the sake of comparison, we also evaluated these activities using the OR6 assay system along with the same culture medium that we used for the ORL8 and ORL11 assays, since we had already confirmed that HCV RNA in OR6 cells was efficiently replicated using this culture medium (data not shown). First, we measured the 50% effective concentration (EC_{50}) of IFN-α against HCV RNA replication. The EC_{50} values of IFN-α in ORL8, ORL11, and OR6 assays were assigned as 0.13, 0.30, and 0.40 IU/ml, respectively, without suppression of cell growth (Fig. 6A). Regarding IFN-β, IFN-γ, and cyclosporine A also, the ORL8 and ORL11 assays were each more sensitive than the OR6 assay (Fig. 6B). It is noteworthy that the EC_{50} values of fluvastatin and simvastatin in the ORL8 and ORL11 assays were fairly lower than those in the OR6 assay (Fig. 6B). In contrast, we observed that the OR6 assay for geldanamycin was slightly more sensitive than the ORL8 or ORL11 assay (Fig. 6B). When the number of cells without treatment was compared to that of cells with treatment, no significant decrease in cell number was observed following treatment with anti-HCV reagents used in Fig. 6B (data not shown). Co-treatment of IFN-α and fluvastatin also demonstrated that the ORL8 and ORL11 assays were much more sensitive than the OR6 assay (Fig. 6C), indicating that these two systems are powerful biosensors of RNA viral replication.

3.5. Persistent reproduction of HCV life cycle in Li23-derived cells

A most interesting point is whether or not infectious HCV is produced in Li23-derived cell lines and thus enables robust HCV RNA replication. To clarify this point, we used HCV-JFH1 (genotype 2a), the only infectious HCV molecular clone identified in a cell culture to date (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al.,

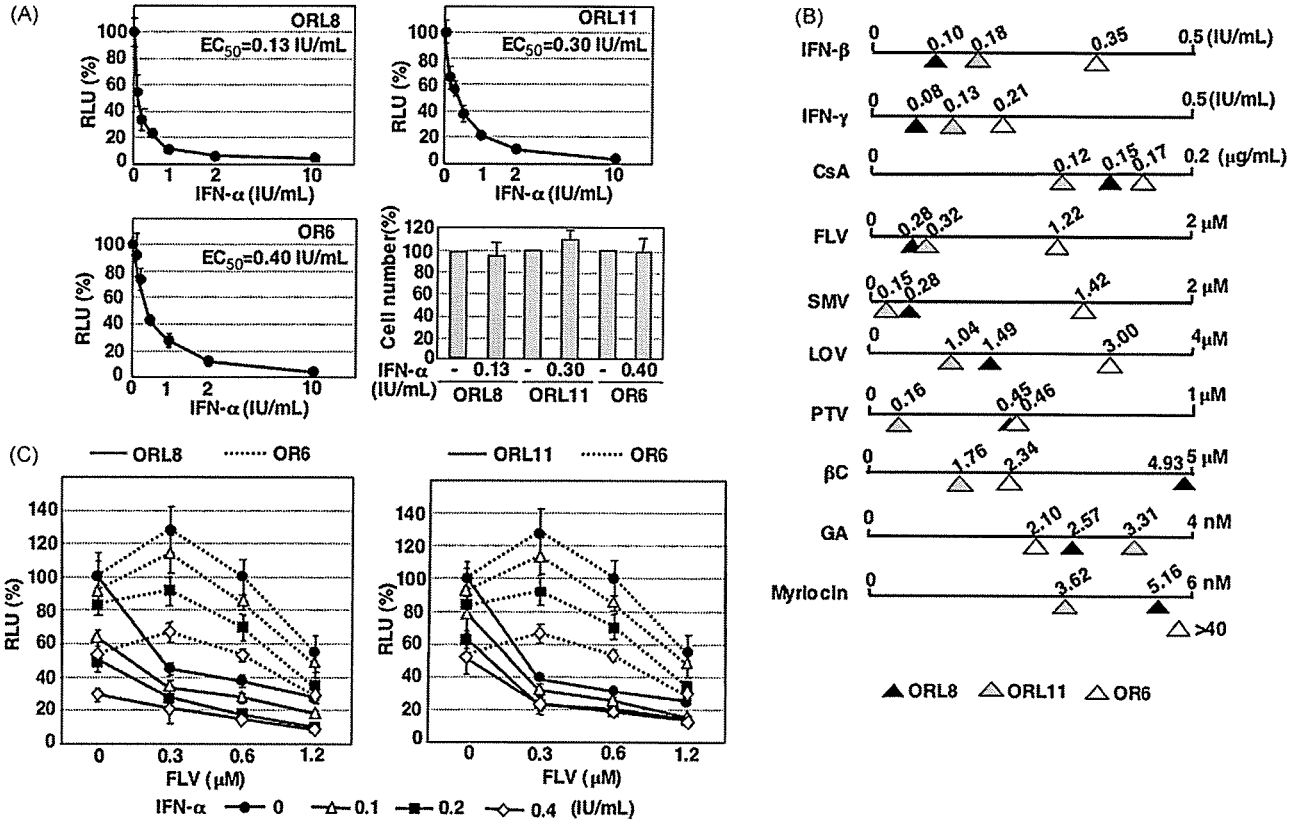

Fig. 6. The diverse effects of anti-HCV reagents in ORL8, ORL11, and OR6 assay systems. (A) IFN-α sensitivities on genome-length HCV RNA replication in ORL8, ORL11, and OR6 assay systems. The ORL8, ORL11, and OR6 cells were treated with IFN-α (0, 0.06, 0.13, 0.25, 0.5, 1, 2, and 10 IU/ml) for 72 h, and then luciferase assay was performed as described in Fig. 5A. ORL8, ORL11, and OR6 cells were cultured in the absence or presence of IFN-α at each 50% effective concentration (EC_{50}) for 72 h, and then the cells were counted as described in Section 2. (B) Diverse EC_{50} values of anti-HCV reagents on genome-length HCV RNA replication in ORL8, ORL11, and OR6 cells. ORL8, ORL11, and OR6 cells were treated with several different concentrations of IFN-β, IFN-γ, CsA, FLV, simvastatin (SMV), lovastatin (LOV), pitavastatin (PTV), β-carotene (βC), geldanamycin (GA), or myriocin for 72 h, after which luciferase assay was performed as described in Fig. 5A. EC_{50} values were calculated from the data of each triplicate assay. (C) ORL8 and ORL11 assay systems are more sensitive than the OR6 assay system in the combination analysis of IFN-α and FLV. ORL8, ORL11, and OR6 cells were treated with a combination of IFN-α (0, 0.1, 0.2, and 0.4 IU/ml) and FLV (0, 0.3, 0.6, and 1.2 μM) for 72 h, after which a luciferase assay was performed as described in Fig. 5A.

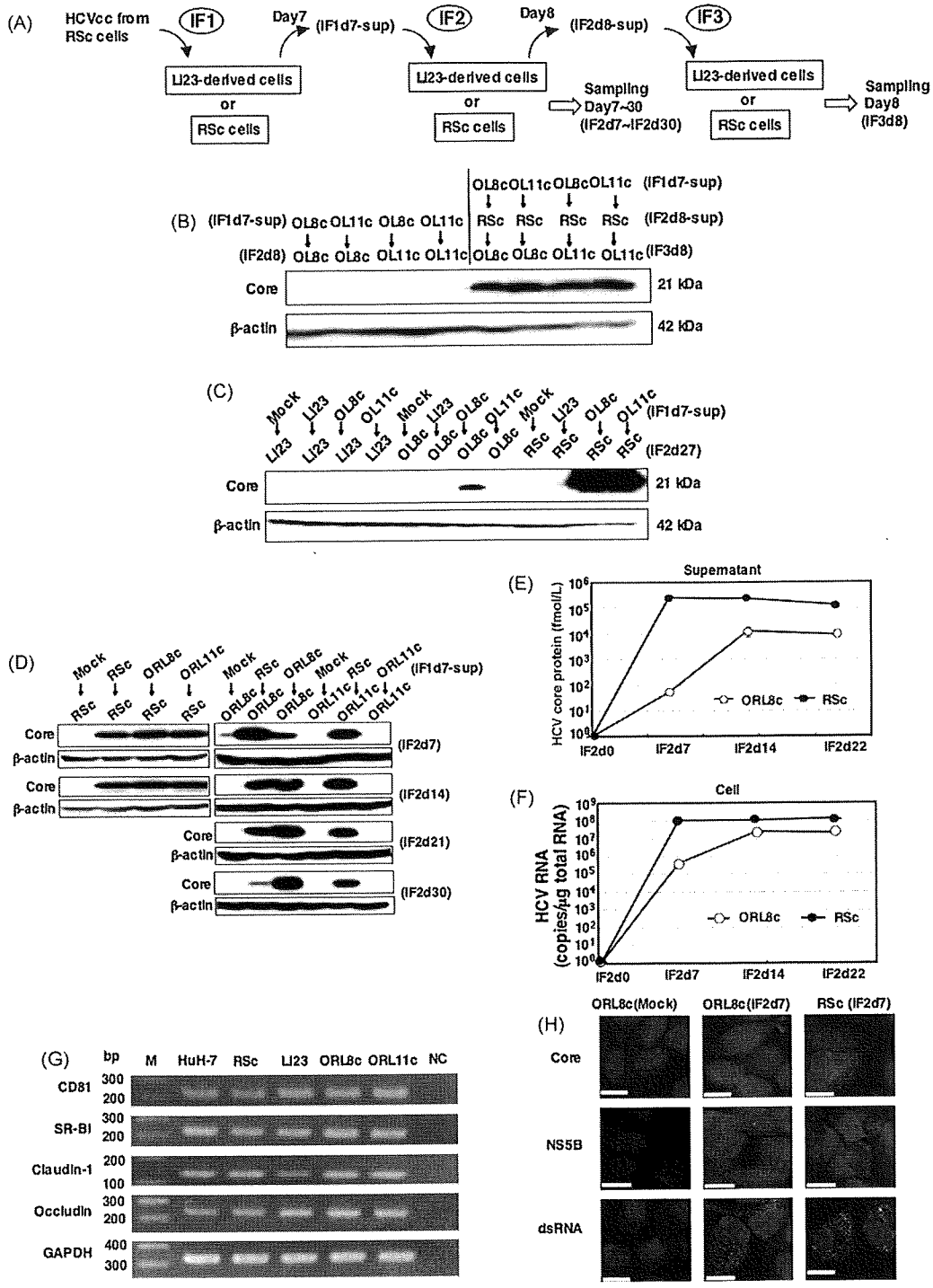

Fig. 7. Reproduction of HCV life cycle in OL8c and ORL8c cells. (A) Flow chart of experiments on HCVcc infection. A time schedule for HCVcc infection and sampling is shown. IF1d7-sup refers to the supernatant of the first-infected cells at 7 days p.i. IF2d7 refers to the secondly infected cells at 7 days p.i. (B) Production of infectious HCV from HCVcc-infected OL8c and OL11c cells. OL8c or OL11c cells were infected with HCVcc produced from RSc cells, and each supernatant at 7 days p.i. (IF1d7-sup) was used for inoculation to OL8c, OL11c, or RSc cells. Western blot analysis was performed to detect HCV core protein in OL8c or OL11c cells at 8 days p.i. (IF2d8). The supernatant (IF2d8-sup) from RSc cells at 8 days p.i. was used for further inoculation to OL8c or OL11c cells. Western blot analysis was performed for the detection of HCV core protein in OL8c or OL11c cells at 8 days p.i. (IF3d8). (C) HCVcc produced from OL8c cells is infectious to OL8c cells. The supernatants (IF1d7-sup) described in (B) were used for inoculation to OL8c and RSc cells. Li23 cells were used as negative controls. Western blot analysis was performed to detect HCV core protein in OL8c, RSc, or Li23 cells at 27 days p.i. (IF2d27). (D) Persistent production of infectious HCVcc from ORL8c cells. ORL8c or ORL11c cells were infected with HCVcc produced from RSc cells, and each supernatant at 7 days p.i. (IF1d7-sup) was used for inoculation to ORL8c or ORL11c cells. RSc cells were used as positive controls. Western blot analysis was performed to detect HCV core protein in ORL8c or ORL11c cells at 7, 14, 21, and 30 days p.i. (IF2). HCV core protein in RSc cells at 7 and 14 days p.i. was also detected by Western blot analysis. (E) Secretion of HCV core protein in culture supernatant. The culture supernatants (IF2d7-, IF2d14-, and IF2d22-sup) of ORL8c or RSc cells were used to determine the levels of core protein by enzyme-linked immunosorbent assay. Experiments were performed in triplicate. (F) Levels of HCV RNA in HCVcc-infected cells. The levels of intracellular HCV RNAs of ORL8c or RSc cells (IF2d7, IF2d14, and IF2d22) were determined by quantitative RT-PCR. Experiments were performed in triplicate. (G) Expression levels of CD81, SR-BI, Claudin-1, and Occludin among RSc, ORL8c, and ORL11c cells. RNA preparation and RT-PCR were performed as described in Fig. 3B. (H) Immunofluorescence analysis of HCVcc-infected cells. ORL8c or RSc cells (IF2d7) were processed and stained with anti-core, anti-NS5B, and anti-dsRNA antibodies and Cy2-conjugated secondary antibody. Mock-infected ORL8c cells served as negative controls. Bar, 20 μm.

2005), and various Li23-derived cell lines obtained in this study (Supplemental Table 2). A flow chart of the expression procedure is shown in Fig. 7A.

Since we detected the transient expression of HCV-JFH1 RNA in OL8c and OL11c cells (Supplemental Fig. 4A), we examined the susceptibility of OL1c, OL2c, OL3c, OL4c, OL8c, OL11c, or OL14c cells to cell culture-generated HCV-JFH1 (HCVcc) produced from HuH-7-derived RSc cells that HCVcc could infect and efficiently replicate (Ariumi et al., 2007, 2008; Kuroki et al., 2009). At 16 days post-infection (p.i.), the core protein was detected in OL2c, OL3c, OL8c, OL11c, and OL14c cells, but not in OL1c and OL4c cells (Supplemental Fig. 4B), indicating that most OLc series cells exhibit good susceptibility to HCVcc. In this context, the supernatant (IF1d7-sup) of HCVcc-infected OL8c or OL11c cells at 7 days p.i. was inoculated to naïve OL8c or OL11c cells; however, we failed to detect the core protein in the cells (IF2d8) at 8 days p.i. (Fig. 7B). However, when the supernatant (IF2d8-sup) of IF1d7-sup-inoculated RSc cells at 8 days p.i. was inoculated to naïve OL8c or OL11c cells, core expression was strongly detected in either case at 8 days p.i. (IF3d8) (Fig. 7B). This suggested that small amount of infectious HCV was produced from OL8c or OL11c cells. Accordingly, the expression of core protein was detected in IF1d7-sup-inoculated OL8c cells, but not in OL11c cells, at 27 days p.i. (IF2d27) (Fig. 7C), indicating that OL8c-derived HCVcc may infect and replicate in naïve OL8c cells. This finding leads to the assumption that ORL8c or ORL11c cells are better than OL8c cells, because ORL8c and ORL11c cells are derived from luciferase reporter full-length HCV RNA-replicating cells (ORL8 and ORL11), and because they each have a more permissible environment for HCV RNA replication. To test this hypothesis, RSc-derived HCVcc was commonly used in order to avoid the issue of uncertain efficiency of RNA transfection to RSc, ORL8c, or ORL11c cells. RSc-derived HCVcc was inoculated to naïve RSc, ORL8c, or ORL11c cells; and RSc, ORL8c, or ORL11c-derived IF1d7-sup was further inoculated to naïve RSc, ORL8c, or ORL11c cells. Expectedly, core expression in the ORL8c cells inoculated with ORL8c-derived IF1d7-sup was strongly detected until at least 30 days p.i. (IF2d30) (Fig. 7D). The level of core protein in the ORL8c cells was equivalent to that in the RSc cells inoculated with RSc, ORL8c, or ORL11c-derived IF1d7-sup (Fig. 7D). Regarding the NS5B expression, similar results were obtained (data not shown). These results suggest that HCV production in ORL8c cells is comparable to that in RSc cells. In contrast, core protein was not detected in ORL11c cells inoculated with ORL11c-derived IF1d7-sup (Fig. 7D). The core protein released into the culture supernatants (IF2d7-, IF2d14-, and IF2d22-sup) of HCVcc-infected ORL8c cells was persistently detected, although at somewhat lower levels than in the RSc cells (Fig. 7E). The level of intracellular HCV RNA in the ORL8c cells was $>10^7$ copies/μg total RNA at IF2d14; this is also somewhat lower than in the RSc cells (Fig. 7F). However, RT-PCR analysis revealed that the expression levels of HCV entry factors (CD81, SR-BI, Claudin-1, and Occuldin) were comparable among HuH-7, RSc, Li23, ORL8c, and ORL11c cells (Fig. 7G). Immunofluorescence analysis showed that the staining levels of dsRNA and HCV proteins were also comparable between HCVcc-infected ORL8c and RSc cells (IF2d7) (Fig. 7H). Colocalization of lipid droplet and HCV core protein was also observed in HCVcc-infected ORL8c and RSc cells (Supplemental Fig. 5), as previously reported (Miyanari et al., 2007). In summary, we demonstrated that ORL8c cells persistently supported the HCV life cycle.

4. Discussion

In this study, we found that human hepatoma Li23-derived cells possess the environments needed for robust genome-length HCV RNA replication and persistent production of infectious HCV. Using Li23-derived cell lines, we developed subgenomic and genome-length HCV RNA replication systems, drug assay systems, and a persistent HCV production system, which correspond to the counterparts of those using HuH-7-derived cell lines (Supplemental Table 2). It is noteworthy that the ORL8c cells cured from ORL8 cells, which were selected by the indicator of HCV RNA replication, showed good potential for producing HCV-JFH1. This finding suggests that the host factors required for robust HCV RNA replication – rather than those for HCV infection or reformation – are key determinants for reproducing the HCV life cycle in cell culture. In fact, we observed similar expression levels of the HCV entry factors between Li23- and HuH-7-derived cells. Therefore, such host factors might be commonly expressed in both ORL8c and RSc cells (Ariumi et al., 2007, 2008; Kuroki et al., 2009).

Our microarray analysis clearly demonstrated that OL8 and OL11 cell lines established in this study were not of HuH-7 cell origin, and revealed that Li23-derived cells possessed rather different expression profiles from those in HuH-7-derived cells (Fig. 3B), although similar liver-specific gene expression profiles were observed in both cell lineages (Fig. 3A). In addition, this analysis revealed that at least OL8 and OL11 cells possessed characteristic expression profiles of the parental Li23 cells, as Oc and OAc cells also showed the HuH-7-type expression profile. Therefore, further comparative studies on the mechanism(s) of HCV proliferation using Li23- and HuH-7-derived cell lines (e.g. ORL8c vs. RSc) may identify new host factor(s) required for efficient HCV proliferation.

A specific combination of adaptive mutations (Q1112R, K1609E, and S2200R) (Abe et al., 2007) is also a key determinant with which to find the Li23 cell line. Until the finding of such a combination of adaptive mutations, we had failed to establish any non-HuH-7-derived cells harboring the HCV replicon. Although it remains unclear what mechanism underlies these adaptive mutations that enhance HCV RNA replication, these mutations might be useful for the development HCV RNA replication systems of various HCV strains.

ORL8 and ORL11 assay systems might become important tools for evaluating or screening anti-HCV reagents, because these assay systems were frequently more sensitive to anti-HCV reagents than the HuH-7-derived OR6 assay system. However, the fact that the ORL8 and ORL11 assays were each more sensitive than the OR6 assay may be due to the fact that OR6 has a higher level of HCV RNA replication than ORL8 and ORL11 cells. Recently, we developed HCV replicon reporter assay systems using four genotype 1b HCV strains (1B-4, KAH5, O, and 1B-5), and found diverse sensitivities against various anti-HCV reagents among the replicons (Nishimura et al., 2009). In that study, we demonstrated that the sensitivities to anti-HCV reagents were not dependent on the replication levels of HCV RNA, and suggested that factor(s) other than the HCV RNA level are involved in conferring sensitivities to anti-HCV reagents including IFN-α (Nishimura et al., 2009). Therefore, the practical use of HuH-7- and Li23-derived assay systems would be very effective for accurately evaluating anti-HCV activity.

Finally, the most important feature of this report is that we were able to persistently produce infectious HCVcc using ORL8c cells. ORL8c-produced HCVcc would be very useful not only for verification of data obtained from HuH-7-derived cells but also for obtaining a variety of new information about the HCV life cycle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2009.08.006.

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Pathogenesis of Hepatitis C Virus Infection in *Tupaia belangeri*▽†

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The lack of a small-animal model has hampered the analysis of hepatitis C virus (HCV) pathogenesis. The tupaia (*Tupaia belangeri*), a tree shrew, has shown susceptibility to HCV infection and has been considered a possible candidate for a small experimental model of HCV infection. However, a longitudinal analysis of HCV-infected tupaias has yet to be described. Here, we provide an analysis of HCV pathogenesis during the course of infection in tupaias over a 3-year period. The animals were inoculated with hepatitis C patient serum HCR6 or viral particles reconstituted from full-length cDNA. In either case, inoculation caused mild hepatitis and intermittent viremia during the acute phase of infection. Histological analysis of infected livers revealed that HCV caused chronic hepatitis that worsened in a time-dependent manner. Liver steatosis, cirrhotic nodules, and accompanying tumorigenesis were also detected. To examine whether infectious virus particles were produced in tupaia livers, naive animals were inoculated with sera from HCV-infected tupaias, which had been confirmed positive for HCV RNA. As a result, the recipient animals also displayed mild hepatitis and intermittent viremia. Quasispecies were also observed in the NS5A region, signaling phylogenic lineage from the original inoculating sequence. Taken together, these data suggest that the tupaia is a practical animal model for experimental studies of HCV infection.

Hepatitis C virus (HCV) is a small enveloped virus that causes chronic hepatitis worldwide (32). HCV belongs to the genus *Hepacivirus* of the family *Flaviviridae*. Its genome comprises 9.6 kb of single-stranded RNA of positive polarity flanked by highly conserved untranslated regions at both the 5′ and 3′ ends (4, 27, 29). The 5′ untranslated region harbors an internal ribosomal entry site (29) that initiates translation of a single open reading frame encoding a large polyprotein comprising about 3,010 amino acids (35). The encoded polyprotein is co- and posttranslationally processed into 10 individual viral proteins (15).

In most cases of human infection, HCV is highly potent and establishes lifelong persistent infection, which progressively leads to chronic hepatitis, liver steatosis, cirrhosis, and hepatocellular carcinoma (9, 16, 21). The most effective therapy for treatment of HCV infection is administration of pegylated interferon combined with ribavirin. However, the combination therapy is an arduous regimen for patients; furthermore, HCV genotype 1b does not respond efficiently (19). The prevailing scientific opinion is that a more viable option than interferon treatment is needed.

The chimpanzee is the only validated animal model for in vivo studies of HCV infection, and it is capable of reproducing most aspects of human infection (5, 18, 23, 28, 35, 36). The chimpanzee is also the only validated animal for testing the authenticity and infectivity of cloned viral sequences (8, 14, 35, 36). However, chimpanzees are relatively rare and expensive experimental subjects. Cross-species transmission from infected chimpanzees to other nonhuman primates has been tested but has proven unsuccessful for all species evaluated (1).

The tupaia (*Tupaia belangeri*), a tree shrew, is a small nonprimate mammal indigenous to certain areas of Southeast Asia (6). It is susceptible to infection with a wide range of human-pathogenic viruses, including hepatitis B viruses (13, 20, 31), and appears to be permissive for HCV infection (33, 34). In an initial report, approximately one-third of inoculated animals exhibited acute, transient infection, although none developed the high-titer sustained viremia characteristic of infection in humans and chimpanzees (33). The short duration of follow-up precluded any observation of liver pathology. In addition to the putative in vivo model, cultured primary hepatocytes from tupaias can be infected with HCV, leading to de novo synthesis of HCV RNA (37). These reports strongly support tupaias as a valid model for experimental studies of HCV infection. However, longitudinal analyses evaluating the clinical development and pathology of HCV-infected tupaias have yet to be exam-

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TABLE 1. Experimental HCV infections performed in this study

Tupaia no.	Inoculum		Biopsy/sacrifice[b]
	Type	Quantity (GE/tupaia)[a]	
Group I[c]			
Tup.4	RCV	1×10^7	84, 94/144 wk p.i.
Tup.5	HCR6	6×10^5	95, 105/155 wk p.i.
Tup.6	HCR6	6×10^5	95, 105/155 wk p.i.
Tup.8	RCV	1×10^7	84, 94/144 wk p.i.
Group II[d]			
Tup.9	Tup.5 (5 wk p.i.)	1×10^2	NT
Tup.10	Tup.5 (5 wk p.i.)	1×10^2	NT
Tup.11	Tup.8 (10 wk p.i.)	1×10^2	NT
Tup.12	Tup.8 (10 wk p.i.)	1×10^2	NT
Tup.13	Tup.4 (8 wk p.i.)	1×10^2	NT
Tup.14	Tup.4 (8 wk p.i.)	1×10^2	NT
Group III[e]			
Tup.15	None		92/100 wk
Tup.17	None		92/100 wk
Tup.38	None		242 wk
Tup.39	None		242 wk

[a] Viral RNA GE/tupaia was estimated by Quantitative real-time RT-PCR (GE, genome equivalents; sensitivity > 10 GE/ml serum).
[b] Liver biopsy was performed at indicated time-point. p.i., postinoculation; NT, not tested.
[c] Group I, primary infection experiment in which 1-year-old animals were inoculated with two different types of inocula.
[d] Group II, reinfection experiment, where HCV RNA-positive sera from Group I experimental infections were passaged to naive animals.
[e] GroupIII, no-infection control.

ined. In the present study, we describe the clinical development and pathology of HCV-infected tupaias over an approximately 3-year time course.

MATERIALS AND METHODS

Animals. Table 1 summarizes the tupaias used in this study. Tupaias born in laboratory captivity were obtained from the Laboratory Animal Center at the Kunming Institute of Zoology (Chinese Academy of Sciences). Tupaias were imported with permission from the Convention on International Trade in Endangered Species of Wild Fauna and Flora (7), quarantined for medical inspection, and housed individually in standard rat cages supplied with filtered air. The animals were fed a daily regimen of eggs, fruit, and the CMS-1 commercial diet for marmosets (CLEA, Japan). Their appetites and feces were carefully monitored. Animal care and experimental handling conformed to study guidelines established by the Subcommittee on Laboratory Animal Care at the Tokyo Metropolitan Institute of Science.

Patient serum used for animal infection. HCV genotype 1b serum, designated HCR6, was obtained from a patient with chronic active hepatitis C. The infectious titer of HCR6 was determined in chimpanzee and Molt4 cells and denoted plasma K (HCR6) by Shimizu et al. (24). The HCR6 serum exhibited a PCR titer of 6×10^6 genome equivalents/ml and an infectious titer of 3.7×10^4 50% chimpanzee infectious doses/ml. Serum aliquots were frozen at −80°C until they were used.

Virion reconstitution of cloned HCV. As described previously, pHCR6 (genotype 1b; 9,611 nucleotides; GenBank accession no. AY045720) is a plasmid carrying HCV genomic cDNA cloned from HCR6 serum (30). pHCR6Rz was designed for precisely trimmed RNA expression, with the entire genomic region of pHCR6Rz recloned under the control of the T7 promoter and the 5′ and 3′ distal ends flanked by hammerhead- and hepatitis D virus ribozyme-encoding sequences, respectively (22, 25).

For molecular reconstitution of HCV particles, pHCR6Rz was transfected into IMY-N9 cells as described previously (12). Briefly, semiconfluent IMY-N9 cells in 100-mm plastic dishes were transfected with 15 μg of plasmid using 40 μl of cationic lipids (DMRIE-C reagent; Life Technology) in accordance with the manufacturer's instructions. Five hours after transfection, the cells were infected with AdexCAT7 (2) (kindly provided by Y. Matsuura) at a multiplicity of infection of 20. After infection, the culture medium was replaced with Hepato-STIM (Becton Dickinson). The culture supernatants were collected at 24 h postinfection and stored at −80°C.

Virus inoculation and collection of serum samples. Animals were infected at 6 months of age. The anesthetic agent, ketamine hydrochloride, was administered intramuscularly at 50 mg/kg body weight prior to virus inoculation and bleeding of the tupaias. The inocula were introduced intravenously at 6×10^5 genome equivalents/animal for patient serum HCR6 and 1×10^7 genome equivalents/animal for reconstituted virions derived from the pHCR6Rz inoculation. Blood samples were drawn from infected and control animals pre- and postinfection. Briefly, the animals were bled weekly for 20 weeks and biweekly thereafter. At each time point, 0.5 ml of blood was drawn from the thigh vein; the sera were separated, aliquoted, and stored for subsequent assays.

Reinfection experiments were performed by transmission of HCV RNA-positive serum from group I (Table 1) to naive animals.

Serum alanine aminotransferase (ALT) concentrations were determined using a Transnase Nissui kit (Nissui Pharmaceutical Co.), standardized, and displayed as IU/liter.

RNA isolation and quantitative RTD-PCR assay for HCV RNA. Serum samples (100 μl) were tested for circulating HCV RNA in vivo using quantitative real-time detection (RTD)-PCR (TaqMan). RNA was extracted from the sera and livers of sacrificed animals using the acid guanidium-phenol chloroform method with tRNA as a carrier (3). Two tupaias (Tup.5 and Tup.6) were inoculated with patient serum HCR6. Another two animals (Tup.4 and Tup.8) were inoculated with reconstituted viral particles (RCV). Tup.15 served as a mock-infected control. Liver specimens (3- to 4-mm^2 blocks) from these tupaias were homogenized with 1.5 ml of 5 M guanidine thiocyanate using a polytron-type homogenizer (Ultra-Turrax T25; IKA Laborteehnik, Staufen, Germany). RNA was then reextracted with 4 M guanidine thiocyanate.

RNA samples were subjected to RTD-PCR on an ABI 7700 sequence detector (Applied Biosystems) as described previously (26). The extracted RNA was dissolved in 200 μl of diethyl pyrocarbonate-treated water containing 10 mM dithiothreitol and 200 units/ml RNase inhibitor in a siliconized tube. RTD-PCR was performed using 1 μg of total RNA, one set of PCR primers, and a probe for a location within the 5′ noncoding region using the EZ *rTth* RNA PCR kit (Perkin Elmer) and the ABI Prism 7700 sequence detector system. A standard curve was constructed using a 10-fold dilution series of in vitro-transcribed and previously titrated synthetic HCV RNA.

Consequently, the quantities represented by genome equivalents correspond to an absolute standard curve (26). All quantitative RTD-PCR assays were performed using duplicate samples, with both negative control serum and HCV-positive serum included. The control sera were diluted before use and were estimated to contain low copy numbers of HCV RNA (100 genome equivalents/ml serum). Samples were deemed positive for HCV RNA if both duplicates yielded PCR-amplified product. Averages of the two estimated values are shown in the figures.

Histological analysis. Tissue samples were carefully collected from anesthetized animals by abdominal incision, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Silver and Sudan IV (Wako Pure Chemical Industries, Ltd.) staining were also carried out to visualize fiber generation and lipid degeneration, respectively. All histological staining was performed in accordance with conventional procedures. The histological status was determined using the modified hepatitis activity index scoring system, which grades necrosis and inflammation on a scale of 0 to 18 (periportal inflammation and necrosis, 0 to 10; lobular inflammation and necrosis, 0 to 4; portal inflammation, 0 to 4) (11). Fibrosis was scored using the Ishak fibrosis scale of 0 to 6 (0, no fibrosis; 1 or 2, portal fibrosis; 3 or 4, bridging fibrosis; and 5 or 6, cirrhosis). The values in each group (Table 2) represent the averages of the scores in five visual fields.

Statistical analysis. The statistical significance of differences between controls and HCV-infected animals was analyzed with the nonparametric Mann-Whitney U test. All comparisons were two tailed. The statistical analysis was conducted with SPSS 12.0 software (SPSS Inc., Chicago, IL).

RESULTS

Inoculation of HCV causes acute hepatitis and transient viremia in tupaias. To begin this study, two distinct but related inocula were chosen for infection of tupaias. Serum from a chronic hepatitis patient (designated HCR6) was chosen for its

TABLE 2. Grading: necroinflammatory scores and fibrosis

Group	Inoculum	Tupaia no.	Grade				Total	Avg	SD	Staging
			A	B	C	D				
94 wk p.i. (biopsy)										
I	HCR 6	Tup.5	0	0	0	0	0	1.3	1.5	0
		Tup.6	1	0	1	0	2			0
	RCV	Tup.4	0	0	0	0	0			0
		Tup.8	0	0	0	3	3			6
	Control	Tup.15	0	0	0	0	0	0	0	0
III		Tup.17	0	0	0	0	0			0
		Tup.38								
		Tup.39								
144 wk p.i. (sacrifice)										
I	HCR 6	Tup.5	1	0	2	3	6	5.5	3.7	0
		Tup.6	3	0	4	3	10			1
	RCV	Tup.4	0	0	0	1	1			0
		Tup.8	1	0	1	3	5			6
	Control	Tup.15						0	0	
III		Tup.17								
		Tup.38	0	0	0	0	0			0
		Tup.39	0	0	0	0	0			0

defined genotype (genotype 1b), and genetic heterogeneity was ascertained by the process of cloning consensus cDNA. The infectivity of this serum was also experimentally defined in chimpanzees; a 50% chimpanzee infectious dose was estimated at 3.7×10^4 50% chimpanzee infectious doses/ml. Furthermore, the consensus genomic sequence of HCV was cloned from the serum (pHCR6; 9,611 bases; GenBank AY045702.1). For the second inoculum (referred to as RCV), clonal viral particles were reconstituted as described in Materials and Methods. This inoculum was expected to be free of neutralizing antibodies and thus was considered potentially more infectious than patient sera. In the case of RCV infection, genetic diversification of viral RNA, also known as quasispecies, can be regarded as a direct indication of de novo synthesis of progenitor virus in vivo.

Either patient serum or cDNA-derived RCV was inoculated into tupaias (Table 1, group I). Two animals (one female and one male) were tested against each inoculum. Age-matched animals were bred as infection-free controls.

All experimental infections are described in Materials and Methods and Table 1. Prior to experimental infection, the normal serum ALT level in tupaias was measured at 22.3 IU/liter ($n = 23$).

Inoculation with patient serum HCR6 caused rapid fluctuations in the serum ALT concentrations, from two- to fivefold, in both inoculated tupaias, suggesting acute hepatitis in vivo (Fig. 1A and B). Correlative quantitative RTD-PCR revealed HCV viremia soon after serum inoculation in Tup.5, which continued to show transient viremia long term. The appearance of viremia sometimes coincided with a steep elevation in the serum ALT (Fig. 1A). Conversely, HCV RNA was not detected in the serum of Tup.6 up to 60 weeks postinoculation and only twice thereafter. Acute-phase ALT elevations (3 to 4 weeks postinoculation) in Tup.6 might represent tight control of HCV infection by the host immune system (Fig. 1B).

Distinct results were obtained for the two animals (Tup.4 and Tup.8) inoculated with RCV. Both animals displayed sustained viremia up to 10 weeks postinoculation (Fig. 1C and D), indicating persistent HCV infection and inability to eradicate the virus. Viremia was detected intermittently throughout the course of infection, sometimes accompanying the elevation of serum ALT. Humoral immune responses in Tup.5 and Tup.6 (see Fig. S1A in the supplemental material) and Tup.4 and Tup.6 (see Fig. S1B in the supplemental material) were indicated.

We performed RTD-PCR to confirm whether HCV could replicate in the tupaias' livers (Tup.4, Tup.5, Tup.6, and Tup.8) and obtained the following results (Fig. 1E): 310 ± 117 copies/μg total RNA in Tup.5, 80 ± 11 copies/μg in Tup.6, 199 ± 77 copies/μg in Tup.4, and 292 ± 48 copies/μg in Tup.8. In contrast, HCV RNA was not detected in the liver of the mock-infected animal (Tup.15).

HCV RNA was also not detected in samples from either preinoculation or age-matched, infection-free control tupaias (Table 1, group III), nor were significant elevations in serum ALT observed for any of the three infection-free controls (data not shown).

HCV causes chronic hepatitis in tupaia liver, leading to fibrosis and cirrhosis. Serum ALT and circulating HCV RNA levels in primary infected tupaias (Table 1, group I) were monitored for 3 years postinoculation. As described above, the magnitudes of serum ALT fluctuations varied substantially among infected animals (Fig. 1A, B, C, and D). Tupaia livers were examined for histological lesions in order to elucidate if HCV caused chronic hepatitis. Liver biopsies via abdominal incisions were performed at 2 years postinoculation. All animals were sacrificed at 3 years postinoculation (4.5 years for uninfected animals). H&E staining of liver specimens from HCV-infected tupaias showed infiltrating lymphocytes within sinusoids and around portal areas, indicating chronic hepatitis in the tupaia livers (Fig. 2B, D, and H). Infiltrating lymphocytes were also observed in limiting plates, indicating ongoing inflammation (Fig. 2G and H). Furthermore, a comparison of liver samples at 2 and 3 years postinoculation revealed that the

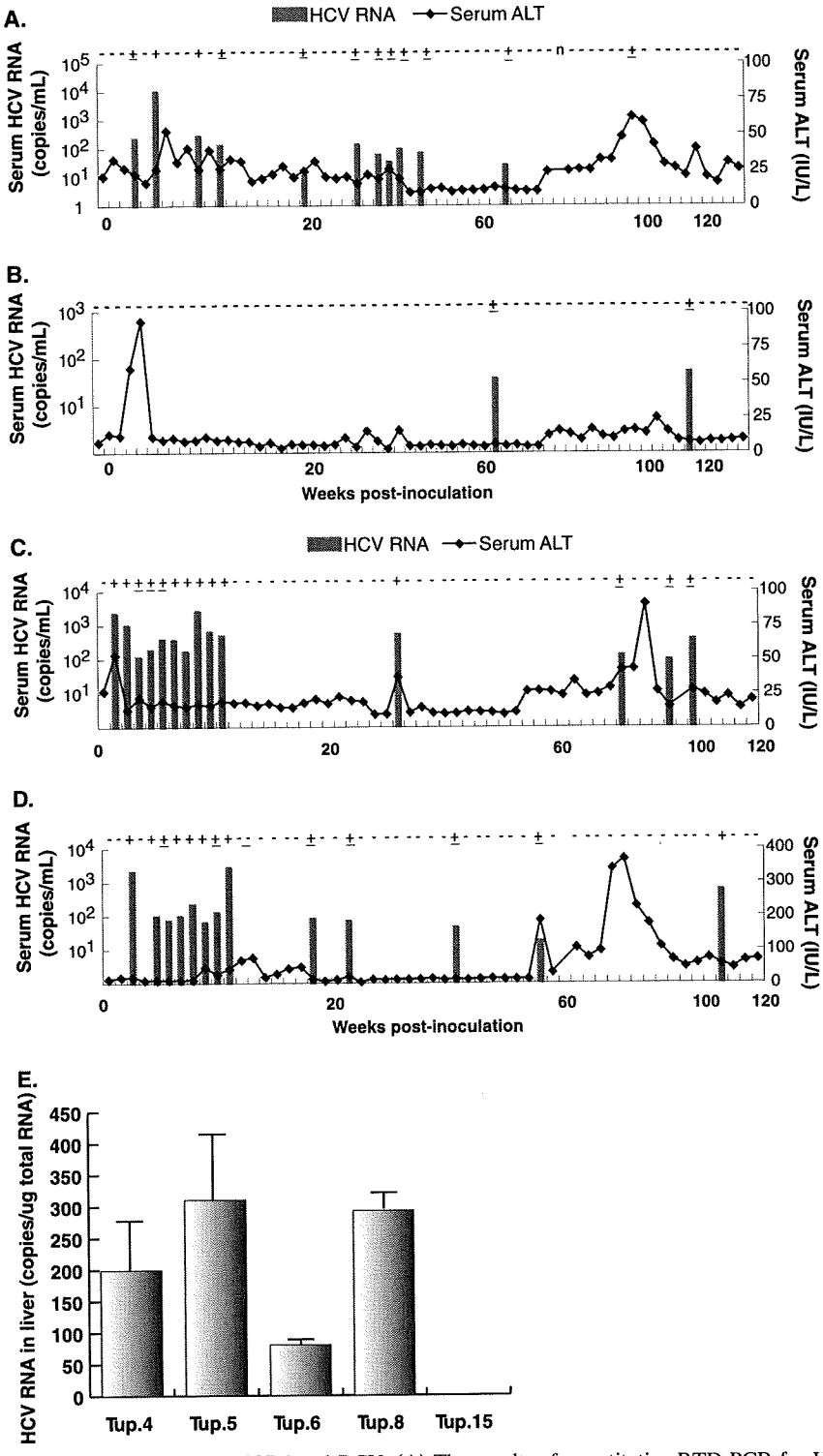

FIG. 1. Course of infection with patient serum HCR6 and RCV. (A) The results of quantitative RTD-PCR for HCV RNA and serum ALT concentrations were combined and plotted to show the course of infection in Tup.5. The bars and the ordinates on the left represent HCV RNA as genome equivalents/ml of serum. The curved line and the ordinates on the right represent serum ALT concentrations as IU/liter serum. (B) Serum HCV RNA and ALT concentrations for infection of Tup.6. (C) The graph for Tup.4. (D) The graph for Tup.8. The vertical axis for serum ALT in this graph is scaled differently from the others because of significant ALT elevation. (E) Quantification of HCV RNA in tupaia liver. HCV RNA in hepatocytes from tupaia (Tup.4, Tup.5, Tup.6, Tup.8, and Tup.15) livers was isolated 172 weeks after HCV infection and quantified by RTD-PCR. As few as 10 copies of the genome were detected, and the quantification range was between 10^1 and 10^8 copies (26).

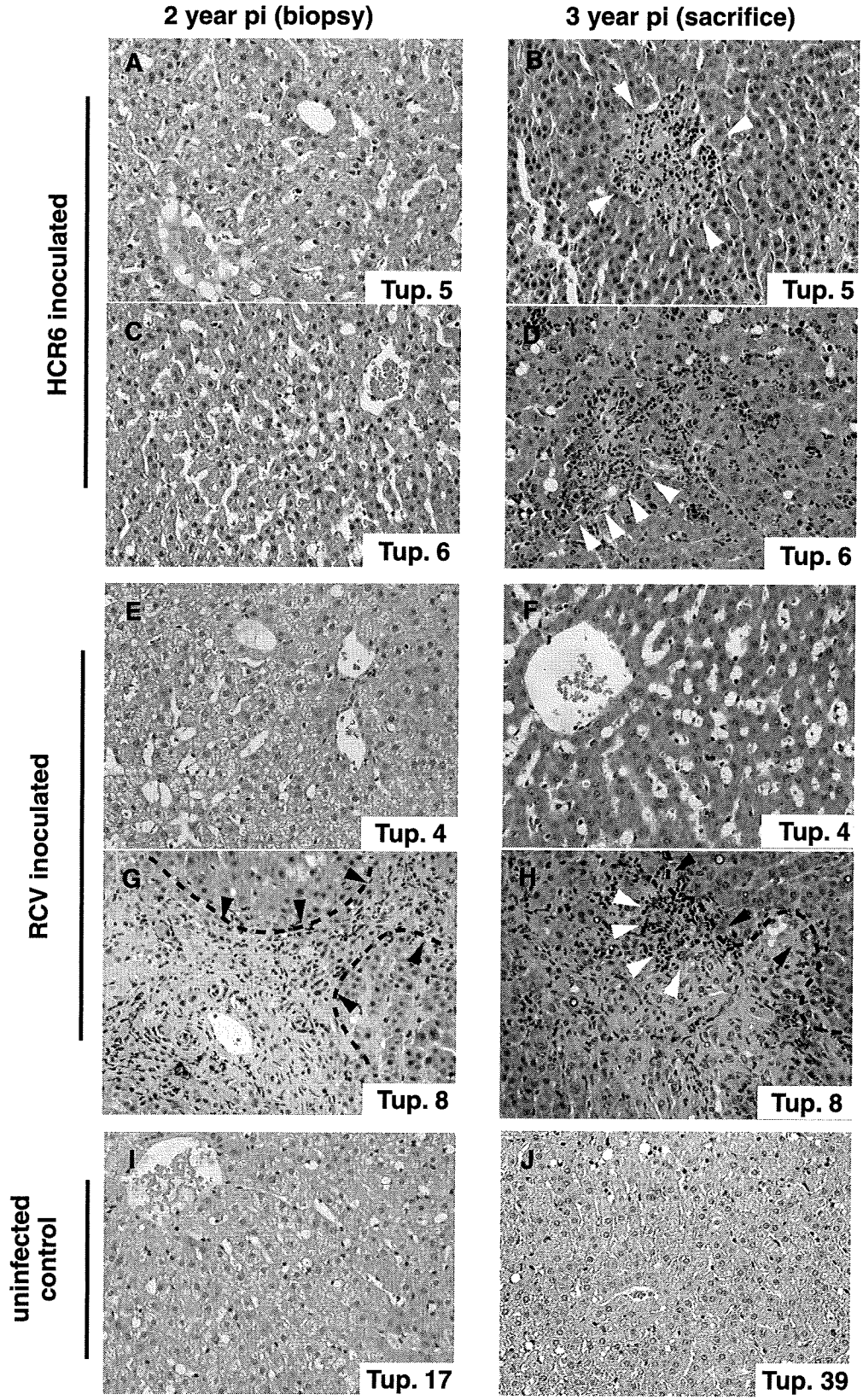

FIG. 2. Micrographs of liver specimens stained with H&E. Liver tissue from HCR6-inoculated tupaias (A to D) and RCV-inoculated tupaias (E to H) was obtained at 2 and 3 years postinoculation (pi). (I and J) Liver specimens from uninfected animals age matched to each inoculated animal were also obtained. The HCV-infected tupaia livers harbored infiltrating lymphocytes (white arrowheads) and fibrosis (broken lines and black arrowheads), which indicate chronic hepatitis.

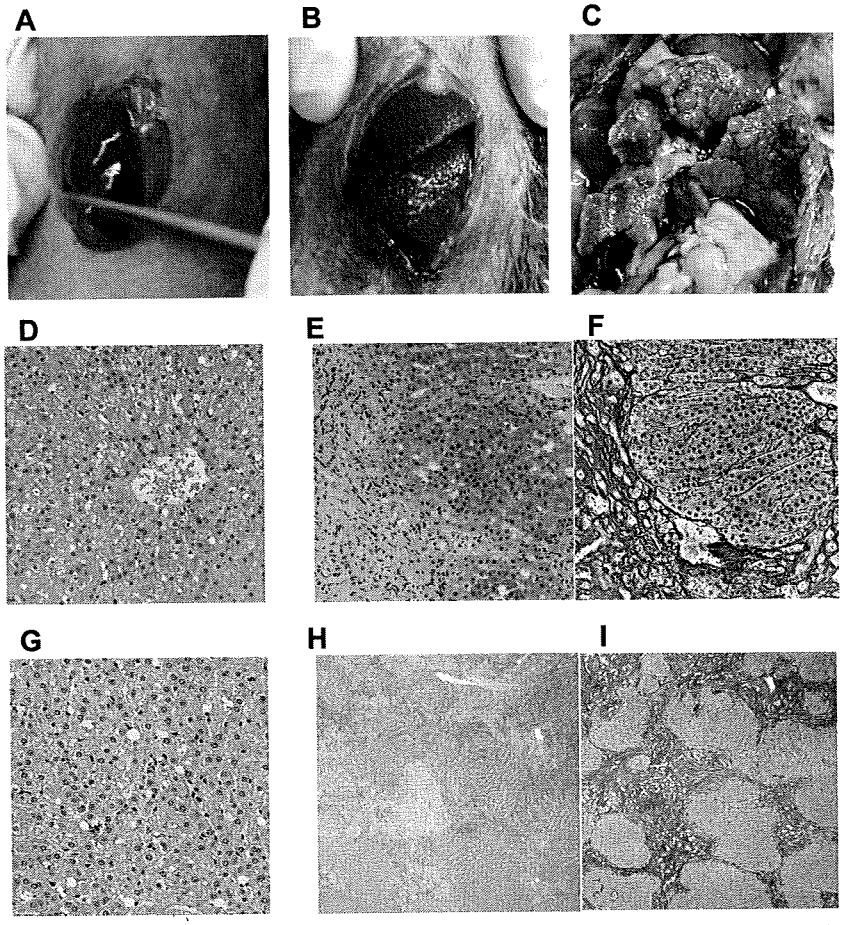

FIG. 3. Macro- and microscopic features of tupaia liver. (A) Infection-free control tupaia (Tup.15; 92 weeks). (B) RCV-infected animal displaying liver cirrhosis (Tup.8; 84 weeks postinoculation). (C) RCV-infected animal with massive surface nodules (Tup.8; 144 weeks postinoculation). (D and G) H&E staining of the uninfected Tup.15 at 92 weeks (D) and the uninfected Tup.39 at 242 weeks (G). (E, F, H, and I) H&E and silver staining of Tup.8 at 84 weeks postinoculation (E and F) or at 144 weeks postinoculation (H and I).

hepatitis had worsened with time in all HCV-infected tupaias (Fig. 2A to H and Table 2).

Fibrosis and cirrhosis were also examined. Mild fibrosis was seen in Tup.6, while severe fibrosis was seen in Tup.8. Cirrhosis was histologically investigated in all animals (Table 2). There was no significant difference between groups I and III at 94 weeks postinfection ($P = 0.194$), but at 144 weeks postinfection, a slight difference was observed ($P = 0.059$; SPSS 12.0). Macroscopic observation of the liver biopsy specimens (taken 2 years postinoculation) indicated liver cirrhosis in Tup.8 (Fig. 3B) compared with Tup.15 (uninfected control) (Fig. 3A), while silver staining of histology samples revealed fibrosis and cirrhotic nodules (Fig. 3E and F). Macroscopic observation upon sacrifice (3 years postinoculation) indicated that liver cirrhosis in Tup.8 had worsened (Fig. 3C). In contrast, age-matched infection-free negative control tupaias displayed none of these pathologies (Fig. 3A, D, and G).

Progressive lipid degeneration was noted in infected tupaias throughout the course of infection (Fig. 4). In particular, Tup.5 displayed microvesicular lipid droplets in the first biopsy specimens (at 2 years), which developed into macrovesicular droplets and foamy degeneration in biopsy specimens at 3 years (Fig. 4C and D). Liver specimens from other infected animals displayed intracellular micro- and macrovesicular lipid droplets in hepatocytes at 3 years postinoculation (Fig. 4F, H, and J). These anomalies were not present in liver specimens from infection-free control animals (Fig. 4A and B).

Transmission of viral-RNA-positive serum to naive animals reproduces acute hepatitis and viremia. To confirm virion regeneration in vivo, and to exclude the possibility of false-positive serum HCV RNA results due to amplification of the original inocula, HCV RNA-positive sera from primary inoculated tupaias were used to inoculate naive tupaias. Three different sera were tested in this passage experiment, with two naive tupaias used as recipient animals for each trial (see Materials and Methods) (Table 1, group II).

In the first reinfection experiment, serum from Tup.5 (originally infected with patient serum HCR6) was collected at 5 weeks postinoculation and used to infect two naive animals. The recipient animals showed intermittent viremia over the subsequent 3 months (Fig. 5A). In the second and third cases of reinfection, sera from Tup.8 at 10 weeks postinoculation and from Tup.4 at 8 weeks postinoculation also induced viremia in the naive inoculated animals, similar to the first reinfection experiment (Fig. 5B and C). Furthermore, the PCR titers of the recipient tupaias were significantly greater than the inoc-

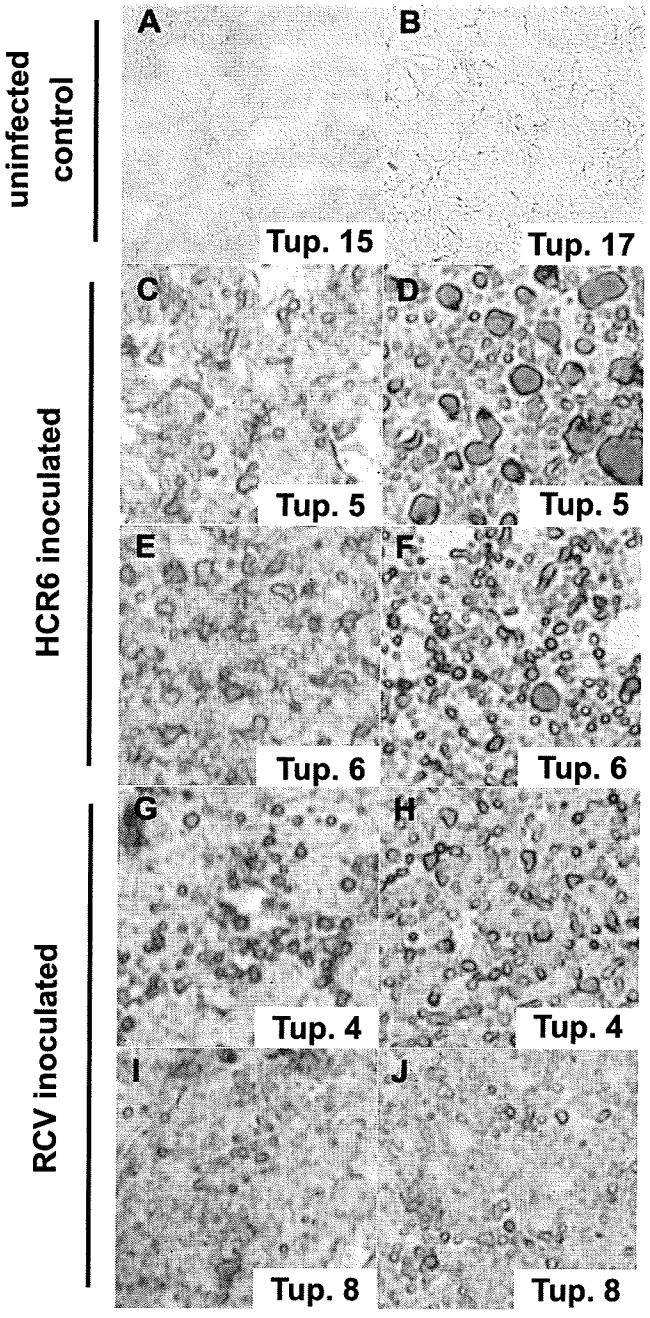

FIG. 4. Sudan IV-stained liver specimens exhibiting fatty liver degeneration. Cryosections of liver stained by Sudan IV as described in Materials and Methods show fatty liver degeneration. The left and right columns display biopsy specimens of infected animals (2 years postinoculation) and animals sacrificed at 3 years postinfection, respectively. (A and B) Uninfected controls at 2 years (Table 1 shows sample timing). (C to F) Patient serum HCR6-infected animals. (G to J) RCV-infected animals.

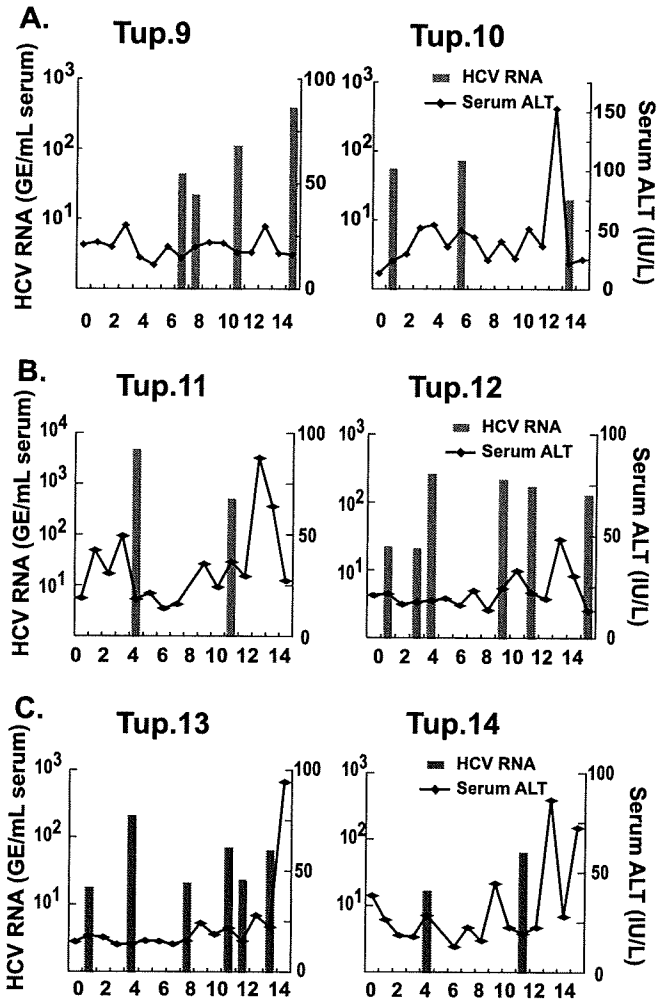

FIG. 5. Results of a reinfection experiment. (A) Quantitative RTD-PCR for HCV RNA and serum ALT levels are shown. Two naive animals were inoculated with tupaia serum (using serum taken at 5 weeks postinoculation from Tup.5, originally inoculated with patient serum HCR6) containing 100 genome equivalents (GE)/ml and were monitored for 15 weeks postinoculation (Table 1). (B) Tupaia serum (taken at 10 weeks postinoculation from Tup.8, originally inoculated with RCV) that was positive for HCV RNA was passaged into two naive animals. The animals were inoculated with tupaia serum at 100 GE/animal and monitored for 15 weeks postinoculation. (C) Tupaia serum (taken at 8 weeks postinoculation from Tup.4, originally inoculated with RCV) that was positive for HCV RNA was passaged into naive animals. The animals were inoculated with serum at 100 GE/animal and monitored for 20 weeks postinoculation.

ulation titers (10^2 genome equivalents/animal) (Table 1). For Tup.11, serum from 4 weeks postinoculation contained almost 10^4 genome equivalents/ml of HCV RNA (Fig. 5B). In addition, significant increases in serum ALT accompanied detection of serum HCV RNA. These results indicate that HCV RNA-positive sera from group I actually contained infectious virion particles. They also suggest that reconstituted HCV particles made from cDNA are infectious in tupaias.

We amplified a portion of the NS5A sequence, which is known as the interferon sensitivity determining region, by reverse transcription-PCR as described in the supplemental material. Each PCR product was subcloned and sequenced to compare the encoded amino acid sequences. For the purposes of this study, animals were inoculated with a molecular clonal virus consisting of a unique viral sequence of cDNA. The interferon sensitivity determining region sequences recovered from an animal infected with clonal inoculum (Tup.8 at 103 weeks postinoculation) were found to be heterogeneous, with