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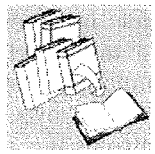
REVIEW



Chemical genetics approach to hepatitis C virus replication: cyclophilin as a target for anti-hepatitis C virus strategy

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SUMMARY

Hepatitis C virus (HCV) is a major causative agent of liver diseases such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Because the current standard therapy, interferon (IFN) or pegylated-IFN alone or in combination with ribavirin, is ineffective on approximately half of the HCV-infected patients, alternative therapeutics are greatly needed. The chemical genetics method is a useful strategy to elucidate molecular mechanisms of the viral life cycle and screen for anti-viral agents. This review focuses on the use of chemical genetics approach to virology, which could be called 'chemical virology', and introduces an example of such analysis. From a cell culture-based screening, an immunosuppressant cyclosporin A (CsA) was identified as an anti-HCV compound. Analysis using CsA as a bioprobe showed that cyclophilin (CyP) B, a cellular target of CsA, regulates the function of HCV RNA polymerase NS5B, which is essential for efficient viral genome replication. By targeting CyP, HCV genome replication was drastically suppressed. Thus, chemical genetics analysis identified CyPB as a cellular cofactor of HCV genome replication and a target for novel anti-HCV agents. Copyright © 2007 John Wiley & Sons, Ltd.

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INTRODUCTION

Hepatitis C virus (HCV), a member of the flaviviridae family, has a positive strand RNA genome [1]. The genome encodes a precursor polyprotein, which is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, envelope (E)1, E2, p7, nonstructural protein (NS)2, NS3, NS4A, NS4B, NS5A and NS5B [2]. NS5B is an RNA-dependent RNA polymerase, which plays a central role in viral genome replication [1].

HCV is a major causative agent of liver diseases such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [3]. HCV infection constitutes a serious health problem worldwide, affecting approximately 170 million individuals and causing approximately 280 000 deaths a year [4]. Currently, anti-HCV therapy has been restricted mainly to treatment with interferon (IFN) or pegylated-IFN alone or in combination with ribavirin (RBV) [4]. Since this treatment eliminates the virus persistently from only about half of HCV-infected patients, however, alternative approaches to eliminating HCV infection are greatly needed [5]. To develop new strategies against HCV, it is essential to analyse the mechanism of HCV replication as well as screening for anti-HCV compounds.

HCV subgenomic replicon system is a system to investigate HCV genome replication in cell culture [6,7]. From biochemical analyses using this replicon system, it is proposed that HCV genome replication occurs in the replication complex (RC), which includes the viral genome RNA and HCV NS proteins [8–12]. The RC is surrounded by a membrane structure and is protected from cellular proteases and nucleases, which seems to contribute to the increase of replication efficiency. The RC is formed on intracellular membranes, including the endoplasmic reticulum (ER). Electron

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Abbreviations used
HCV, hepatitis C virus; IFN, interferon; CsA, cyclosporin A; CyP, cyclophilin; E, envelope; NS, nonstructural protein; RBV, ribavirin; RC, replication complex; ER, endoplasmic reticulum; HMG, 3-hydroxy-3-methyglutaryl; CN, calcineurin; P-gp, P-glycoprotein

microscopy analyses have demonstrated that HCV proteins induce alterations in membrane structures around the ER, which is related to the formation of the RC [9,13]. Genetic analyses have shown that the amino acid substitutions that increase replication efficiency often occur in the viral genome [14–16]. Although the mechanisms responsible for the increase of replication efficiency by such adaptive mutations are not well understood, the S2204I mutation within the NS5A coding region, one of the most frequently observed adaptive mutations, has been found to suppress hyperphosphorylation of NS5A and be related to replication efficiency [17]. Moreover, molecular biological analyses have shown a series of cellular factors interacting with HCV proteins that may be involved in HCV genome replication [18]. Thus, the mechanisms of HCV genome replication can be better understood by different approaches.

CHEMICAL GENETICS FOR INVESTIGATING HCV REPLICATION

Chemical genetics, which has been proposed and developed during the late 1990s, is a research field in which chemical compounds are used to understand and control cellular and physiological functions of protein [19]. This technique involves analysis of phenotypic changes of a target cell that occur upon treatment with a compound that binds to and changes the function of the target molecule, in a manner similar to introducing a mutation into the protein when using classical genetics techniques (see below and Figure 1). In addition to biochemical, molecular biological and virological techniques, chemical genetics approaches can be applied and be useful to analysis of the regulatory mechanisms of viral life cycles, which are regulated by not only the virus itself, but also various cellular factors. The experimental flowchart for such analysis is summarised in Figure 1. First, screening is undertaken to identify compounds that affect the activity of a virological phenotype, such as viral genome replication, cytopathic effect, viral adhesion to cells, etc. (Fig. 1A). Second, the cellular target of the compound is identified by biochemical and molecular biological assays (Figure 1B). Finally, by examining the relationship between the 'phenotype' and the 'target', the molecular mechanism underlying the viral life cycle can be elucidated (Figure 1C).

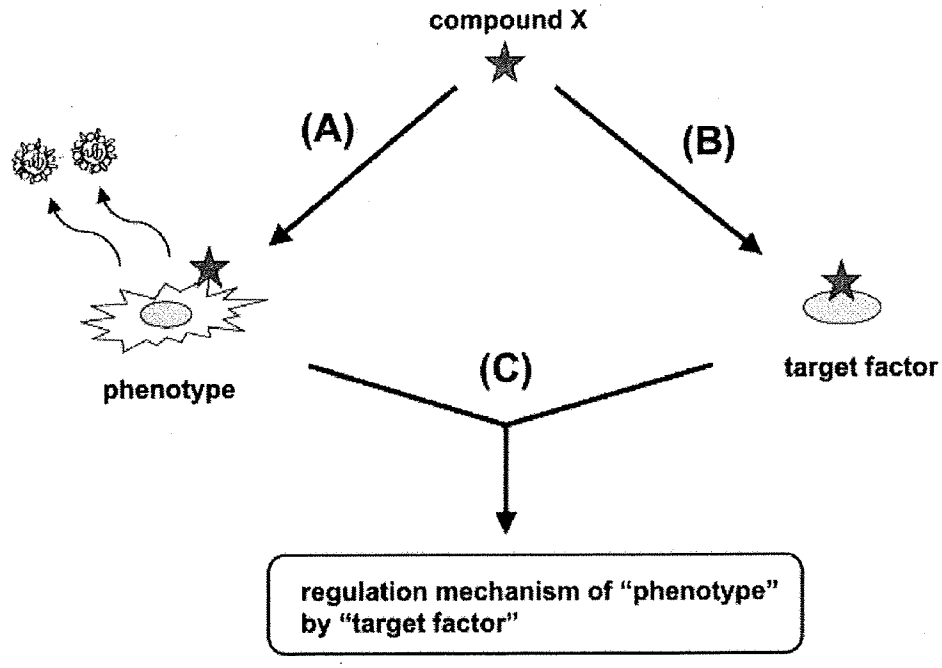

Figure 1. Schematic representation of chemical genetics analysis of viruses. (A) Screening for compounds that change viral phenotypes, such as viral genome replication. (B) Identification of the cellular target factor(s) of the compounds obtained in (A). (C) Analysis of the regulation of the 'phenotype' [observed in subpart (A)] by the 'target factor' [identified in subpart (B)]

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Mechanisms discovered using this method are expected to serve as targets for anti-viral strategies. Further, compounds found during screening may be useful as lead compounds for anti-viral drug development. Using such forward chemical genetics, cellular factors that play significant roles in HCV genome replication have been identified in at least three series of studies, as discussed below.

F-box and leucine-rich repeat protein 2 (FBL2)

The regulation of NS5A by FBL2 was demonstrated by studying the observation that lovastatin suppressed HCV genome replication. Ye *et al.* [20] reported that inhibitors of protein geranylgeranylation, including the 3-hydroxy-3-methyglutaryl (HMG) CoA reductase inhibitor lovastatin and an inhibitor of protein geranylgeranyl transferase I, suppressed HCV genome replication, accompanied by the dissolution of the RC. Kapadia and Chisari [21] also showed that lovastatin inhibited HCV genome replication. Addition of geranylgeraniol to lovastatin-treated cells restored viral replication [20]. From these results, the authors suggested that the geranylgeranylation of a host protein should be important for HCV genome replication. This geranylgeranylated protein has been identified as FBL2, a member of the F-box protein family [22]. FBL2 interacted with NS5A in a geranylgeranylation-dependent manner, and the knockdown of endogenous FBL2 or expression of a dominant negative form of FBL2 inhibited HCV genome replication. These results suggest that FBL2 regulates HCV genome replication through the geranylgeranylation-dependent interaction with NS5A. Further analysis is required to elucidate the mechanism by which FBL2 regulates the function of NS5A.

Sphingolipid

Screening using the subgenomic replicon system identified a lipophilic long-chain base compound, named NA255, from fungal metabolites as an inhibitor of HCV genome replication [23]. NA255 prevented the synthesis of sphingolipids, major components of the lipid rafts. It was suggested that sphingomyelin binds to NS5B and recruits it to the lipid rafts on the ER membrane, on which the HCV RC is assembled. NA255 was suggested to disrupt the association of HCV proteins to the RC.

Cyclophilin B

Through the forward chemical genetics approach, we found that an immunosuppressant cyclosporin A (CsA) possesses anti-HCV activity [24]. Analysis using CsA as a bioprobe suggested that cyclophilin (CyP) B, a cellular target of CsA, could play a critical role in HCV genome replication [25]. CyPB stimulated the RNA binding activity of NS5B via molecular interaction with NS5B and we showed that this regulatory mechanism could serve as a target for the development of anti-HCV agents. Our series of studies on CyPB are described below to illustrate the usefulness of the chemical genetics for analysing HCV life cycles.

CYCLOPHILIN B AS A REGULATOR OF HCV GENOME REPLICATION DEMONSTRATED BY CHEMICAL GENETICS

Suppression of HCV genome replication by cyclosporin A

Screening for compounds suppressing HCV genome replication was performed using the HCV subgenomic replicon system. Treatment with 100 IU/mL IFN-α for 7 days as a positive control decreased HCV RNA in the cells to around 1/400 (Figure 2A). Of many compounds tested, CsA, given at 1 μg/mL for 7 days, decreased HCV RNA to about 1/500 (Figure 2A) [24]. CsA also reduced the expression of HCV-encoded proteins to undetectable levels without affecting cellular protein expression (Figure 2B), and suppressed HCV RNA synthesis. These anti-viral effects were observed without cytotoxicity. CsA treatment inhibited the multiplication of HCV genomic RNA in a time course of *in vitro* infection experiment using the plasma derived from an HCV-infected patient [24]. These data indicate that CsA suppresses HCV genome replication in cell culture.

CyPB plays an important role in HCV genome replication

The next step was to identify the CsA target factor involved in HCV genome replication as shown in Figure 1B. CsA has three major cellular targets: CyP, the calcineurin (CN)/NF-AT pathway, and P-glycoprotein (P-gp) [25–27]. CsA binds

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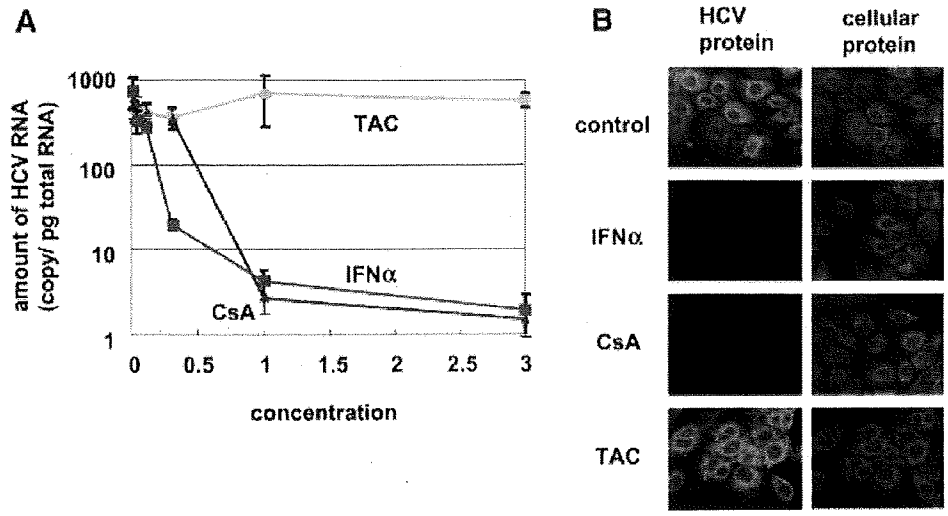

Figure 2. Suppression of HCV genome replication by cyclosporin A (CsA). (A) HCV RNA was quantified by real time RT-PCR analysis in cells treated with interferon (IFN)α (×100 IU/mL), CsA (μg/mL), or tacrolimus (TAC) (μg/mL) at the indicated doses for 7 days. (B) HCV NS5A as an HCV protein (green) and PDI as a cellular protein (red) were detected by indirect immunofluorescence analysis in cells treated without (control) or with 100 IU/mL IFNα, 1 μg/mL CsA or 1 μg/mL TAC for 7 days. (Reprinted from reference [24] by American Association for the study of Liver Diseases, with permission from Elsevier)

directly to peptidyl prolyl cis–trans isomerase, CyP, and inhibits its enzymatic activity. The CsA/CyP complex subsequently interacts with and inhibits CN, a phosphatase involved in the activation of transcription factor NF-AT. The enzymatic inhibition of CN by CsA prevents the nuclear translocation and activation of NF-AT, which is essential for the T-cell immune response. The immunosuppressive function of CsA is mediated by the inhibition of the CN/NF-AT pathway. As the third action, CsA inhibits the activity of P-gp, a transporter on the plasma membrane. To determine which inhibitory action of CsA mediates the suppression of HCV genome replication, a series of CsA derivatives with activity against only some targets was used. Inhibition of CyP, but neither the inhibition of the CN/NF-AT pathway nor the P-gp activity, correlated with the suppression of HCV genome replication [25]. In support of this, other CyP inhibitors, sanglifehrins, also decreased HCV RNA in cells [25]. Thus, CyP plays a role in HCV genome replication.

CyP is a protein family consisting of at least 15 subtypes in mammals [28]. RNAi analysis showed that the specific knockdown of CyPA, CyPC, CyPE or CyPH did not affect HCV genome replication, while downregulation of CyPB decreased replication [25]. This indicates that CyPB specifically regulates HCV genome replication.

CyPB stimulates the RNA binding activity of NS5B

Finally, experiments were performed to elucidate the molecular mechanism by which CyPB associates with the viral life cycle (Figure 1C). First, we analysed binding between CyPB and HCV proteins. GST pull-down assays showed that recombinant CyPB interacted with NS5B but not with NS3, NS4B or NS5A protein [25]. In contrast, CyPA did not bind any HCV protein. Endogenous CyPB and NS5B were associated in co-immunoprecipitation analysis. This CyPB–NS5B interaction was dissociated following treatment with CsA. It is known that both NS5B and the HCV RNA are mainly localised to the cytoplasmic surface of the ER [25]. It was demonstrated that a fraction of CyPB was also localised on the cytoplasmic face of the ER membrane, where it co-localised with NS5B and HCV RNA [25]. Moreover, NS5B and CyPB formed a complex with HCV RNA in cells. Functionally, it was shown that CyPB regulated the RNA binding activity of NS5B. Specific knockdown of CyPB or treatment with CsA reduced the RNA-bound NS5B in the RC. Further, *in vitro*

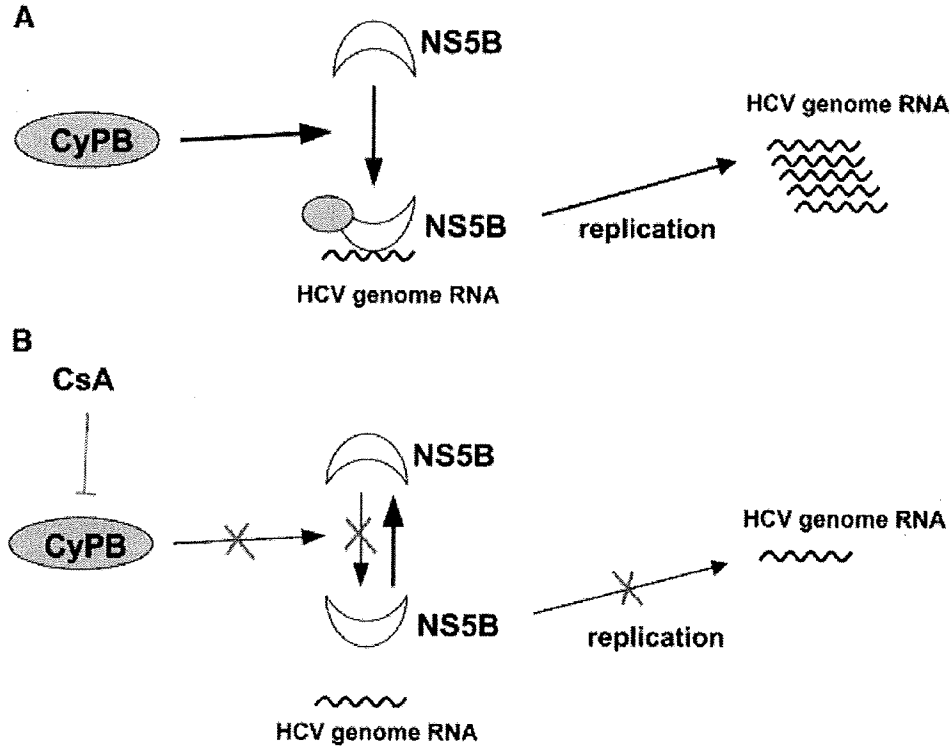

Figure 3. Schematic representation of the regulation of NS5B by cyclophilin (CyP) B. (A) In the normal state (in the absence of CsA), HCV RNA polymerase NS5B interacts with cellular CyPB. Through this interaction, the RNA binding activity of NS5B is increased, resulting in efficient replication of the HCV genome. (B) In the presence of CsA, the association of NS5B with CyPB is disrupted. The activity of NS5B is decreased and the level of HCV genome replication is reduced. Thus, CsA suppresses HCV genome replication

RNA binding assays demonstrated that the addition of recombinant CyPB augmented the RNA binding affinity of NS5B.

These findings are summarised in Figure 3. In the normal state (without CsA) (Figure 3A), NS5B interacts with cellular CyPB. This interaction augments the RNA binding activity of NS5B and drives efficient HCV genome replication. In the presence of CsA (Figure 3B), however, the association of CyPB and NS5B is disrupted. Without the stimulation of RNA binding activity by CyPB, NS5B functions less efficiently in replication, resulting in reduced HCV genome replication. Thus, regulation of the NS5B RNA binding activity by CyPB is essential for efficient replication of the HCV genome [25].

CyP as a target for the development of anti-viral agents

As described above, the regulation of NS5B by CyPB has now been revealed. Further studies were performed to determine whether this mechanism might be targeted for the development of anti-HCV agents. Although CsA had strong anti-HCV activity in hepatocytes, this compound simultaneously exerts immunosuppressive activity by acting on T cells. Therefore, CsA itself may be a double-edged sword in the settings of the clinical treatment of HCV. Ideally, compounds might be found that lack immunosuppressive activity and that more strongly inhibit the CyPB–NS5B interaction. NIM811, a CsA derivative in which MeLeu at Position 4 is replaced by Melle, fulfils both of these criteria [29,30]. This compound completely lacks immunosuppressive function and has an approximately twofold stronger binding affinity to CyP [29]. The suppressive effect of NIM811 on HCV genome replication was greater than that of CsA, especially at relatively low doses as shown in Figure 4A [31,32]. Cotreatment with NIM811 and IFN-α achieved an approximately 2-log further reduction of replication compared with IFN-α treatment alone (Figure 4B). A 3-week treatment of the cells with NIM811 reduced the intra-

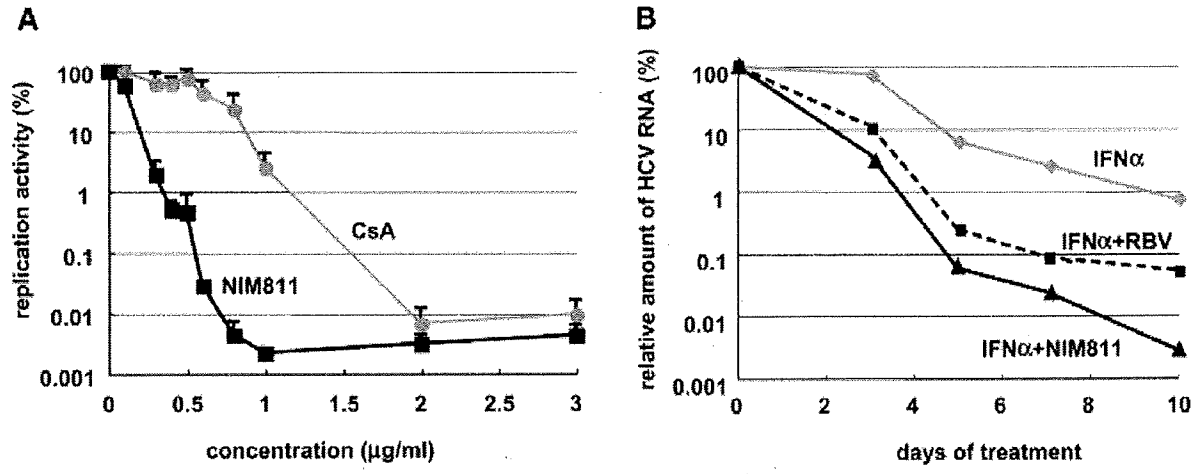

Figure 4. Suppression of HCV genome replication by NIM811. (A) Replication was monitored by the activity of luciferase expressed from a luciferase-containing replicon in cells treated with CsA or NIM811 at the indicated doses for 7 days. (B) HCV RNA was quantified in cells treated with 10 IU/mL IFNα alone, 10 IU/mL IFNα and 200 μM RBV or 10 IU/mL IFNα and 1 μg/mL NIM811 for the indicated number of days. (Reprinted from reference [31], with permission from Elsevier)

cellular HCV RNA below the detection level. Cotreatment with 1 μg/mL NIM811 and 10 IU/mL IFN-α for 10 days showed more than a 4-log reduction of HCV RNA, a far more dramatic reduction than that seen with IFN-α and RBV (Figure 4B) [31]. These results indicate that CyP can serve as a molecular target for the development of anti-HCV agents.

Effects of CyP inhibitors on HCV-infected patients

The anti-HCV effect of CsA has also been reported by several other groups [33–37]. Moreover, our study has elucidated the molecular mechanism of the regulation of HCV genome replication by CyPB. By targeting this mechanism, we identified NIM811 as a candidate anti-HCV agent. Clinical trials to evaluate NIM811 [31,35] as well as DEBIO-025, another non-immunosuppressive CsA derivative [37], are now underway. In regards to the effect of CsA itself on HCV-infected patients, it has been reported that cotreatment of IFN-α and CsA increases the sustained virological response compared with IFN-α monotherapy (55 vs. 32%) [38]. It was also reported that CsA treatment decreased HCV RNA to undetectable levels in 5/8 of patients who had HCV recurrence following liver transplantation and did not respond to IFN-α and RBV cotreatment [39]. Thus, the effects of cyclosporins on patients seem to be expectable, but these effects must be extensively investigated.

CONCLUSION

As discussed above, chemical compound-based virological analyses, which may be called 'chemical virology', can lead to novel findings regarding the mechanisms of the viral life cycle. Such analytical strategies can identify (1) viral replication mechanisms, (2) new function of cellular proteins, (3) molecular targets useful for the development of anti-viral compounds and (4) lead compounds for anti-viral agents. Among the many methods used to study viruses, chemical genetics may become a powerful tool to understand viral mechanisms at the molecular level.

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Serum-derived hepatitis C virus infectivity in interferon regulatory factor-7-suppressed human primary hepatocytes

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See Editorial, pages 1–5

***Background/Aims*: The development of an efficient in vitro infection system for HCV is important in order to develop new anti-HCV strategy. Only Huh7 hepatocyte cell lines were shown to be infected with JFH-1 fulminant HCV-2a strain and its chimeras. Here we aimed to establish a primary hepatocyte cell line that could be infected by HCV particles from patients' sera.**

***Methods*: We transduced primary human hepatocytes with human telomerase reverse transcriptase together with human papilloma virus 18/E6E7 (HPV18/E6E7) genes or simian virus large T gene (SV40 T) to immortalize cells. We also established the HPV18/E6E7-immortalized hepatocytes in which interferon regulatory factor-7 was inactivated. Finally we analyzed HCV infectivity in these cells.**

***Results*: Even after prolonged culture HPV18/E6E7-immortalized hepatocytes exhibited hepatocyte functions and marker expression and were more prone to HCV infection than SV40 T-immortalized hepatocytes. The susceptibility of HPV18/E6E7-immortalized hepatocytes to HCV infection was further improved, in particular, by impairing signaling through interferon regulatory factor-7.**

***Conclusions*: HPV18/E6E7-immortalized hepatocytes are useful for the analysis of HCV infection, anti-HCV innate immune response, and screening of antiviral agents with a variety of HCV strains.**

***Keywords*: Immortalization; Primary hepatocytes; HCV infection; IRF-7; IRF-3; HPV18/E6E7; Innate immune response**

1. Introduction

Infection with Hepatitis C virus (HCV) is a serious problem worldwide since 3% of the world's population is chronically infected [1]. Chronic HCV may lead to liver cirrhosis and hepatocellular carcinoma. Current standard therapy utilizes the combination of pegylated interferon-α and ribavirin, which results in a sustained response in only 30–60% of patients [2–5]. Many patients, however, do not qualify for or tolerate standard therapy [6]. Thus, it is important to develop an efficient in vitro infection system for HCV to facilitate the discovery of new anti-HCV strategies. Only Huh7 cell line is permissive for replication, infection and release of the fulminant hepatitis-derived HCV-2a (JFH-1) *strain* and its chimeric derivatives [7–9]. No other hepatocyte cell lines are able to support HCV replication efficiently.

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Normal human hepatocytes are the ideal system in which to study HCV infectivity. When cultured in vitro, however, they proliferate poorly and divide only a few times [10]. Continuous proliferation could be achieved however by introducing oncogenes, such as Simian virus large tumor antigen (SV40 T) [11]. This often resulted in tumor development [12] together with numerical (aneuploidy) and structural (aberrations) chromosome abnormalities [13]. The human papilloma virus E6E7 genes (HPV/E6E7) immortalized multiple cell types that were phenotypically and functionally similar to the parental cells [14–20]. As yet, no human hepatocytes have been immortalized with HPV18/E6E7.

We established a human primary non-neoplastic hepatocyte cell line transduced with the HPV18/E6E7 that retained primary hepatocyte characteristics even after prolonged culture, and were more prone to HCV infection than those cells immortalized with SV40 T antigen. We further improved the susceptibility of HPV18/E6E7-immortalized hepatocytes to HCV infectivity by impairing interferon regulatory factor-7 (IRF-7) expression. These cells are useful to assay infectivity of HCV strains other than JFH-1, HCV replication, innate immune system engagement of HCV, and screening of anti-HCV agents. This infection system using non-neoplastic cells also suggested that IRF-7 plays an important role in eliminating HCV infection.

2. Materials and methods

2.1. Cell cultures

We obtained the approval of the Ethical Committee of Kyoto University for the use of human hepatocytes and sera obtained from HCV-positive patients. Informed consent was obtained from both the hepatocyte donor and HCV-positive patients. Primary hepatocytes (P.H.) were cultured as described [21]. HeLa, 293, Huh-7.5, and PH5CH8 cells were cultured as previously described [22]. For three-dimensional (3D) cultures, Mebiol Gel (Mebiol Inc.) was prepared according to the manufacturer's instructions.

2.2. Plasmids construction

The SV40 T, hTERT and HPV/E6E7 fragments from pAct-SVT, PCX4neo/hTERT, and pLXSN-E6E7 plasmids were inserted into pCSII-EF-RFA plasmid creating the pCSII-EF-SVT, pCSII-EF-hTERT, and pCSII-EF-E6E7 plasmids, respectively. The full-length IRF-3 and IRF-7 genes were cloned by RT-PCR using total RNA isolated from 293 cells as a template and were inserted into pcDNA3 vector. Dominant-negative forms of IRF-3 (DNIRF-3) and IRF-7 (DNIRF-7) were constructed by PCR amplification of the coding region for amino acid residues 108–427 of IRF-3 and 237-514 of IRF-7, respectively. The amplified IRF-3 fragment was cloned into pcDNA3 in frame with a FLAG epitope tag generating pcFLAG-DNIRF-3. The amplified IRF-7 fragment was cloned into pLXSH in frame with HA epitope tag generating pLXSH-HA-DNIRF-7. The pIFNβ promoter-luc and pIFNα promoter-luc plasmids were gifts from Dr. Taniguchi of the Tokyo University. The psiRNA-hIRF-3 and psiRNA-hIRF-7 plasmids were purchased from Invivogen (USA).

2.3. Immunoblot analysis

Immunoblot analysis was performed as described previously [22]. We used anti-SV40 T (Santa Cruz), anti-HPV18/E7 (Santa Cruz), anti-tubulin (Sigma), anti-FLAG (Sigma), and anti-HA (Sigma) antibodies.

2.4. Transfection, small interfering RNA silencing and luciferase assays

Transfection of plasmid DNA was performed using Effectene transfection reagent (Qiagen) as recommended by the manufacturer. The pLXSH-HA-DNIRF-7 plasmid was transfected into the HuS-E/2 clone; transfectants were selected in 100 μg/ml hygromycin B (Gibco). The psiRNA-hIRF-3 and psiRNA-hIRF-7 plasmids were separately transfected into HuS-E/2 cells followed by Zeocin (250 μg/ml) selection. After two weeks of continuous selection, cells were infected with HCV. Luciferase assays were conducted as previously described [22]. The results are presented as relative light units (RLU) normalized to the total content of protein in the cell lysates.

2.5. Reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR

Using 250 ng of total RNA as a template, we performed RT-PCR with a one-step RNA PCR kit (Takara) according to the manufacturer's instructions. The primer sets and reaction conditions used are detailed in Table 1. To measure HCV-RNA titers by real-time RT-PCR, we collected RNA from infected wells. Five hundred nanograms of total cellular RNA was analyzed for the quantity of HCV-RNA as previously described [23].

2.6. HCV infection experiment

HCV infection experiment from serum was done as mentioned before [22]. HCV-infected-serums were titrated and 1×10^5 HCV-RNA copies/ml were used for each infection experiment. Concentrated culture medium for HCV/JFH1-producing cells was prepared as previously described [7]. HCV titer in the concentrated medium was measured, adjusted and added to the cells as mentioned above.

2.7. Blocking of HCV infectivity by anti-CD81

Inhibition of HCV infectivity was performed by blocking CD81 as previously described [7].

3. Results

3.1. Establishment of immortalized primary human hepatocytes

Primary hepatocytes were isolated from liver tissue obtained from a 9-year-old male patient with Primary Hyperoxaluria who had undergone liver transplantation. Hepatocytes were left unmanipulated or transduced with CSII-EF-hTERT alone or in combination with CSII-EF-SVT or CSII-EF-E6E7 to enhance the efficiency of immortalization. After six weeks only cells transduced by the combination of hTERT and either LT or HPV18/E6E7 continued to proliferate. Initially appearing colonies with a growth advantage were picked up and expanded. SV40 T-immortalized cell clones were named HuS-T cells and given numbers from 1 to 7,

Table 1
Primer sequences and RT-PCR parameters

Genes	Primer sequence 5′–3′	PCR parameters[a]
HGF	F: AGGAGCCAGCCTGAATGATGA R: CCCTCTGATGTCCCAAGATTAGC	95, 56, 72 1 min, 45 s, 1 min
TGFα	F: ATGGTCCCCTCGGCTGGA R: GGCCTGCTTCTTCTGGCTGGCA	95, 59, 72 45 s, 30 s, 1 min
TGFβ1	F: GCCCTGGACACCAACTATTGCT R: AGGCTCCAAATGTAGGGGCAGG	95, 58, 72 45 s, 30 s, 1 min
TGFβ2	F: GATTTCCATCTACAAGACCACGAGGGACTTGC R: CAGCATCAGTTACATCGAAGGAGAGCCATTCG	95, 58, 72 45 s, 30 s, 1 min
HGFR	F: TGGTCCTTGGCGTCGTCCTC R: CTCATCATCAGCGTTATCTTC	95, 54, 72 30 s, 45 s, 1 min
EGFR	F: CTACCACCACTCTTTGAACTGGACCAAGG R: TCTATGCTCTCACCCCGTTCCAAGTATCG	95, 58, 72 45 s, 30 s, 1 min
TGFβ1R	F: CGTGCTGACATCTATGCAAT R: AGCTGCTCCATTGGCATAC	95 s, 54, 72 30 s, 45 s, 1 min
TGFβ2R	F: TGCACATCGTCCTGTGGAC R: GTCTCAAACTGCTCTGAAGTGTTC	95, 58, 72 45 s, 30 s, 1 min
FGFR	F: ATGTGGAGCTGGAAGTGCCTC R: GGTGTTATCTGTTTCTTTCTCC	95, 54, 72 30 s, 45 s, 1 min
IGF-1R	F: ACCCGGAGTACTTCAGCGCT R: CACAGAAGCTTCGTTGAGAA	95, 54, 72 30 s, 45 s, 1 min
HNF1α	F: GTGTCTACAACTGGTTTGCC R: TGTAGACACTGTCACTAAGG	95, 52, 72 45 s, 30 s, 1 min
HNF1β	F: GAAACAATGAGATCACTTCCTCC R: CTTTGTGCAATTGCCATGACTCC	95, 52, 72 1 m, 45 s, 1 min
HNF3β	F: CACCCTACGCCTTAACCAC R: GGTAGTAGGAGGTATCTGCGG	95, 56, 72 1 m, 45 s, 1 min
HNF4	F: CTGCTCGGAGCCACAAAGAGATCCATG R: ATCATCTGCCACGTGATGCTCTGCA	95, 58, 72 45 s, 30 s, 1 min
Albumin	F: AGTTTGCAGAAGTTTCCAAGTTAGTG R: AGGTCCGCCCTGTCATCAG	95, 55, 72 45 s, 30 s, 1 min
Apolipoprotein-a	F:AGGCTCGGCATTTCTGGCAG R: TATCCCAGAACTCCTGGGTC	95, 55, 72 45 s, 30 s, 1 min
HTF	F: TCGCTACAGCCTTTGCAATG R: TTGAGGGTACGGAGGAGTTCC	95, 55, 72 45 s, 30 s, 1 min
E-cadherin	F: TCCATTTCTTGGTCTACGCC R: TTTGTCCTACCGACTTCCAC	95, 55, 72 45 s, 30 s, 1 min
CYP 1B1	F: CACCAAGGCTGAGACAGTGA R: GCCAGGTAAACTCCAAGCAC	94, 57, 72 30 s, 30 s, 1 min
CYP 2C9	F: GGACAGAGACGACAAGCACA R: TGGTGGGGAGAAGGTCAAT	94, 57, 72 30 s, 30 s, 1 min
CYP 2B	F: GGCACACAGCCAAGTTTACA R: CCAGCAAAGAAGAGCGAGAG	94, 57, 72 30 s, 30 s, 1 min
CYP 3A4	F: TGTGCCTGAGAACACCAGAG R: GCAGAGGAGCCAAATCTACC	94, 57, 72 30 s, 30 s, 1 min
CYP 2E1	F: CCGCAAGCATTTTGACTACA R: GCTCCTTCACCCTTTCAGAC	94, 57, 72 30 s, 30 s, 1 min
CYP 1A1	F: AGGCTTTTACATCCCCAAGG R: GCAATGGTCTCACCGATACA	94, 57, 72 30 s, 30 s, 1 min
GAPDH	F: CCATGGAGAAGGCTGGGG R: CAAAGTTGTCATGGATGACC	95, 8, 72 45 s, 30 s, 1 min

Table 1 (*continued*)

Genes	Primer sequence 5′–3′	PCR parameters[a]
CD81	F: CTCAACTGTTGTGGCTCCAAC R: CCAATGAGGTACAGCTTCCC	95, 55, 72 45 s, 30 s, 1 min
TLR3	F: GATCTGTCTCATAATGGCTTG R: GACAGATTCCGAATGCTTGTG	95, 55, 72 45 s, 30 s, 1 min
TLR7	F: CCAGACATCTCCCCAGCGTC R: GGCAAAACAGTAGGGACGGC	95, 55, 72 45 s, 30 s, 1 min
TLR8	F: CTGTGAGTTATGCGCCGAAG R: CGGGATTTCCGTTCTGGTGC	95, 55, 72 45 s, 30 s, 1 min
Myd88	F: GGTCTCCTCCACATCCTCCC R: CCAGCTTGGTAAGCAGCTCG	95, 55, 72 45 s, 30 s, 1 min
IRF3	F: GAACCCCAAAGCCACGGATC R: CCTCCCGGGAACATATGCAC	95, 55, 72 45 s, 30 s, 1 min
IRF7	F: GTGCTGTTCGGAGAGTGGCTC R: CAGCCCAGGCCTTGAAGATG	95, 55, 72 45 s, 30 s, 1 min

CYP, cytochrome P450; EGFR, epidermal growth factor receptor; F, forward primer; FGFR, fibroblast growth factor receptor; GAPDH, glyceraldehyde phosphate dehydrogenase; HGF, hepatocyte growth factor: HGFR, hepatocyte growth factor receptor; HNF, hepatocyte nuclear factor; HTF, human transferrin; IGF-1R, insulin-like growth factor-type I receptor; IRF, interferon regulatory factor; R, reverse primer; TGF, transforming growth factor; TGFR, transforming growth factor receptor; TLR, toll like receptor.

[a] Temperatures are tabulated in the first lane in degrees celsius and the corresponding times in the second lane. Performing one-step RT-PCR, reverse transcription was carried out at 42 °C for 20 min with a pre-PCR denaturation at 95 °C for 10 min.

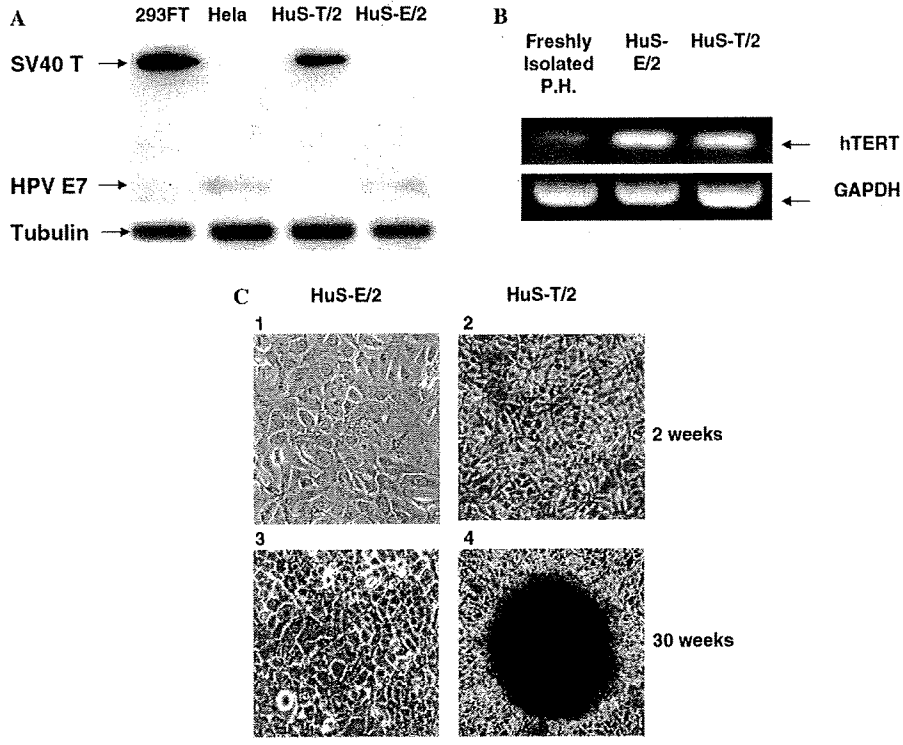

Fig. 1. (A) Immunoblot detection of SV40 T and HPV E7 expression in HuS-T/2 and HuS-E/2 cells, respectively. 293-FT and HeLa cells were used as positive controls for SV40 T and HPV E7 expression, respectively. The specific bands representing the targets are indicated. Detection of tubulin expression in all cells served as an internal control. (B) Human Telomerase Reverse Transcriptase (hTERT) expression was examined by RT-PCR in freshly isolated hepatocytes and the HuS-E/2 and HuS-T/2 cell lines. GAPDH expression was used as an internal control. The hTERT-specific bands are shown. (C) Morphological characteristics of HuS-E/2 and HuS-T/2 cells after two (panels 1 and 2) and 30 (panels 3 and 4) weeks in culture. [This figure appears in colour on the web.]

while the HPV18/E6E7-immortalized clones were named HuS-E cells and given numbers from 1 to 4. Expression of SV40 T and HPV E7 proteins was detected in the appropriate cells by immunoblot analysis (Fig. 1A). In both immortalized cell lines, expression of hTERT-mRNA was enhanced in comparison to non-transduced, freshly isolated hepatocytes as determined by RT-PCR (Fig. 1B). HuS-E cells were larger in size and exhibited slower growth than HuS-T cells (Fig. 1C).

3.2. Characterization of HuS-E and HuS-T immortalized hepatocytes

The HuS-E/2 and HuS-T/2 clones demonstrated the highest expression of hepatocyte-specific markers and transcription factors by RT-PCR (data not shown); these cells were used as representative for each group in this study. To address if HuS-E/2 and HuS-T/2 maintained similar characteristics as primary hepatocytes, they were both cultured continuously for 30 weeks and the expression profiles of a variety of growth factors (Fig. 2A), growth factor receptors (Fig. 2B), hepatocyte-specific nuclear factors (Fig. 2C), albumin, apolipoprotein-A1, transferrin (Fig. 2D), cytochrome p450 (CYP) genes (Fig. 2E), and GAPDH were compared with freshly isolated primary hepatocytes after isolation or two weeks of culture, Huh-7.5 cells, and 293 cells. After two weeks in culture, the expression of nearly all examined genes was similar between freshly isolated hepatocytes and the HuS-E/2 cell line. HuS-E/2 cells, however, exhibited higher expression of TGFβ2 (Fig. 2A), TGFβ2R, and HGFR (Fig. 2B) and lower expression of CYP 3A4 and 2C9 (Fig. 2E) in comparison to freshly isolated hepatocytes. Primary hepatocytes displayed reduced expression of TGFβ1 and TGFβ2 (Fig. 2A) and a loss of CYP1A1 expression (Fig. 2E) after two weeks of culture. HuS-E/2 cells exhibited higher expression of HGF (Fig. 2A), HGF receptor (Fig. 2B), HNF-4, (Fig. 2C), albumin, apolipoprotein-A1, HTF, and E-cadherin (Fig. 2D) in comparison to HuS-T/2 cells. Expression of CYP 3A4 (Fig. 2E) was lost from both HuS-T/2 and HuS-E/2 cells, while HuS-T/2 cells also lost the expression of HNF-1α (Fig. 2D), and CYPs 2B, 2E1 (Fig. 2E).

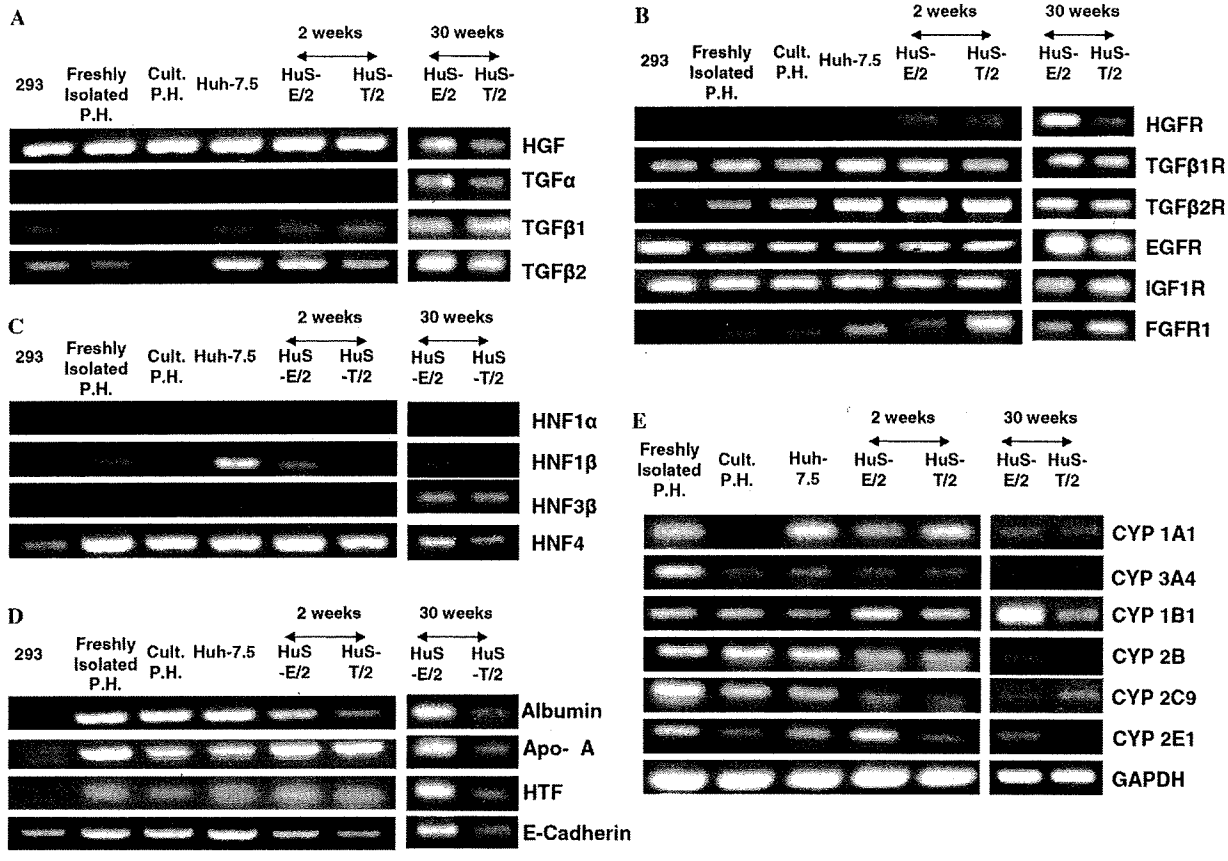

Fig. 2. Expression of the genes encoding growth factors (A), growth factor receptors (B), hepatocyte-specific nuclear factors (C), hepatocyte differentiation and functional markers (D), and CYP enzymes (E) in 293 cells, freshly isolated primary hepatocytes (P.H.), primary hepatocytes cultured for two weeks (Cult. P.H.), Huh-7.5 cells, and HuS-E/2 and HuS-T/2 cells cultured for two and 30 weeks were investigated by RT-PCR. The bands representing specific targets are indicated in the representative reactions.

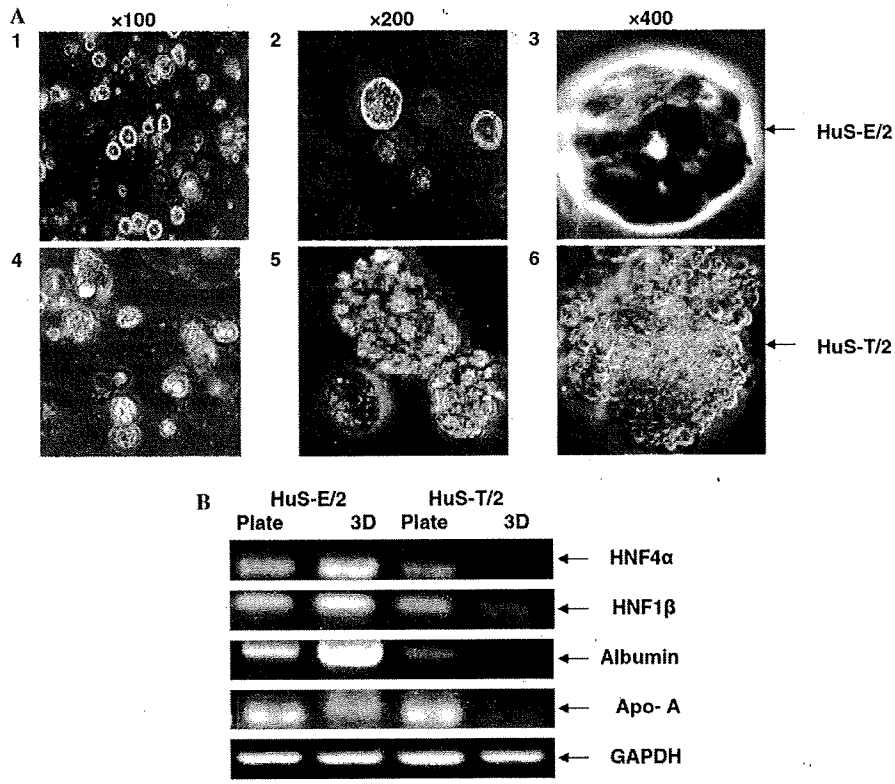

Fig. 3. (A) The morphology of HuS-E/2 and HuS-T/2 cells in 3D culture. HuSE/2 and HuS-T/2 cells were cultured in Mebiol Gel in 12-well plates at a concentration of 5×10^5 cells/well. The microscopic characteristics of these cells after one week of 3D culture are shown. (B) The expressions of HNF4α, HNF1β, albumin, and apo-A by HuS-E/2 and HuS-T/2 cells in both flat and 3D cultures are detailed. After one week of culture of HuS-E/2 and HuS-T/2 cells in flat and 3D cultures, the expressions of HNF4α, HNF1β, albumin, and apo-A were measured by RT-PCR in 250 ng total RNA.

HuS-T/2 but not in HuS-E/2 cells showed a transformed-like character starting from the 13th week of culture. This was demonstrated by continuing proliferation after confluence, pile-up formations (Fig. 1C), and proliferating in serum-deprevied condition. However, HuS-E/2 cells did not show any transformed-like characters even after 30 weeks of culture.

3.3. The characteristics of HuS-E and HuS-T immortalized hepatocytes in 3D culture

After one week in 3D culture, HuS-E/2 (Fig. 3A, panels 1, 2, and 3) cells adopted a donut-shaped structure with a central pore, while HuS-T/2 cells (Fig. 3A, panels 4, 5, and 6) displayed irregular mass formations (similar to the growth pattern of Huh-7.5 cells in 3D culture (data not shown)). In 3D culture, while the expression of HNF4, HNF1β, and albumin was enhanced in HuS-E/2, it was decreased in HuS-T/2 cells (Fig. 3B).

3.4. HCV infection to HuS-E/2

We further assessed the HCV infectivity of HuS-E- and HuS-T-derived clones by infection with HCV-1b-infected serum. Of the three HuS-E clones examined, HuS-E/2 clone demonstrated the highest infectability with HCV genotype 1b in comparison to Huh-7.5, PH5CH8 (Fig. 4A), and HuS-T cells (data not shown), which were excluded from further experiments.

3.5. Anti-CD81 blocked HCV infectivity

CD81 is involved in the entry of HCV pseudoparticles [24] and in vitro-synthesized JFH-1 [7]. To determine if authentic viral particles follow the same route of entry when infecting HuS-E/2 cells, we first examined the CD81 expression by RT-PCR. Both HuS-E/2 and HuS-T/2 cells expressed similar amounts of CD81 as freshly isolated hepatocytes and Huh-7.5 cells (Fig. 4B). Antibodies against CD81 reduced HCV infectivity of HuS-E/2 cells from the levels seen using a non-specific control antibody, confirming the importance of CD81 in HCV infectivity (Fig. 4C).

3.6. IFNα blocked HCV infectivity

We treated HuS-E/2 cells with HCV-containing serum. Cells were then cultured in fresh medium supplemented

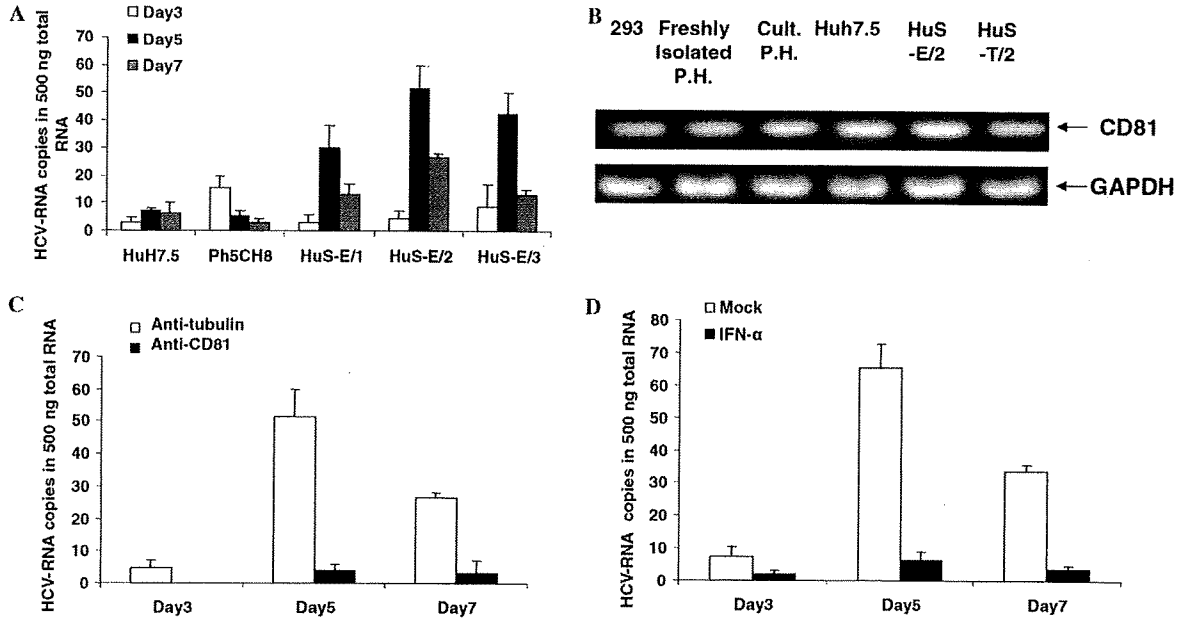

Fig. 4. (A) Serum from an HCV patient was used to infect Huh-7.5 cells, PH5CH8 cells, and three HPV E6E7-immortalized clones (HuS-E/1-3) for 24 h. After washing three times in phosphate-buffered saline (PBS), cells were cultured in fresh medium. Cells were then harvested and lysed at the indicated time points. The quantity of HCV genome RNA per 500 ng total RNA was determined by real-time RT-PCR analysis. (B) HuS-E/2 and HuS-T/2 cells both expressed CD81. Expression of CD81 (upper panel) and GAPDH as an internal control (lower panel) in 293 cells, freshly isolated P.H., cultured P.H., and Huh-7.5, HuS-E/2, and HuS-T/2 cells was investigated by RT-PCR. (C) Anti-CD81 antibodies blocked HCV infectivity. HCV infection was performed as described in (A) with the addition of CD81-specific (black bar) or anti-tubulin antibodies (control, white bar). (D) IFNα inhibits HCV multiplication in HuS-E/2 cells infected with HCV-containing serum. HuS-E/2 cells were infected with HCV as described in (A). After washing three times with PBS, cells were cultured in fresh medium supplemented with (black bar) or without (white bar) 100 U/ml IFNα.

without or with 100 U/ml IFNα. The enhancement of the HCV-RNA genome titers on the fifth day (about 10-fold) was not observed in cells treated continuously with IFNα (Fig. 4D). This result suggests that IFNα inhibited HCV replication in infected HuS-E/2 cells.

3.7. The effect of blocking IRF-3 and IRF-7 signaling on HCV infectivity

Production of interferon-alpha (IFNα) and interferon-beta (IFNβ) limits viral replication and spread, providing one of the most effective innate antiviral responses [25]. Signaling through IRF-3 and IRF-7 plays important roles in the stimulation of IFN-α/β production [25]. To determined which molecules (IRF-3 or IRF-7) play an important role in modulation of the innate immune response against HCV infection in these cells, we first detected intrinsic expression of double-stranded RNA-stimulated Toll-like receptor (TLR) 3, the downstream effector IRF-3, single-stranded RNA-stimulated TLR7, and 8, and the downstream effectors MyD88 and IRF-7 by RT-PCR. TLR3 exhibited very low expression in freshly isolated hepatocytes, Huh-7.5, HuS-E/2, and HuS-T/2 cells, while TLR7, TLR8, MyD88, and IRF-7 were easily detectable in both freshly isolated and immortalized cell lines (Fig. 5A).

The abilities of DNIRF-3 and DNIRF-7 to inhibit IFNβ and IFNα production by HuS-E/2 cells infected with Sendai virus were confirmed using assays of IFNβ or IFNα promoter-driven luciferase reporters. DNIRF-3 exhibited strong inhibition of IFNβ production (Fig. 5B) and weaker inhibition of IFNα transcription (Fig. 5C), while DNIRF-7 strongly inhibited IFNα production (Fig. 5C) and only weakly inhibited IFNβ production (Fig. 5B).

We then assessed the inhibition of HCV infectivity by DNIRF-3 and DNIRF-7. Transient transfection with DNIRF-3, DNIRF-7, or an empty vector was performed prior to HCV infection. Using Effectene reagent, the efficiency of plasmid transfection into HuS-E/2 cells was approximately 70% (data not shown). While there was no significant effect of DNIRF-3 on HCV infectivity, DNIRF-7 demonstrated a marked increase in HCV titers on days 3 and 5 after infection in comparison to control cells (Fig. 5D). To confirm that the enhancement of HCV replication by DNIRF-7 is not mediated by the impairment of IRF-3 signaling by heterodimeric interactions between IRF-3 and DNIRF-7, we performed siRNA inhibition of IRF-3 and IRF-7. The reduction of IRF-3 and IRF-7 expression by siRNA was obvious by RT-PCR (Fig. 5E). siRNA-mediated suppression of either IRF-3 or IRF-7 inhibited IFNβ and IFNα production

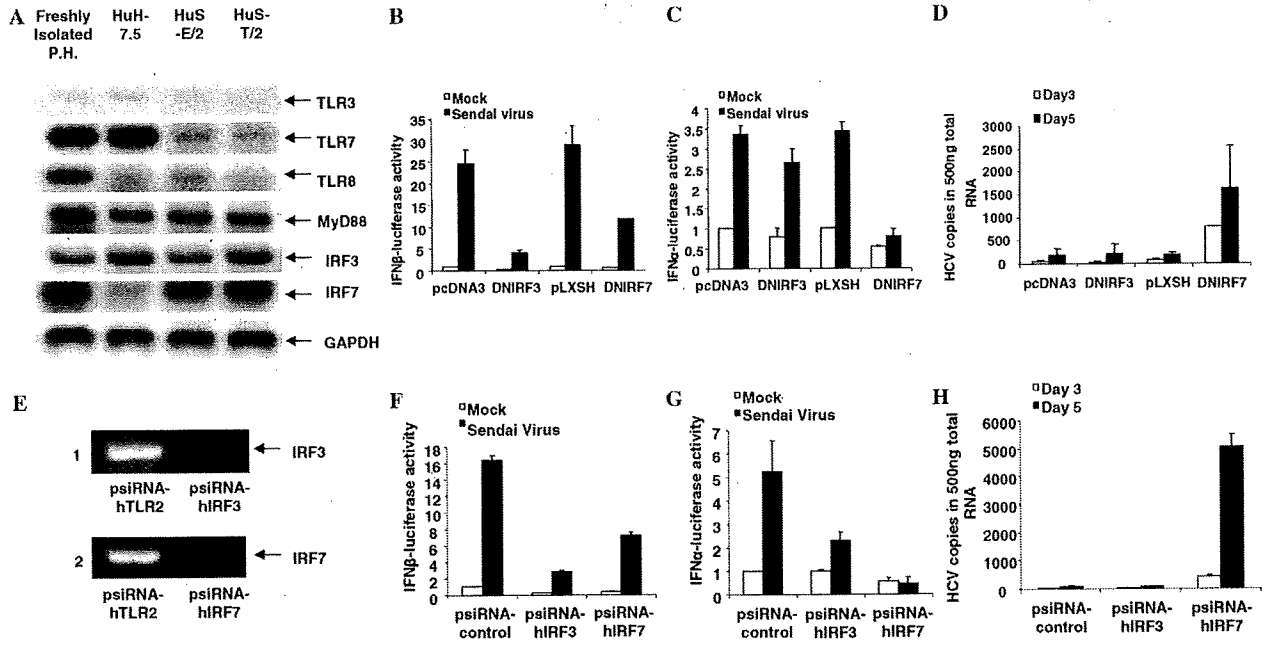

Fig. 5. (A) We examined the expression of TLR3, TLR7, TLR8, MyD88, IRF-3, and IRF-7, as well as GAPDH as an internal control in freshly isolated primary hepatocytes and Huh-7.5, HuS-E/2, and HuS-T/2 cells was investigated by RT-PCR. (B and C) HuS-E/2 cells were cotransfected with pIFNβ-luc (B) or pIFNα-luc (C) with an expression plasmid encoding DNIRF-3, DNIRF-7, or the appropriate empty vector (pcDNA3 and PLXSH, respectively). Twenty-four hours later, cells were infected (black bar) with Sendai virus or mock-infected (white bar), then analyzed for luciferase activity after 12 h. (D) IRF-7, but not IRF-3, suppression enhanced HCV infectivity of HuS-E/2 cells. HuS-E/2 cells were transiently transfected with empty pcDNA3, DNIRF-3, empty pLXSH, or DNIRF-7 plasmids. Twenty-four hours later, serum from a patient with HCV was used to infect transfected cells for 24 h. After washing, cells were cultured in fresh medium. The cells were then harvested and lysed at the indicated time points. The quantity of HCV genome RNA per 500 ng total RNA was determined by real-time RT-PCR analysis. (E) IRF-3 and IRF-7 levels were suppressed by specific siRNAs. HuS-E/2 cells were transfected with control psiRNA-hTLR2, psiRNA-hIRF-3, or psiRNA-hIRF-7, then selected with Zeocin at 250 μg/ml. Two weeks later, cells were harvested and assessed for the expression of IRF-3 and IRF-7 by RT-PCR. (F and G) HuS-E/2 cells were transfected with control psiRNA-hTLR2, psiRNA-hIRF-3, or psiRNA-hIRF-7, followed by selection in Zeocin at 250 μg/ml. Two weeks later, cells were cotransfected with pIFNβ-luc (F) or pIFNα-luc (G). Twenty-four hours later, cells were infected (black bar) with Sendai virus or mock-infected (white bar), then analyzed for luciferase activity after 12 h. (H) Transfected cells were infected with serum from HCV patient; HCV infectivity was assessed as described above.

in HuS-E/2 cells infected with Sendai virus in patterns similar to the effects seen following DNIRF-3 and DNIRF-7 expression, respectively (Figs. 5F and G). Blockade of IRF-7 expression resulted in a significantly higher titer of HCV after infection, while IRF-3 down-regulation did not have any significant effect on HCV titers (Fig. 5H). The enhancement of IRF-7 silencing by siRNA improved the infectivity of HCV (data not shown). These results suggest that IRF-7 plays the major role in the innate immune response to HCV in HuS-E/2 cells.

3.8. Establishment of stable DNIRF-7 expressing clones derived from HuS-E/2 cells

Since DNIRF-7 enhanced HCV infectivity, we transduced the plasmid encoding DNIRF-7 and a hygromycin-B resistance gene, into HuS-E/2 cells. Following selection with hygromycin-B, we obtained the HuS-E7/DN22 and HuS-E7/DN24 clones. As detected by RT-PCR, both clones demonstrated similar expression levels of albumin, apolipoprotein-A1, and HNF4 as the parental HuS-E/2 cells (Fig. 6A). The HuS-E7/DN24 clone exhibited stronger expression of DNIRF-7 than the HuS-E7/DN22 clone by immunoblotting (Fig. 6B). The induction of IFNα in HuS-E7/DN24 in response to infection with an RNA virus (Sendai virus) was low in comparison to the parental HuS-E/2 and HuS-E7/DN22 clones, as detected by IFNα-luciferase reporter assay (Fig. 6C). HuS-E7/DN24 also exhibited a higher HCV infectability in comparison to parental HuS-E/2 cells and the HuS-E7/DN22 clone (Fig. 6D).

3.9. Infection of HuS-E7/DN24 cells with different HCV genotypes

Huh7.5 and HuS-E7/DN24 cells were separately infected with serums derived from 3 different HCV-patients or by JFH-1 concentrated medium (HCV-2a). Two serums were infected by HCV-1b, while the third by HCV-2b. Inoculated virus titer was adjusted to be the same in all cases. Except for JFH-1, which efficiently

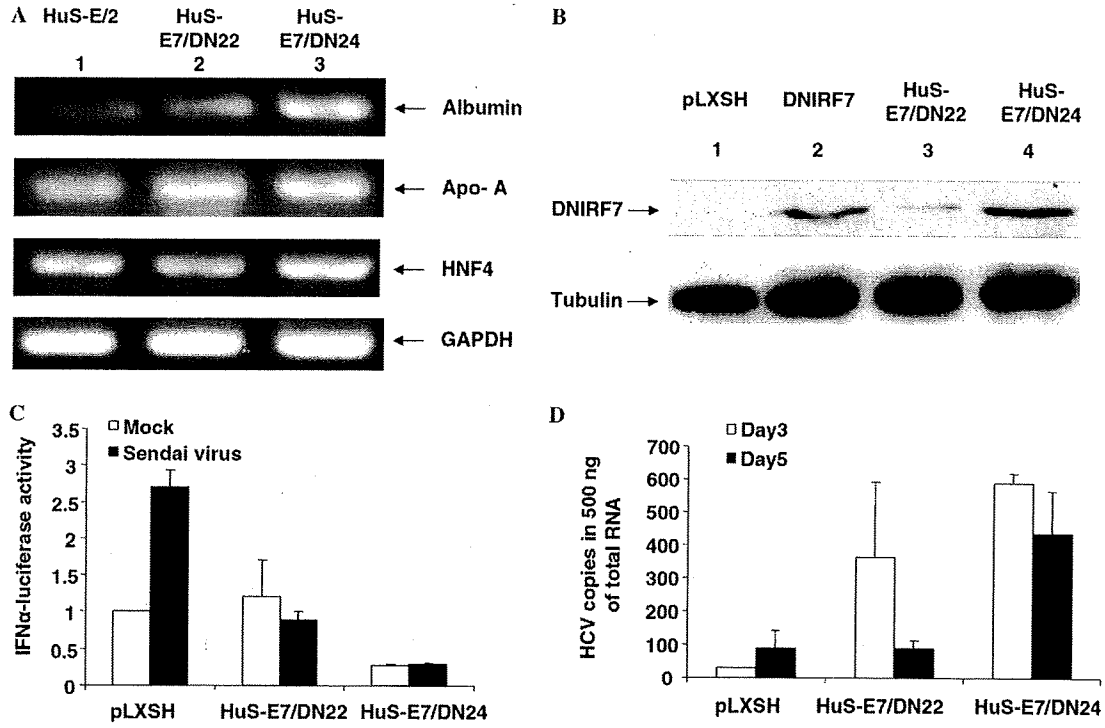

Fig. 6. (A) The pLXSH-HA-DNIRF-7 plasmid was transfected into HuS-E/2 cells, followed by selection in 100 μg/ml Hygromycin B. Two clones, HuS-E7/DN22 (lane 2) and HuS-E7/DN24 (lane 3), were obtained. We investigated the expression of albumin, apo-A, HNF4, and GAPDH as an internal control in parental HuS-E/2, HuS-E7/DN22, and HuS-E7/DN24 hepatocytes cultured for two weeks by RT-PCR. (B) Expression of HA-tagged DNIRF-7 (upper panel) and tubulin (control, lower panel) was detected by immunoblotting analysis. HuS-E/2 cells transiently transfected with either empty pLXSH vector (lane 1) or pLXSH-HA-DNIRF-7 (lane 2) were used as negative and positive controls, respectively, after 48 h. (C) HuS-E/2, HuS-E7/DN24, and HuS-E7/DN22 cells were transfected with IFNα-luc. HuS-E/2 cells were also cotransfected with pLXSH. All of these cells were then infected (black bar) or with Sendai virus or mock-infected, then analyzed for luciferase activity after 12 h. (D) HuS-E7/DN24 cells exhibited high infectivity to HCV samples derived from patient serum. HuS-E/2 cells were transiently transfected with empty pLXSH. Twenty-four hours later, serum from a recurrently transplanted HCV patient was used to infect transfected cells and HuS-E7/DN22 and HuS-E7/DN24 cells for 24 h. After washing three times, cells were cultured in fresh medium. Cells were then harvested and lysed at the indicated time points.

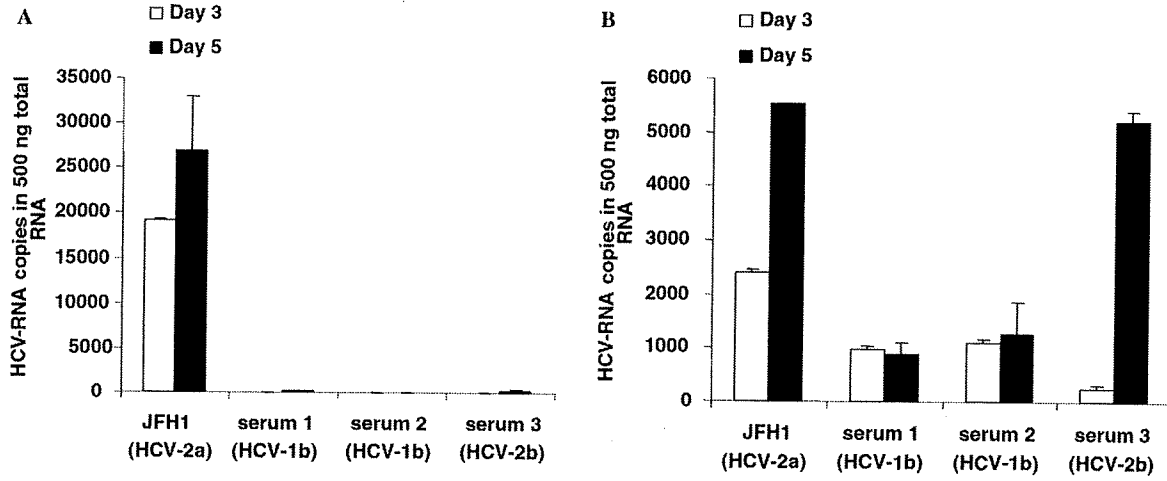

Fig. 7. The infectability of Huh-7.5 and HuS-E7/DN24 cells to different HCV genotypes. Huh-7.5 (A) and HuS-E7/DN24 (B) cells were infected with same titer of JFH1 (HCV-2a), two different HCV-1b serums and one HCV-2b serum. After removing the infected medium, the cells were washed in PBS and recultured in fresh medium. Cells were harvested and lysed at the indicated time points. The quantity of HCV genome RNA per 500 ng RNA was detected by real-time RT-PCR analysis.

replicated in Huh7.5 cells (Fig. 7A), HuS-E7/DN24 cells showed a higher and reproducible infectability for the different HCV strains than Huh7.5 cells (Fig. 7B). Similar higher infectability of HuS-E7/DN24 cells was observed with HCV-4a genotype (unpublished data). These results suggest that the high infectability of Huh-7.5 with JFH-1 is specific among the combinations of HCV strains and cell lines; while HuS-E7/DN24 cells were generally permissive to HCV-infected serum independent of HCV strains.

4. Discussion

This study demonstrates that ectopic expression of the HPV18/E6E7 genes in combination with hTERT could efficiently immortalize mature human hepatocytes, generating a cell line with stable expression of hepatocyte markers and functions for more than 30 weeks in culture. HuS-E/2 cells continuously exhibited higher expression of both HGF and HGFR than HuS-T/2 cells. This result suggests that HPV18/E6E7-immortalized hepatocytes maintain responsiveness to paracrine signals capable of inducing cell differentiation to a greater extent than SV40 T-immortalized hepatocytes. This conclusion is further supported by the increased expression of HNF4 in HuS-E/2 cells in comparison to HuS-T/2 cells. HNF4 is a major hepatocyte transcription factor, required for hepatocyte differentiation and liver-specific gene expression [26]. HNF4 drives hepatocytes differentiation by acting upstream in a transcription factor cascade that included HNF1α [27]. HuS-E/2 cells continued to express HNF1α throughout prolonged culture, while HuS-T/2 cells lost expression completely. Maintenance of hepatocellular functions was demonstrated by continuous and high expression of albumin, apolipoprotein-A, human transferrin, and E-cadherin by HuS-E/2 in comparison to HuS-T/2 cells. These differences became more pronounced in the late passages. In a similar manner, HuS-E/2 cells continued to express all of the examined CYP genes, with the exception of CYP 3A4, while HuS-T/2 cells lost expression of CYP 3A4, 1B, and 2E1 completely and displayed markedly lower expression of CYP 1B1 than HuS-E/2 cells. Thus, human hepatocytes immortalized by HPV E6/E7 transfection are phenotypically similar to primary hepatocytes, even during extended cultures.

Recently, it was reported that the JFH-1 strain and derived chimeras could only infect and propagate efficiently in Huh7.5.1 and Huh7.5 cells, both of which are subclones of Huh7 cells [7–9]. This limitation, however, may be specific to the JFH-1 **strain**, which may not accurately reflect the course of other HCV strains' infection. Thus, usage of HCV particles isolated from patient serum could be more useful to study authentic HCV infection. Using sera from HCV patients as a source of infective virus, HPV18/E6E7-immortalized cell lines exhibited higher reproducible susceptibility to HCV infection than HuS-T, PH5CH8, and Huh-7.5 cell lines.

IRF3 and IRF7 play an important role in the activation of interferon signaling [28]. We suppressed the functions of IRF-3 or IRF-7 to assess their role in HCV infectivity. In fact, we observed significant increase of HCV replication in HuS-E/2 cells bearing dominant-negative IRF7 that impaired IFN signaling. The suppression of IRF-3, however, did not have any significant effect on HCV infectivity or replication in this cell line. This may result from the blockade of IRF-3 activation by an HCV NS3/4A serine protease [29] through at least two independent pathways that inhibit the TLR3-dependent and RIG-I-dependent signaling pathways [29–33]. Although HCV was shown to inhibit basal expression levels of IRF-7 at both mRNA and protein levels and it was shown that NS5A suppresses IRF-7-induced IFNα promoter activation [34], Stimulation of TLR7 was shown to activate IRF-7 and induce suppression of HCV replicon levels in Huh-7 cells [35]. This suggests that the inhibition of IRF7 by HCV is not complete. Using IRF-7-deficient (IRF-7−/−) mice, Honda [36] demonstrated that the transcription factor IRF-7 is essential for the induction of IFNα/β genes. We established a clone stably expressing DNIRF-7 (HuS-7E/DN24), which demonstrated higher infectivity with different HCV strains than the parental HuS-E/2 clone.

In summary, we have established a human hepatocyte-derived cell line that maintains the characteristic features of primary hepatocytes by transduction with HPV18/E6E7. This cell line is highly infectable by HCV, which suggests that these cells may be useful to characterize the molecular mechanisms involved with HCV infection and to develop novel HCV treatment modalities.

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