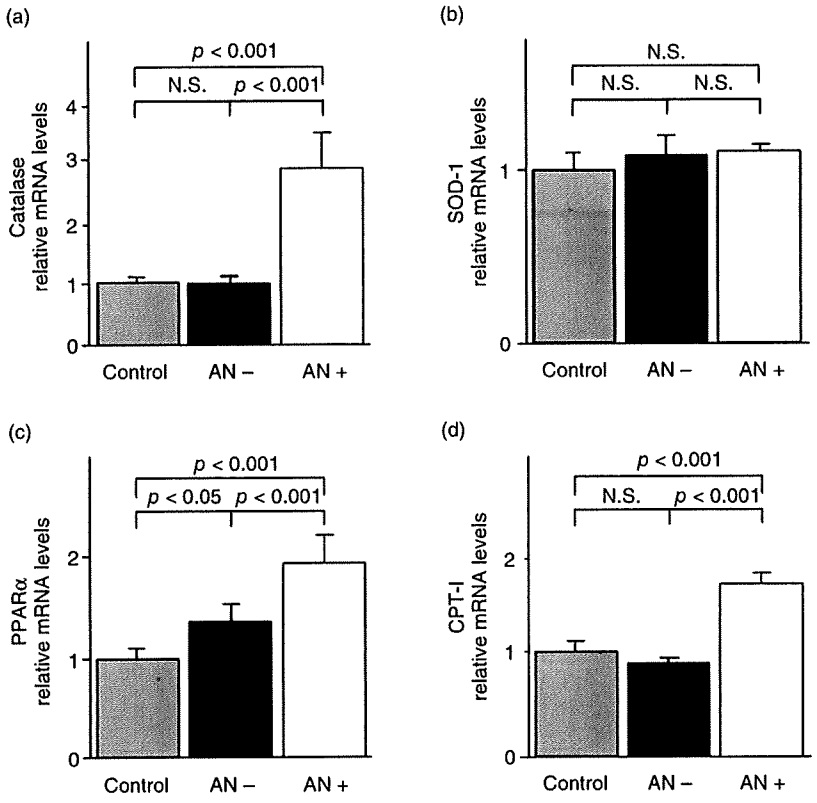

Figure 7 The mRNA expression levels of catalase, SOD-1, PPARα and CPT-I in mouse liver cell line, Hepa 1–6 cells treated with or without recombinant adiponectin. The mRNA expression levels of (a) catalase, (b) SOD-1, (c) PPARα and (d) CPT-I in mouse liver cell line, Hepa 1–6 cells treated with (AN+) or without (AN–, control) recombinant adiponectin for 24 h. Control is basal mRNA expression levels of Hepa 1–6 cells (gray column). The mRNA expression levels were normalized relative to the level of GAPDH mRNA expression and expressed in arbitrary units. For all panels, data are mean ± SD, and the *P*-values represent the results of analysis of variance with the Fisher's PLSD test.

viously that lack of adiponectin increased serum TNF-α in the CDAA diet-fed mice.[26] In the present study, we found that lack of adiponectin was associated with overexpression of MCP-1 and underexpression of M2-macrophage markers, IL-10 and arginase-1, in mice liver. These findings indicate that adiponectin deficiency reduces the recruitment of "M2-polarized Kupffer cells" into mice liver. In addition, adiponectin might affect not only Kupffer cell recruitment to, but also Kupffer cell phenotype within, the liver.

In this study, steatohepatitis progression was successfully prevented in KO mice by adenovirus-mediated adiponectin expression. Upregulation of adiponectin signaling could be useful therapeutically by increasing plasma adiponectin levels or development of adiponectin receptor agonists. However, with regard to hyperadiponectinemia, direct administration of adiponectin protein to individuals with liver diseases might not be a good strategy because of difficulties in maintaining high plasma concentrations. Thiazolidinediones, known as PPARγ ligands, elevate the promoter activity of adiponectin and increase plasma concentration of adiponectin.[41] Adiponectin promoter has a functional PPAR-responsive element (PPRE) site.[42] Not only PPARγ, but also PPARα ligands increase the expression of adiponectin through PPRE site located on its promoter region.[43] Furthermore, PPARα agonist increases the expression of both AdipoR1 and AdipoR2 in adipocytes and macrophages.[44] These findings indicate that dual activation of PPARγ and PPARα intensifies adiponectin actions by increasing plasma levels of adiponectin and by increasing adiponectin receptors.

There are some limitations in this study to investigate the effect of adiponectin on NASH progression. The MCD diet-induced mice steatohepatitis presents hepatic steatosis, inflammation, and fibrosis with enhanced oxidative stress, and this picture resembles the human NASH liver. However, "MCD-induced steatohepatitis" is, strictly speaking, different from metabolic syndrome-related NASH in human. The critical difference of this model compared to NASH in the pathogenesis of steatohepatitis is that mice of this model gradually lose weights (visceral and subcutaneous fat) during the MCD food intake and are free of insulin resistance that is

thought to be one of the most important factors in the pathogenesis of NASH. In this study, we used MCD diet-induced mice steatohepatitis model as a human NASH-like model without insulin resistance. To investigate the precise mechanism of NASH, the development of new mice NASH models will be needed.[45]

In conclusion, lack of adiponectin accelerated, and adiponectin administration prevented steatohepatitis progression in mice. One of the mechanisms in this phenomenon is reduction in hepatic antioxidant enzymes, catalase and SOD-1, resulting in enhanced oxidative stress in KO mice. Another possible mechanism is the effects of adiponectin on recruitment and phenotype polarization of Kupffer cells. Further studies are needed to elucidate the precise mechanism of the effect of adiponectin on macrophage phenotype polarization. Collectively, augmentation of the function of adiponectin could be a useful preventive approach against NASH progression.

ACKNOWLEDGMENTS

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Delayed liver regeneration after partial hepatectomy in adiponectin knockout mice

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ABSTRACT

We previously demonstrated that adiponectin has anti-fibrogenic and anti-inflammatory effects in the liver of mouse models of various liver diseases. However, its role in liver regeneration remains unclear. The aim of this study was to determine the role of adiponectin in liver regeneration. We assessed liver regeneration after partial hepatectomy in wild-type (WT) and adiponectin knockout (KO) mice. We analyzed DNA replication and various signaling pathways involved in cell proliferation and metabolism. Adiponectin KO mice exhibited delayed DNA replication and increased lipid accumulation in the regenerating liver. The expression levels of peroxisome proliferator-activated receptor (PPAR) α and carnitine palmitoyltransferase-1 (CPT-1), a key enzyme in mitochondrial fatty acid oxidation, were decreased in adiponectin KO mice, suggesting possible contribution of altered fat metabolism to these phenomena. Collectively, the present results highlight a new role for adiponectin in the process of liver regeneration.

The liver is one of few adult organs that can regenerate following acute injury, such as toxins, viral hepatitis and hepatic resection. To understand the mechanism of liver regeneration, many investigators have induced liver regeneration by two-thirds partial hepatectomy (PHx) in rodent models [1]. Liver regeneration induced by PHx results in synchronous induction of hepatocyte DNA replication and mitosis. This proliferative response is initiated by the release of various cytokines and growth factors. During the regeneration process, immediate early transcription factors such as c-Jun, JunB or c-Myc are activated to promote hepatoproliferation [2].

Adiponectin (also known as ACRP30 and GBP28) is a plasma protein secreted from adipose tissue with known anti-diabetic and anti-atherogenic properties [3,4]. Adiponectin is expressed in the plasma (range, 5–30 μg/ml), and low levels are found in obesity and type 2 diabetes [3]. So far, two types of receptors for adiponectin, AdipoR1 and AdipoR2, have been identified [4]. It is reported that AdipoR1 is expressed mainly in skeletal muscles, whereas AdipoR2 is expressed mainly in the liver [4]. Mouse genetic analysis also demonstrated that AdipoR1 is strongly related to the activation of AMP-activated protein kinase (AMPK) signaling pathways, whereas AdipoR2 is linked to the stimulation of peroxisome proliferator-activated receptor (PPAR) α signaling pathways [5]. Studies from our laboratory have demonstrated that adiponectin has anti-fibrogenic and anti-inflammatory effects in mouse models of hepatic fibrosis [6] and acute hepatitis [7], respectively. Although hypoadiponectinemia is associated with several liver diseases [8], the role of adiponectin in liver regeneration remains unclear so far.

In this present study, we demonstrated that adiponectin KO mice exhibit delayed liver regeneration and show accumulation of triglyceride in hepatocytes after PHx. The results suggest that these changes might be attributed to impaired fatty acid oxidation.

Materials and methods

Mice. Generation of mice carrying homozygote mutation for mouse adiponectin gene, which was bred into the C57BL/6 background for five generations, was described previously [9]. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

Partial hepatectomy. Adiponectin KO mice and wild-type (WT) littermates (males, weighing 22–30 g, 12 weeks of age) were subjected to 70% PHx to induce liver regeneration. PHx was performed according to the method of Higgins and Anderson [10]. Six to eight mice at each time points were sacrificed at 0, 12, 24, 48, 72, and 96 h after PHx. At the time of sacrifice, the mice were weighed, blood was harvested from the inferior vena cava and the remnant liver was removed en bloc. The removed livers were rapidly weighed and frozen in liquid nitrogen and stored at −80 °C.

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Sections from the removed liver were fixed in 10% buffered formalin for further histological analysis.

BrdU labeling and immunohistochemical staining. Hematoxylin and eosin (H&E) stained liver sections were assessed for hepatocyte lipid accumulation. Hepatocyte DNA replication was assessed by nuclear incorporation of bromodeoxyuridine (BrdU), a thymidine analog incorporated into newly synthesized DNA, on immunostained liver sections using Cell Proliferation Kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). BrdU (30 mg/kg body weight) was injected intraperitoneally 4 h before harvesting the remnant regenerating livers. In each regenerating livers, we counted the number of BrdU-positive nuclei per 1000 hepatocytes to calculate the BrdU labeling index.

Western immunoblotting. For Western immunoblot analysis, total extracts prepared from liver tissues were separated on sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) and transferred onto polyvinylidene fluoride membrane as described previously [6]. We used primary antibodies specific to cyclin D1 (Santa Cruz Biotechnologies, Santa Cruz, CA). The density of each band was measured by a densitometer.

Real-time RT-PCR. Total RNA extraction from whole liver, reverse-transcription polymerase chain reaction (RT-PCR) and real-time PCR were performed as described previously [7]. The Quantitect gene assay kit was used for analysis of murine cyclin A2, cyclin B1, carnitine palmitoyl transferase (CPT-1), PPARα, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative gene expression was quantified using GAPDH as an internal control.

Analysis of hepatic triglyceride content. Total lipids were extracted from the liver as described previously [11]. Hepatic triglyceride contents were measured by using Wako Test kit (Wako Pure Medical, Tokyo, Japan), and the results were expressed in μg per mg liver weight.

Statistical analysis. The results are presented as means ± SD. Differences between two groups were examined for statistical significance using the Mann–Whitney U test. A P value less than 0.05 denoted the presence of a statistically significant difference.

Results

Adiponectin KO mice show delayed liver regeneration after PHx

To determine the role of adiponectin in liver regeneration, we compared the kinetics of DNA replication in hepatocytes and liver restitution in WT and KO mice at 24–96 h after PHx. DNA replication was monitored by immunohistochemical staining of BrdU nuclear incorporation. The restitution of liver mass was assessed by liver/body weight ratio. Compared with WT mice, the BrdU labeling index of KO mice was significantly lower at 48 and 72 h but was significantly higher at 96 h after PHx (Fig. 1A and B). These results showed that adiponectin KO livers show a lag in DNA replication after PHx, compared with the wild-type mice. We also found that the liver/body weight ratio was significantly lower in KO mice at 48 and 72 h after PHx than in WT mice (Fig. 1C). These results suggest delayed DNA replication of hepatocytes and impaired liver restitution after PHx in KO mice.

Underexpression of cell cycle regulators in regenerating livers of adiponectin KO mice

Next, we investigated the roles of cell cycle regulators; cyclin D1, cyclin A2, and cyclin B1, in impaired liver regeneration of KO mice. We evaluated the expression of cyclin D1 protein in the regenerating livers by Western immunoblotting at 0 and 24 h after PHx. Densitometric analysis showed a significantly low expression

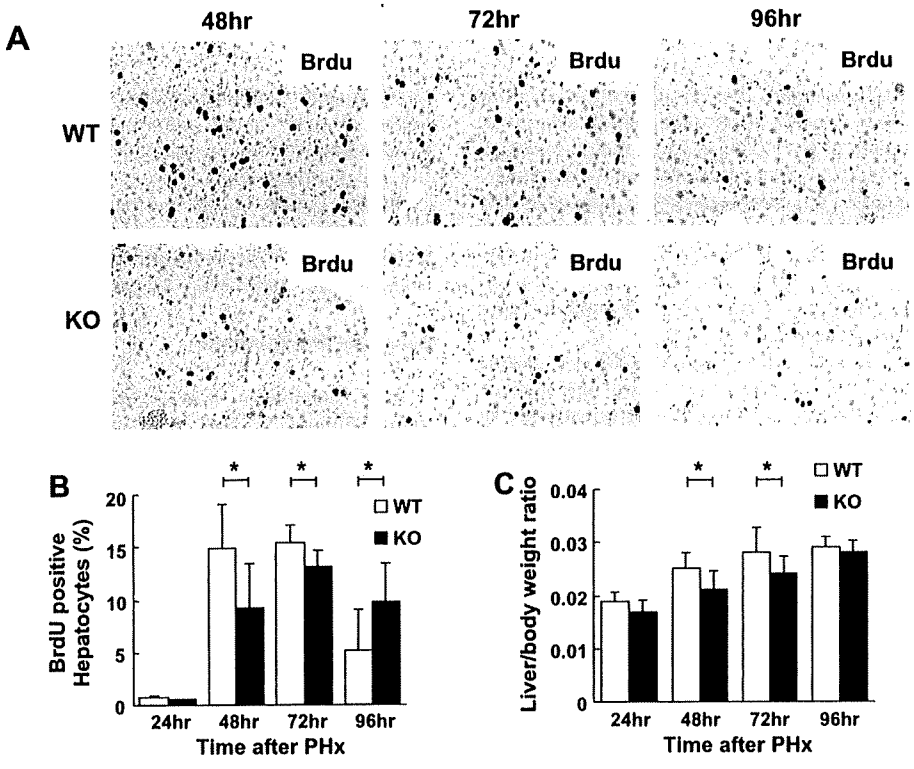

Fig. 1. Delayed hepatocytes proliferation and restitution of liver mass after PHx in adiponectin KO mice. (A) Representative micrographs of liver sections harvested from WT and KO mice after PHx, immunostained with BrdU antibody. (B) Quantitative analysis of BrdU labeling index (percentage of BrdU-positive hepatocytes) in the regenerating liver. (C) Serial changes in liver/body weight ratio after PHx. Data are means ± SD values of 6–8 mice per group and time point. *$P < 0.05$.

of cyclin D1 protein in KO mice compared with WT mice at 24 h after PHx (Fig. 2A and B). Furthermore, the mRNA levels of cyclin A2 and cyclin B1 measured by real-time RT-PCR in KO mice were significantly lower at 48 and 72 h after PHx than in WT mice (Fig. 2C and D).

Increased fatty change and triglyceride content in regenerating livers after PHx in adiponectin KO mice

Lipid droplets accumulate in hepatocytes of the regenerating livers and this process is known to be essential for hepatocyte proliferation during liver regeneration [12]. We examined whether adiponectin deficiency affects this process by histological analysis and measurement of triglyceride content in the regenerating livers of WT and KO mice after PHx. We assessed lipid droplet formation in H&E stained liver sections at 0, 48, and 72 h after PHx. Lipid droplets were found in many hepatocytes at 48 and 72 h after PHx in both genotypes. At 72 h after PHx, KO mice showed increased fat accumulation compared with WT mice (Fig. 3A). Quantitative analysis confirmed that hepatic triglyceride content were significantly higher in KO mice than WT mice at 72 h after PHx (Fig. 3B).

Reduced mRNA expression levels of fatty acid oxidation-related genes in regenerating livers after PHx in adiponectin KO mice

To determine the molecular mechanism responsible for the increased hepatic fat accumulation in KO mice after PHx, we investigated fatty acid metabolism during liver regeneration by analyzing the mRNA expression level of CPT-1, a rate-limiting enzyme of fatty acid β-oxidation, at 0, 12, 24, 48, and 72 h after PHx. We also examined the mRNA expression level of PPARα, which is involved in fatty acid metabolism, at 0, 24, 48, 72, and 96 h after PHx. Real-time RT-PCR analysis showed downregulation of CPT-1 and PPARα in KO mice compared with WT mice at all time points. The differences in CPT-1 expression between the control and KO mice were significant at 24 and 72 h after PHx (Fig. 4A). Likewise, the differences in PPARα expression between the two groups were significant at 72 and 96 h after PHx (Fig. 4B). These results indicate that impaired fatty acid oxidation results in increased fat accumulation in the regenerating livers of adiponectin KO mice.

Discussion

In this study, we tested the hypothesis that adiponectin influences hepatocyte proliferation during liver regeneration. Our results in mice demonstrated that adiponectin deficiency caused delays in hepatocyte proliferation and restitution of liver mass during liver regeneration.

During the first few hours after PHx, various genes involved in cytokine signaling are induced to prime the resting hepatocytes for cell division [1]. TNFα and IL-6 are strongly induced in this process [1] and mouse genetic studies confirmed that liver regeneration is impaired in mice with homozygote mutation of IL-6 or TNF receptor type 1 [2]. Previous studies also demonstrated that leptin KO mice show disturbed liver regeneration and low expression of TNFα and IL-6 after carbon tetrachloride-induced liver injury [13]. Furthermore, we reported previously that adiponectin has anti-inflammatory properties as it suppressed the secretion of TNFα from Kupffer cells in lipopolysaccharide- and D-galactosamine-induced liver injury model in mice [7]. To further analyze the contribution of adiponectin to the priming phase of liver regeneration, we first determined whether impaired liver regeneration in adiponectin KO mice is due to abnormal production of these cytokines. However, there was no significant difference in serum levels and hepatic expression levels of TNFα and IL-6 between adiponectin KO and WT mice (data not shown). This was also confirmed by the fact that the expression levels of immediate early genes were not altered in the regenerating adiponectin KO livers (data not shown). These data suggest that adiponectin is not involved in the priming phase of liver regeneration.

It is reported that overexpression of cyclin D1 is associated with the onset of hepatocyte DNA replication in rodent models of liver regeneration [14]. We next examined the expression of cyclin D1 in the regenerating livers and showed a significant reduction of cyclin D1 expression in adiponectin KO mice compared with wild-type.

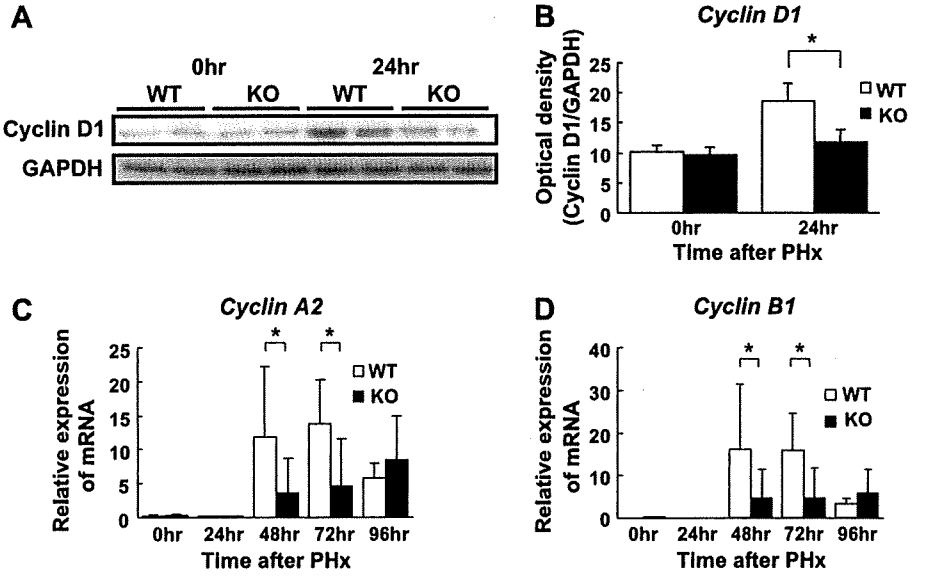

Fig. 2. Reduced expression levels of cell cycle regulatory proteins in adiponectin KO livers after PHx. (A) Western immunoblot analysis of cyclin D1 expression in the liver. Representative blots are presented. (B) Results of densitometric analysis. Data were expressed relative to GAPDH expression. Expression levels of cyclin A2 (C) and cyclin B1 (D) mRNAs in the regenerating liver after PHx measured by real-time RT-PCR. Data were expressed relative to GAPDH expression. Data are means ± SD values of 6–8 mice per group and time point. $^{*}P < 0.05$.

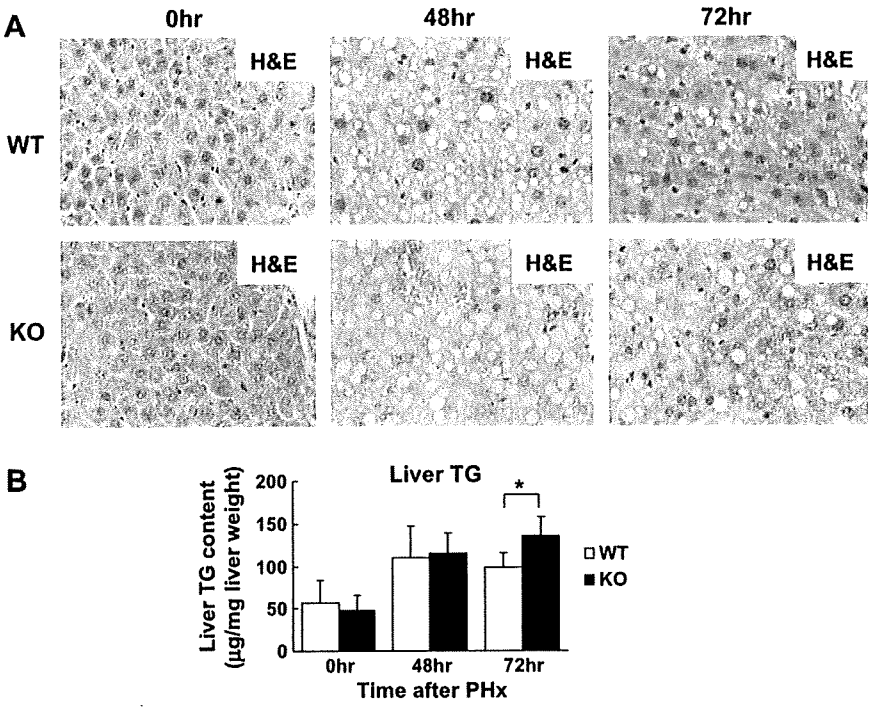

Fig. 3. Increased lipid droplet formation and triglyceride accumulation in hepatocytes of adiponectin KO mice after PHx. (A) Microphotographs of liver sections harvested from WT and KO mice after PHx stained with hematoxylin and eosin. Magnification, 200×. (B) Triglyceride contents in the regenerating livers. Data are means ± SD values of 6–8 mice per group and time point. *$P < 0.05$.

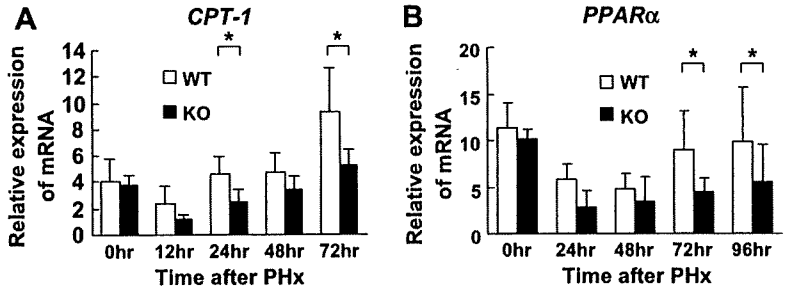

Fig. 4. Decreased expression levels of CPT-1 and PPARα in the regenerating liver of adiponectin KO mice after PHx. The expression levels of CPT-1 (A) and PPARα (B) mRNAs in the regenerating liver of WT and KO mice after PHx by real-time RT-PCR. Data were expressed relative to GAPDH expression. Data are means ± SD values of 6–8 mice per group and time point. *$P < 0.05$.

We also showed reduced expression of cyclin A2 in adiponectin KO regenerating livers, indicating that adiponectin deficiency caused disturbances of the G1/S transition (DNA replication) of cell cycle progression during liver regeneration. Stimulation of Cdk1–cyclin B1 complex kinase activity is essential for G2/M transition (mitosis) during liver regeneration [15]. The current data also demonstrated decreased expression of cyclin B1 in adiponectin KO livers, suggesting that adiponectin deficiency resulted in abnormal S-phase progression and entry into mitosis in the process of liver regeneration.

The liver regeneration process requires complex signaling that includes the mobilization of nutrients and hepatocyte proliferation [16,17]. Accumulation of triglycerides in the liver manifests as fat droplet formation transiently after PHx and is believed to be essential for proper liver regeneration. Interestingly, a recent study showed that liver regeneration was impaired in mice lacking Caveolin1, an essential component of caveolae in the plasma membrane [12]. Impaired liver regeneration in Caveolin1 KO mice was associated with disrupted lipid droplet formation and was rescued by glucose feeding in advance, suggesting the important role of free fatty acid as an energy source during liver regeneration [12]. In the present study, we also observed fat accumulation in the regenerating livers and this phenomenon was more evident in hepatocytes of adiponectin KO than WT mice. This phenomenon was confirmed by increased triglyceride content in the regenerating livers of adiponectin KO mice. In the liver, adiponectin increases fatty acid oxidation through the AdipoR2–PPARα signaling pathway, which was recently confirmed in various mouse genetic models [4]. Therefore, we tested the hypothesis that the increased fat accumulation in adiponectin KO livers is due to impairment of fatty acid catabolism, leading to impaired energy supply required for proper hepatocyte proliferation. Although the expression levels of AdipoR1 and AdipoR2 in the regenerating livers of two genotypes were not different (data not shown), the expression of PPARα was reduced in the regenerating livers of adiponectin KO mice.

PPARα is known to stimulate the expression and enzyme activity of CPT-1, a rate-limiting enzyme of fatty acid oxidation, which regulates the uptake of acyl-CoA into the mitochondria [18]. Our study also showed reduced expression of CPT-1 in the regenerating livers of adiponectin KO, indicating that adiponectin deficiency caused impaired mitochondrial fatty acid oxidation.

Interestingly, there were no significant differences in serum glucose, free fatty acid and triglyceride levels in the partially hepatectomized mice of both genotypes (data not shown). These results support our hypothesis that delayed regeneration of adiponectin KO liver is mainly due to impaired mitochondrial fatty acid oxidation in hepatocytes of the regenerating liver.

The PPARα is the downstream target of adiponectin–AdipoR signaling pathway [5]. Homozygote mutation in mouse PPARα gene is reported to result in delayed liver regeneration with increased lipid accumulation in hepatocytes [19]. In addition, PPARα is known to be associated with cell proliferation through the regulation of cell cycle regulators, such as cyclin D1 [20]. These findings are in agreement with our results in adiponectin KO mice. Take together, low expression of PPARα might cause altered fat metabolism and cell cycle control in adiponectin KO mice during liver regeneration.

Clinically, low serum adiponectin levels are reported in obese patients with fatty liver [21], suggesting the possible contribution of adiponectin in the mechanism of impaired regeneration of fatty liver disease, such as non-alcoholic fatty liver disease and alcoholic liver disease. Further studies are needed to enhance our understanding of impaired liver regeneration.

In conclusion, this study provides new data linking adiponectin to liver regeneration.

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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

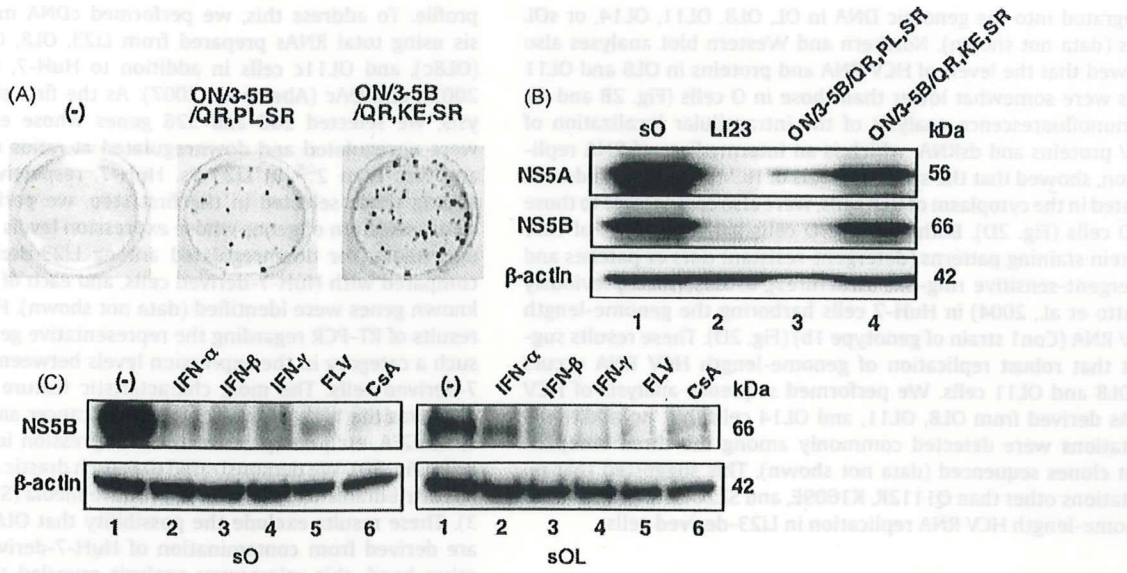

Fig. 1. Li23-derived cells harboring HCV replicon. (A) G418-resistant colonies from Li23 cells transfected with replicon RNA. ON/3-5B RNA with three additional mutations (ON/3-5B/QR,PL,SR or ON/3-5B/QR,KE,SR) was transfected into Li23 cells. The panels show G418-resistant colonies (57 colonies/μg RNA 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

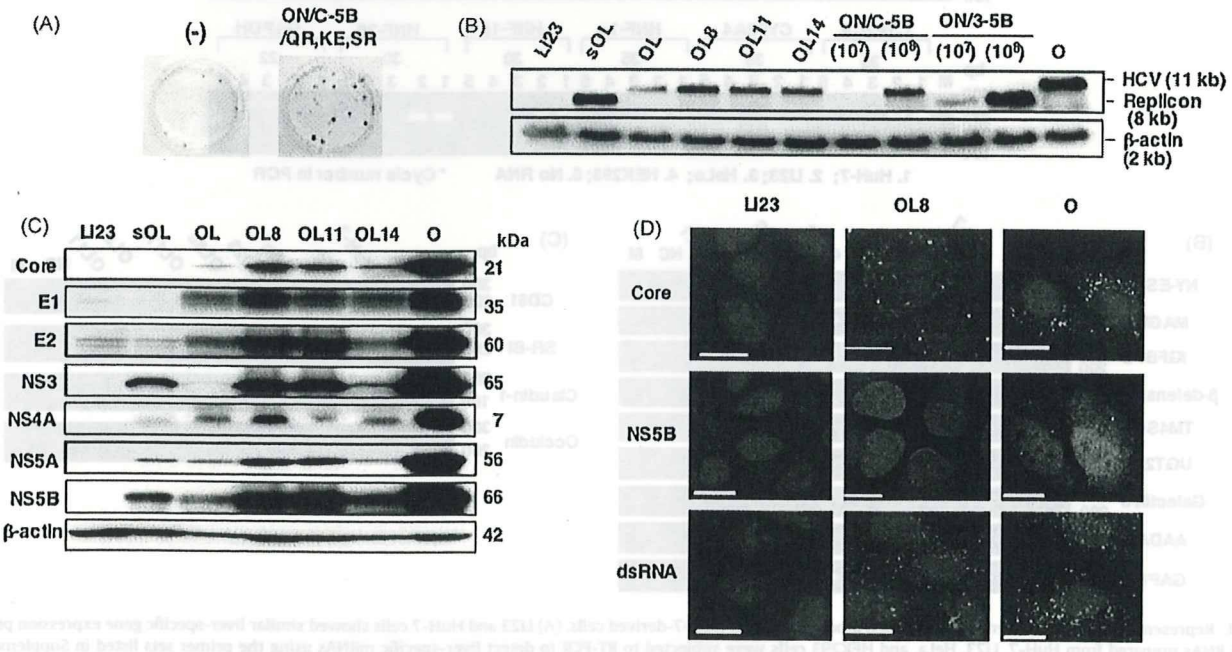

Fig. 2. Establishment of Li23-derived cell lines harboring replicative genome-length HCV RNA. (A) G418-resistant colonies from sOLc cells transfected with genome-length HCV RNA (ON/C-5B/QR,KE,SR). The panels show G418-resistant colonies (100 colonies/μg RNA) that were stained with Coomassie brilliant 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).

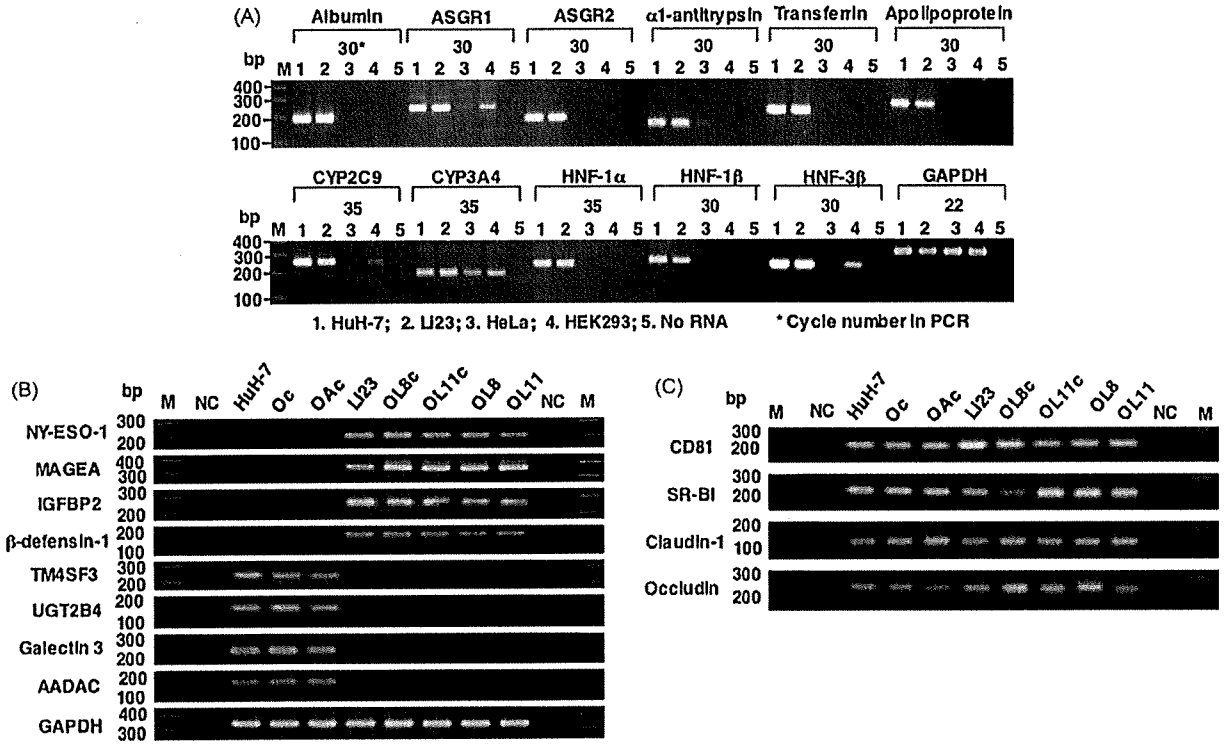

Fig. 3. Representative genes differentially expressed among Li23- and HuH-7-derived cells. (A) Li23 and HuH-7 cells showed similar liver-specific gene expression profiles. Total RNAs prepared from HuH-7, Li23, HeLa, and HEK293 cells were subjected to RT-PCR to detect liver-specific mRNAs using the primer 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).

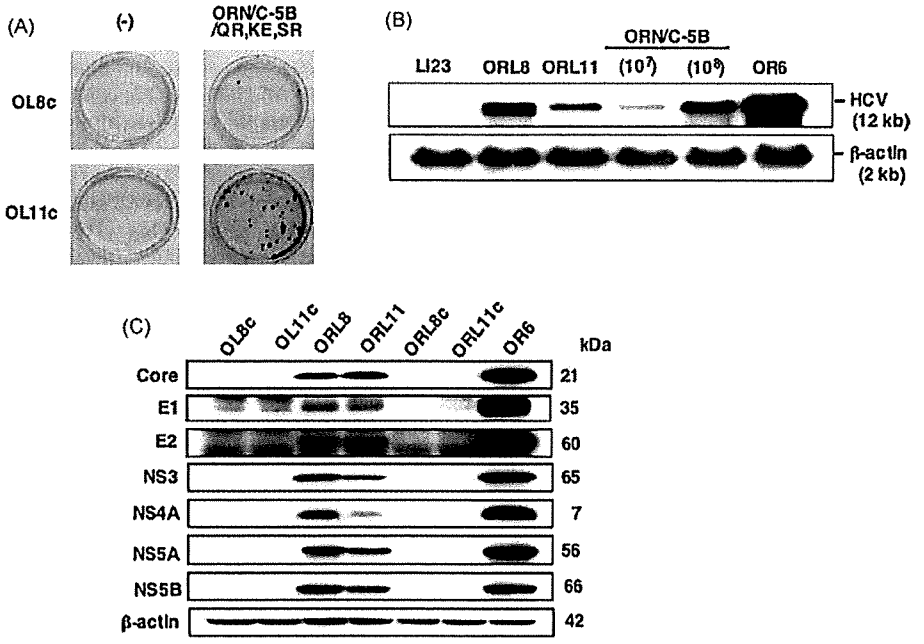

Fig. 4. Establishment of OL8- and OL11-derived cell lines harboring replicative genome-length HCV RNA encoding renilla luciferase. (A) G418-resistant colonies from OL8c or OL11c cells transfected with genome-length HCV RNA (ORN/C-5B/QR,KE,SR) encoding renilla luciferase gene. The panels show G418-resistant 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

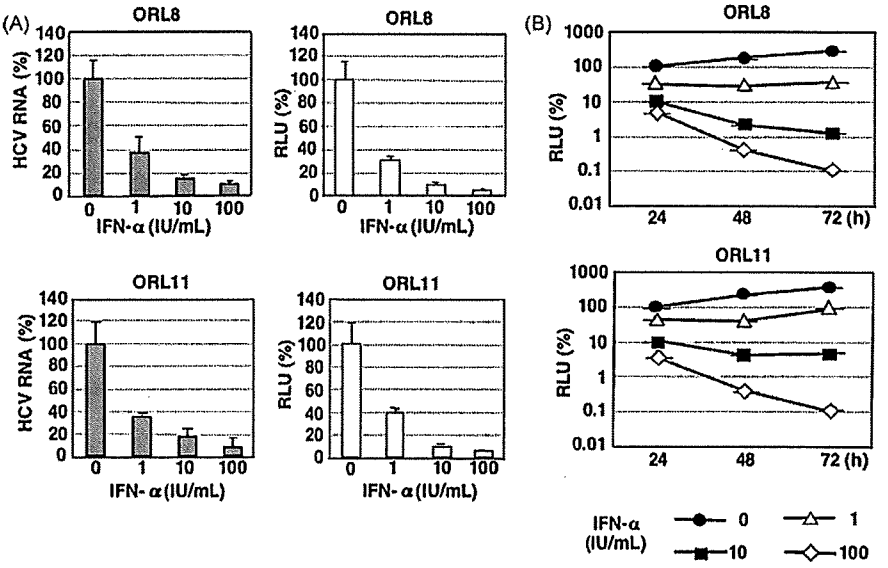

Fig. 5. ORL8 and ORL11 reporter assay system to monitor genome-length HCV RNA replication. (A) Renilla luciferase activity is correlated with HCV RNA level. The ORL8 (upper panels) and ORL11 (lower panels) cells were treated with IFN-α (0, 1, 10, and 100 IU/ml) for 24 h, and then luciferase reporter 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.,

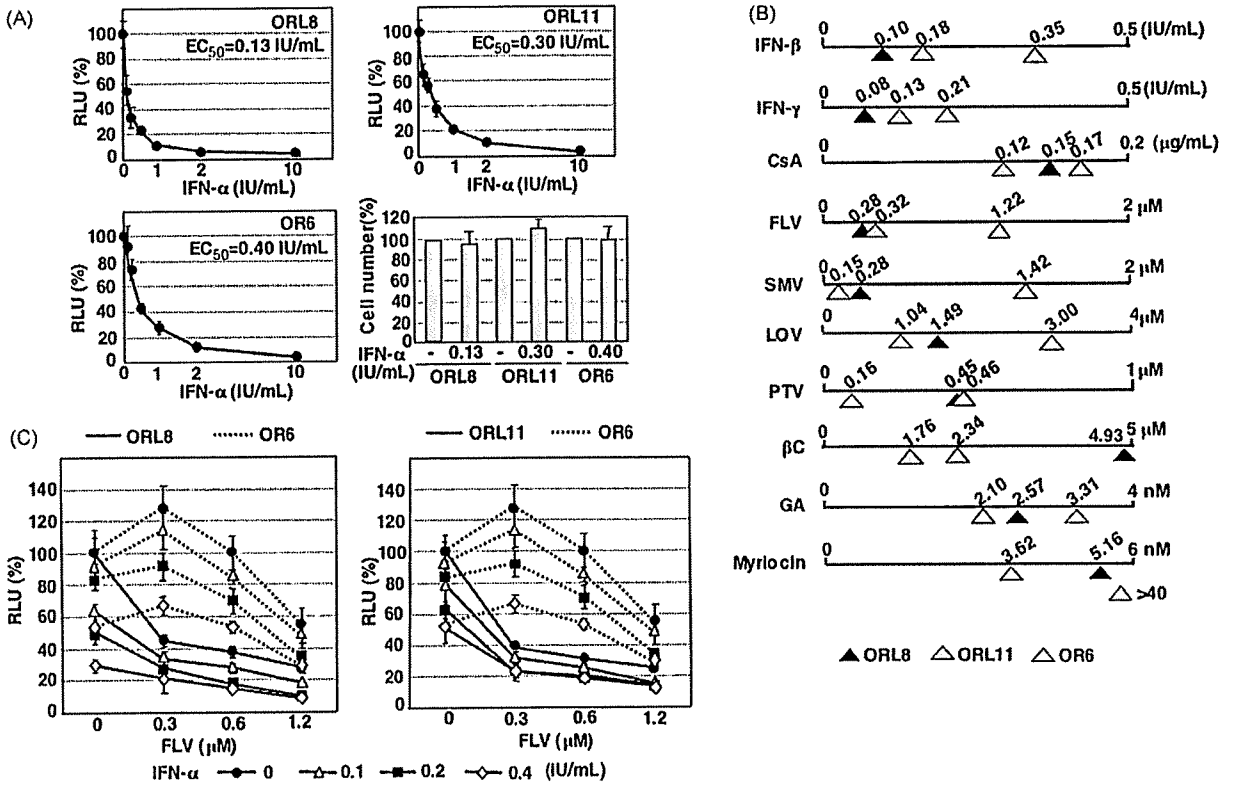

Fig. 6. The diverse effects of anti-HCV reagents in ORL8, ORL11, and OR6 assay systems. (A) IFN-α sensitivities on genome-length HCV RNA replication in ORL8, ORL11, and OR6 assay systems. The ORL8, ORL11, and OR6 cells were treated with IFN-α (0, 0.06, 0.13, 0.25, 0.5, 1, 2, and 10 IU/ml) for 72 h, and 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.

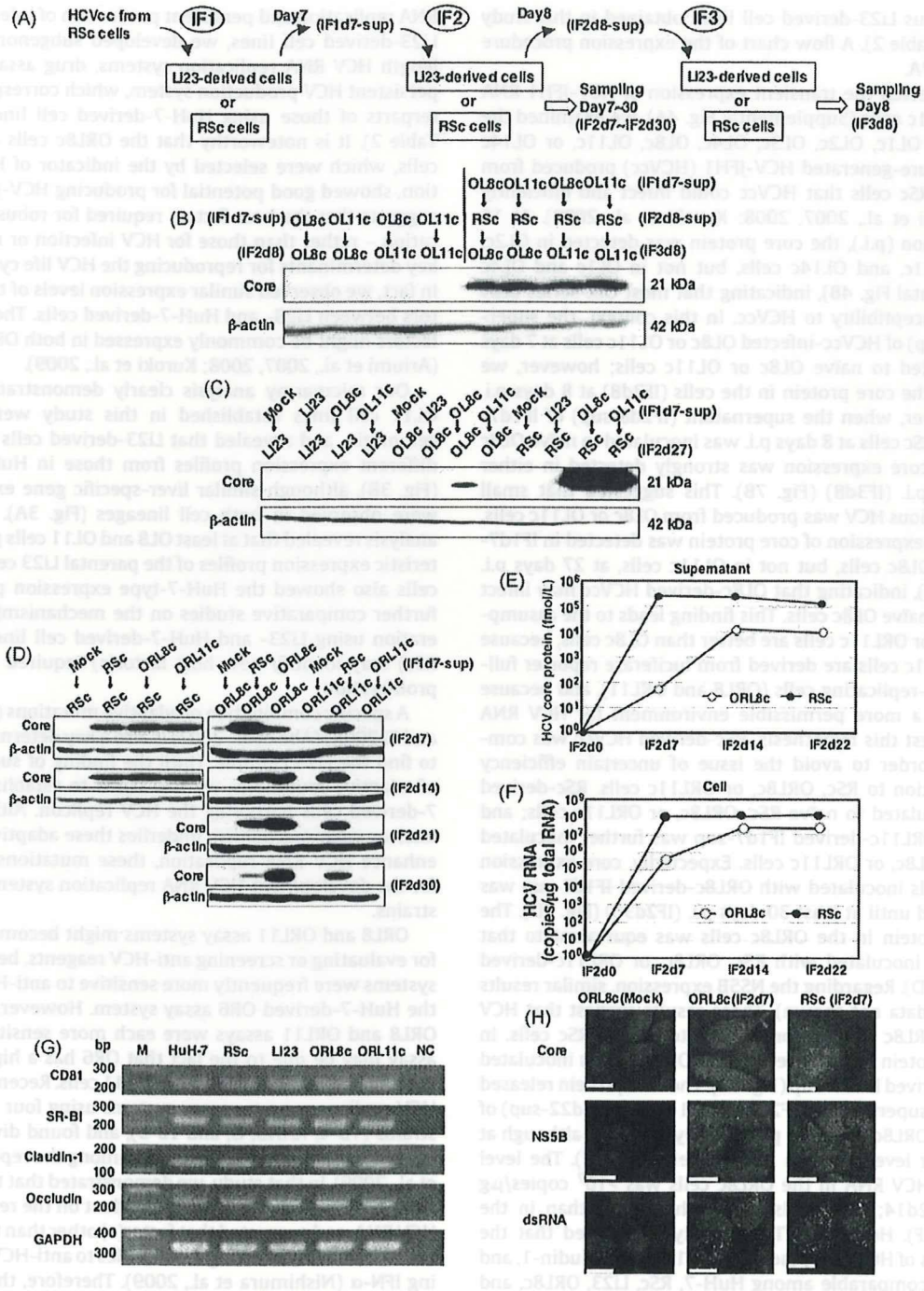

Fig. 7. Reproduction of HCV life cycle in OL8c and ORL8c cells. (A) Flow chart of experiments on HCVcc infection. A time schedule for HCVcc infection and sampling is shown. IF1d7-sup refers to the supernatant of the first-infected cells at 7 days p.i. IF2d7 refers to the secondly infected cells at 7 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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ORIGINAL ARTICLE

HCV genotype 1b chimeric replicon with NS5B of JFH-1 exhibited resistance to cyclosporine A

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Abstract Cyclosporine A (CsA) is a well-characterized anti-HCV reagent. Recently it was reported that the genotype 2a JFH-1 strain was more resistant than genotype 1 HCV strains to CsA in a cell culture system. However, the JFH-1 responsible region for the resistance to CsA remains unclear. It was also demonstrated that in genotype 1b HCVs, NS5B interacts with cyclophilin (CyP). To clarify whether or not NS5B of JFH-1 is significant for CsA resistance, we developed a chimeric replicon with NS5B of JFH-1 in the genotype 1b backbone. The chimeric replicon was more resistant to CsA than the parental genotype 1b replicon. Furthermore, reduction of CyPA had a greater effect on HCV RNA replication and sensitivity to CsA than reduction of CyPB. Here, we demonstrated that NS5B of JFH-1 contributed to this strain's CsA-resistant phenotype. NS5B and CyPA are significant for determining HCV's sensitivity to CsA.

Introduction

The combination of a pegylated interferon (IFN) with ribavirin (RBV) is the current standard therapy for chronic hepatitis C and yields a sustained virological response (SVR) rate of about 55% [6]. This means that about 45% of patients with chronic hepatitis C are still threatened by the progress of the disease to cirrhosis and hepatocellular carcinoma. To find a more effective therapy, several anti-HCV reagents have been reported using HCV replicon systems [11, 14]. Especially, cyclosporine A (CsA), which is widely used as an immunosuppressive reagent, and its derivatives, which lack immunosuppressive activity, possess anti-HCV activity [8, 18, 19]. These reagents will help to improve the SVR rate.

Cyclophilins (CyPs), CsA ligands, are a family of cellular enzymes possessing peptidyl-prolyl isomerase activity. CyP family members play significant roles in numerous cellular processes, including transcriptional regulation, immune response, protein secretion and mitochondrial function [7]. CsA possesses three major cellular targets: CyP, the calcineurin-nuclear factor of activated T-cells pathway and P-glycoprotein [7]. The mechanism of anti-HCV activity of CsA is through disassociation between CyP and HCV nonstructural protein 5B (NS5B), an RNA-dependent RNA polymerase [20]. Fernandes et al. [5] also reported that NS5A was significant in the sensitivity of HCV to CsA. However, the role of CyPs as a cellular target of CsA in HCV RNA replication remains controversial [17, 20, 21]. While genotype 1a and 1b HCV strains were highly sensitive to CsA, a genotype 2a strain, JFH-1, was less sensitive to CsA [12, 21]. Moreover, in genotype 1b HCV, interaction between CyPB and HCV NS5B is required for robust HCV RNA replication [10].

To investigate whether or not NS5B of JFH-1 is an important factor for determining sensitivity to CsA, we engineered a 1b/2a chimeric HCV subgenomic replicon derived from genotype 1b HCV-O RNA, in which NS5B and a 3′-untranslated region (UTR) were replaced with

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those of HCV JFH-1 RNA. This replicon system enables to investigate the direct effect of NS5B on the CyPs.

We report here that NS5B of JFH-1 contributes to the CsA-resistant phenotype of this strain. Furthermore, CyPA but not CyPB is essential for HCV RNA replication in 1b and 1b/2a chimeric replicon-harboring cells. Finally, supplementation with vitamin E (VE) negates the anti-HCV activity of CsA in the presence or absence of CyPs. These results contribute to our understanding of the mechanism(s) that mediate the efficacy of CsA's anti-HCV activity.

Materials and methods

Cell culture

293FT cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum. The HuH-7-derived OR6c cells were cultured as previously described [10]. The cells harboring the subgenomic replicon were maintained in the culture medium containing G418 (0.3 mg/ml; Promega, Madison, WI).

Reagents

IFN-α, IFN-γ, and VE were purchased from Sigma-Aldrich (St. Louis, MO). CsA was purchased from Calbiochem (San Diego, CA). Pitavastatin (PTV) was purchased from KOWA Co., Ltd. (Tokyo, Japan). Mizoribine (MZB) and RBV were kindly provided by Asahi Kasei Pharma (Tokyo, Japan) and Yamasa Corporation (Choshi, Japan), respectively.

Plasmid construction

The plasmid of pORN/3-5B/QR,KE,RS/5B(J) was based on pORN/3-5B/QR,KE,RS [1] and was constructed by replacing the NS5B coding region and 3′UTR with the corresponding JFH-1 sequence. The NS5A/NS5B junction was set after amino acid 2419 of HCV-O and generated by polymerase chain reaction (PCR). The sequence numbering for coding and non-coding regions was based on a sequence from GenBank: HCV-O (accession no. **AB191333**) and JFH-1 (accession no. **AB047639**). Retroviral vector pCX4bsr [2] was used as an expression vector. To obtain full-length CyPA and CyPB cDNAs, reverse transcription (RT)-PCR with KOD-plus DNA polymerase (Toyobo, Osaka, Japan) was performed as previously described [4]. The pCX4bsr/Myc-CyPA and pCX4bsr/Myc-CyPB plasmids expressing Myc-tagged CyPA and CyPB, respectively, were obtained by inserting the PCR products of full-length CyPA and CyPB into the MluI-NotI sites of the pCX4bsr/Myc vector. Expression plasmids for HA-tagged NS5B (HCV-O and JFH-1) were generated by insertion of PCR fragments encoding each HCV protein into the MluI-NotI sites of the pCX4bsr/HA vector. The sequences of all constructed plasmids were confirmed by the sequencing analysis as described previously [1].

RNA synthesis, RNA transfection, and selection of G418 cells

Plasmid DNAs were linearized by XbaI and used for the RNA synthesis with the T7 MEGAScript kit (Ambion, Austin, TX). In vitro transcribed RNA was transfected into OR6c cells by electroporation [9]. The transfected cells were selected in culture medium containing G418 (0.3 mg/ml) for 3 weeks.

RNA interference, lentiviral vector construction

A detailed description of methods of RNA interference and lentiviral vector construction is available in Supplementary Materials.

Western blot analysis

Western blot analysis was performed as described previously [1]. The antibodies used in this study were those against NS3 (Novocatra Laboratories, UK), NS5A (a generous gift from Dr. A Takamizawa, Research Foundation for Microbial Diseases, Osaka University), NS5B (1b, a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), NS5B (1b/2a) [15], CyPA (BIOMOL, Plymouth Meeting, PA), CyPB (Affinity BioReagents, Rockford, IL), and β-actin (AC-15; Sigma).

Immunoprecipitation

Immunoprecipitation was performed as described previously [10]. Briefly, pre-cleared cell lysates were incubated with an anti-Myc antibody (PL14; MBL, Nagoya, Japan). Immunocomplexes were recovered by adsorption to protein G-Sepharose resin (GE Healthcare Bioscience, Uppsala, Sweden). After three washes with lysis buffer, the immunoprecipitates were analyzed by immunoblot analysis using anti-Myc and anti-HA (3F10; Roche, Mannheim, Germany) antibodies.

Evaluation of sensitivity of anti-HCV reagents

The cells were plated onto 24-well plates ($1.5–2 \times 10^4$ cells/well). After 24 h culture, the culture medium was replaced with anti-HCV reagent containing medium. After 72 h additional culture, the cells were washed with