

(Fig. 2A), and the Pearson's correlation coefficient for this data set (93 proteins/genes) was 0.73. Next, we divided 93 proteins into those related to metabolism and others biological processes. The HCC/non-HCC ratios of protein expression for metabolism-related proteins showed substantial correlation with those of mRNA (Fig. 2B,  $r = 0.9$ ), whereas those of other proteins were poorly correlated (Fig. 2C,  $r = 0.36$ ). Extreme care must be taken in a direct comparison of proteomic data with transcriptome

because of multiple layers of discrepancies caused by the distinct sensitivities of cDNA array hybridization and 2-DE, the inability of a cDNA array to distinguish mRNA isoforms and post-translational modifications of proteins. Nevertheless, our results suggest that the expression of considerable portion of proteins with metabolic function listed here is regulated at transcriptional level. On the other hand, post-transcriptional and/or post-translational processes seem to be involved in the regulation of expression level for proteins with other cellular functions as a whole. Four proteins (albumin, transferrin, peptidylprolyl isomerase A, and carbonic anhydrase 1) showed apparent poor correlation in protein and mRNA expression profiles (Table 3 and Fig. 2). Transcriptional control might have little effect on the expression changes of these proteins between HCC and non-HCC.

A number of proteins were expressed as multiple spots on 2-DE gels and most multi-spotted proteins showed little spot-to-spot variations in the averaged HCC/non-HCC ratio. Although we do not know how these multiple spots were generated, many of them might be due to the conformational equilibrium of proteins under electrophoresis rather than to any post-translational modifications [28]. On the other hand, the HCC/non-HCC expression ratios of several multi-spotted proteins varied from spot to spot, and three proteins (transferrin, fumarate hydratase, and tumor rejection antigen gp96) were categorized as these multi-spotted proteins (Table 4).

For example, gp96 was detected in two spots (spot #436 and 537) with distinct molecular mass and  $pI$  and they showed different HCC/non-HCC expression ratio (Fig. 3A and B and Table 4). The expression of these two isoforms was observed to change in the opposite direction between non-HCC and HCC: #436 was up-regulated in HCC (HCC/non-HCC ratio: 1.96) while #537 was down-regulated (HCC/non-HCC ratio: 0.79) (Table 4 and Fig. 3C and D). Gp96 is a glycoprotein present in endoplasmic reticulum and is supposed to function as a molec-

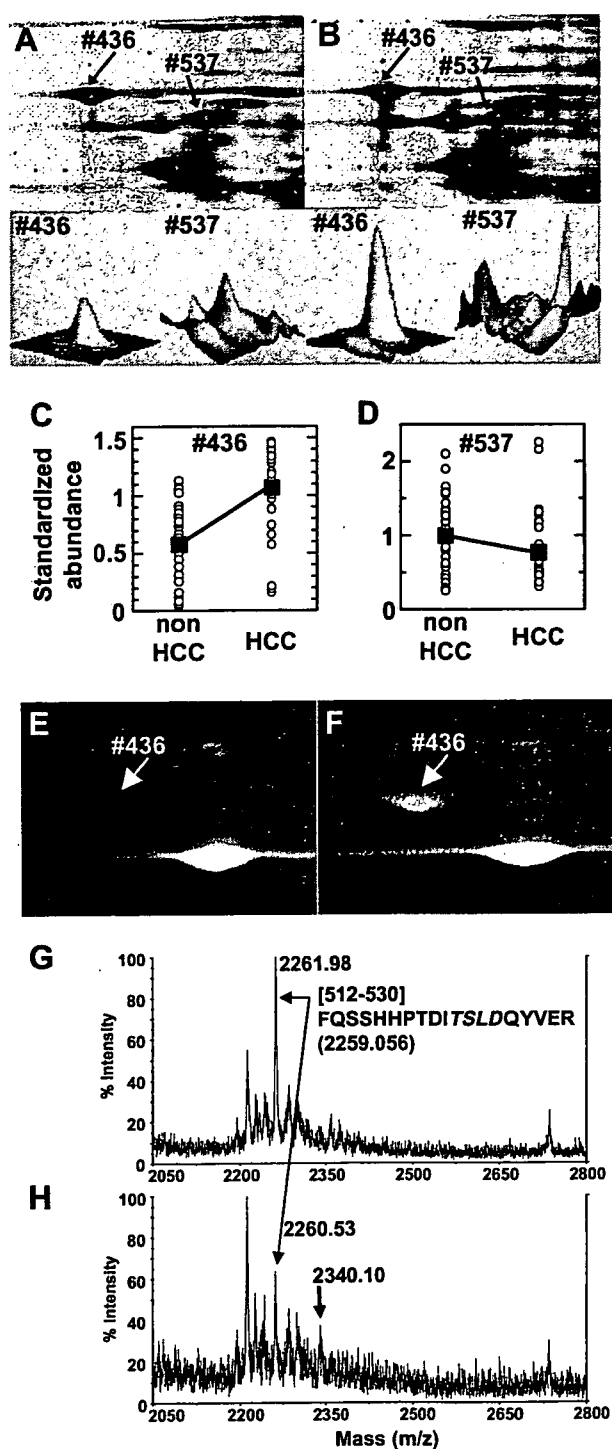


Fig. 3. Comparison of expression profiles of two gp96 spots between HCC and non-HCC. The expression profile and phosphorylation of tumor rejection antigen gp96 in HCC and non-HCC was investigated. Magnified gel images and 3D views of two gp96 spots in non-HCC (A) and HCC (B) were shown. Differences in expression level of two gp96 spots, #436 (C) and #537 (D), between non-HCC and HCC were shown. The open circle indicates the standardized abundance of the individual spot in each sample. The closed square represents the averaged abundance of each gp96 spot. Magnified gel images of non-HCC (E) and HCC (F) stained with ProQ. The #436 spot was positively stained with ProQ, while unambiguous staining of the #537 spot was not observed. Tryptic peptides prepared from the spot #436 were analyzed by MALDI-TOF mass spectrometry in the positive ion mode (G) and the negative ion mode (H). A peak of 2261.98 detected in positive ion mode corresponds to the amino acid sequence from 512 to 530. In addition to the original peak ( $m/z$ : 2260.53), a peak mass shifted by +80 Da was detected in the negative ion mode. A predicted phosphorylation consensus motif for protein kinase CK2 is indicated in italics (G).

ular chaperone and intracellular  $\text{Ca}^{2+}$  regulator [29,30]. Several previous reports have shown that gp96 is glycosylated and phosphorylated, and exists as heterogeneous molecular entities with various molecular weights [31]. In order to know whether gp96 spots were phosphorylated or not, we stained the 2-DE gels with ProQ Diamond which is a dye specific to proteins phosphorylated on serine, threonine or tyrosine residues [32], and has been used successfully to visualize phosphoproteins [33]. We found that the spot #436 was positively stained with ProQ (Fig. 3E and F). We further tried to detect possible phosphorylated peptides in the tryptic digests prepared from #436 by MALDI-TOF-MS according to Nabetani et al. [21]. Searching for those peaks that had relatively stronger intensities in negative ion mode than in positive ion mode, we found two peaks as candidates for acidically modified peptides. They were assigned to the peptides SILFVPT-SAPR (amino acid sequence: 385–395, data not shown) and FQSSHPTDITSLDQYVER (aa512–530). Fig. 3G and H show the unmodified peak and the acidically modified peak (mass shifted by +80 Da in negative ion mode) of the latter peptide, respectively. This peptide contained a predicted phosphorylation consensus motif, [Ser or Thr]-X-X-[Asp or Glu], for protein kinase CK2 (Fig. 3G) which was suggested to phosphorylate gp96 [34]. These results together with ProQ staining indicated that at least one gp96 isoform was phosphorylated and was up-regulated in HCC. Over-expression of gp96 in HCC has been reported previously [35], though the reports that showed over-expression of its phosphorylated form are rare. Further investigation into biological meaning of gp96 phosphorylation may provide us important information about HCC development.

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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.bbrc.2007.11.101.

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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.

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**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 stan-

dard therapy utilizes the combination of pegylated interferon- $\alpha$  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 pFNβ promoter-luc and pFNα promoter-luc plasmids were gifts from Dr. Taniguchi of the Tokyo University. The psiRNA-hIRF-3 and psiRNA-hIRF-7 plasmids were purchased from Invivo-gen (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  $1 \times 10^5$  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 <sup>a</sup>
HGF	F: AGGAGCCAGCCTGAATGATGA R: CCTCTGATGTCCCAAGATTAGC	95, 56, 72 1 min, 45 s, 1 min
TGF $\alpha$	F: ATGGTCCCCTCGGCTGGA R: GGCCTGCTTCTTCTGGCTGGCA	95, 59, 72 45 s, 30 s, 1 min
TGF $\beta$ 1	F: GCCCTGGACACCAACTATTGCT R: AGGCTCCAAATGTAGGGGCAGG	95, 58, 72 45 s, 30 s, 1 min
TGF $\beta$ 2	F: GATTTCCATCTACAAGACCACGAGGGACTTGC R: CAGCATCAGTTACATCGAAGGAGAGCCATTCCG	95, 58, 72 45 s, 30 s, 1 min
HGFR	F: TGGTCCTTGGCGTCTGCTCTC 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
TGF $\beta$ 1R	F: CGTGCTGACATCTATGCAAT R: AGCTGCTCCATTGGCATAAC	95 s, 54, 72 30 s, 45 s, 1 min
TGF $\beta$ 2R	F: TGCACATCGTCTGTGGAC R: GTCTCAAACCTGCTCTGAAGTGTTTC	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
HNF1 $\alpha$	F: GTGTCTACAACCTGGTTTGCC R: TGTAGACACTGCACTAAGG	95, 52, 72 45 s, 30 s, 1 min
HNF1 $\beta$	F: GAAACAATGAGATCACTTCCTCC R: CTTTGTGCAATTGCCATGACTCC	95, 52, 72 1 m, 45 s, 1 min
HNF3 $\beta$	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: AGTTTGCGAGAAGTTTCCAAGTTAGTG 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: TTTGTCTACCGACTTCCAC	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: CCGCAAGCATTITGACTACA R: GCTCCTTACCCTTTCAGAC	94, 57, 72 30 s, 30 s, 1 min
CYP 1A1	F: AGGCTTTTACATCCCAAGG 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 <sup>a</sup>
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: CGGGATTTCGGTCTCGGTGC	95, 55, 72 45 s, 30 s, 1 min
Myd88	F: GGTCTCCTCCACATCCTCCC R: CCAGCTTGTAAGCAGCTCG	95, 55, 72 45 s, 30 s, 1 min
IRF3	F: GAACCCCAAAGCCACGGATC R: CCTCCGGGAACATATGCAC	95, 55, 72 45 s, 30 s, 1 min
IRF7	F: GTGCTGTTCCGAGAGTGGCTC 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.

<sup>a</sup> 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.

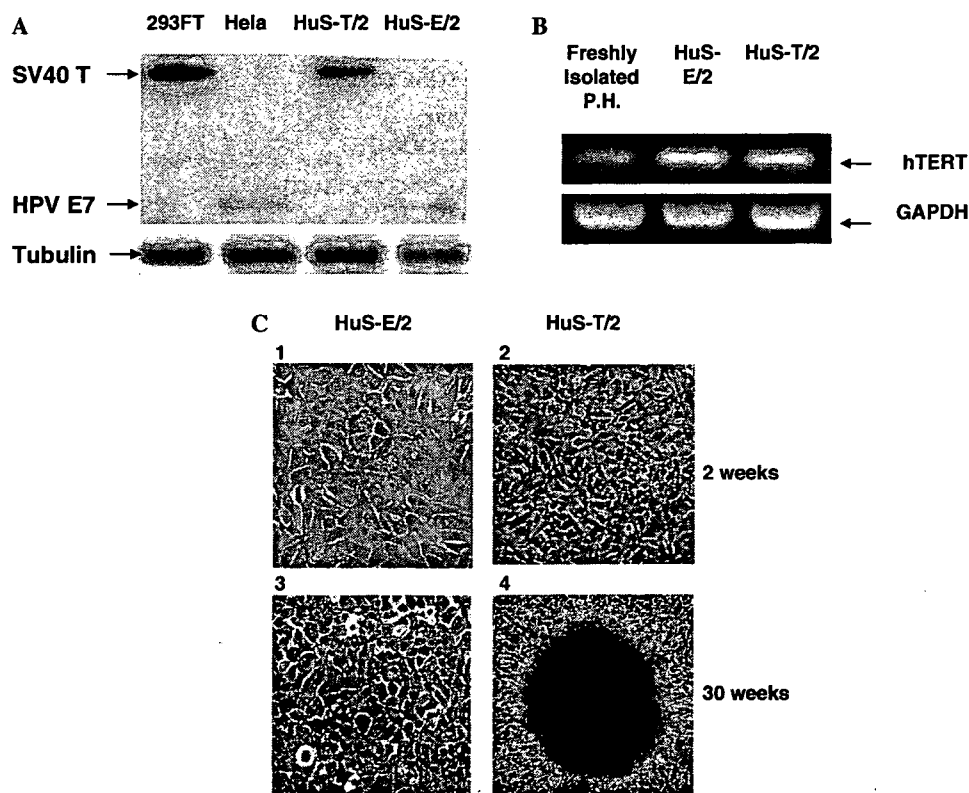


Fig. 1. (A) Immunoblot detection of SV40 T and HPV E7 expression in HuS-T/2 and HuS-E/2 cells, respectively. 293-FT and HeLa cells were used as positive controls for SV40 T and HPV E7 expression, respectively. The specific bands representing the targets are indicated. Detection of tubulin expression 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 TGF $\beta$ 2 (Fig. 2A), TGF $\beta$ 2R, 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 TGF $\beta$ 1 and TGF $\beta$ 2 (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-1 $\alpha$  (Fig. 2D), and CYPs 2B, 2E1 (Fig. 2E).

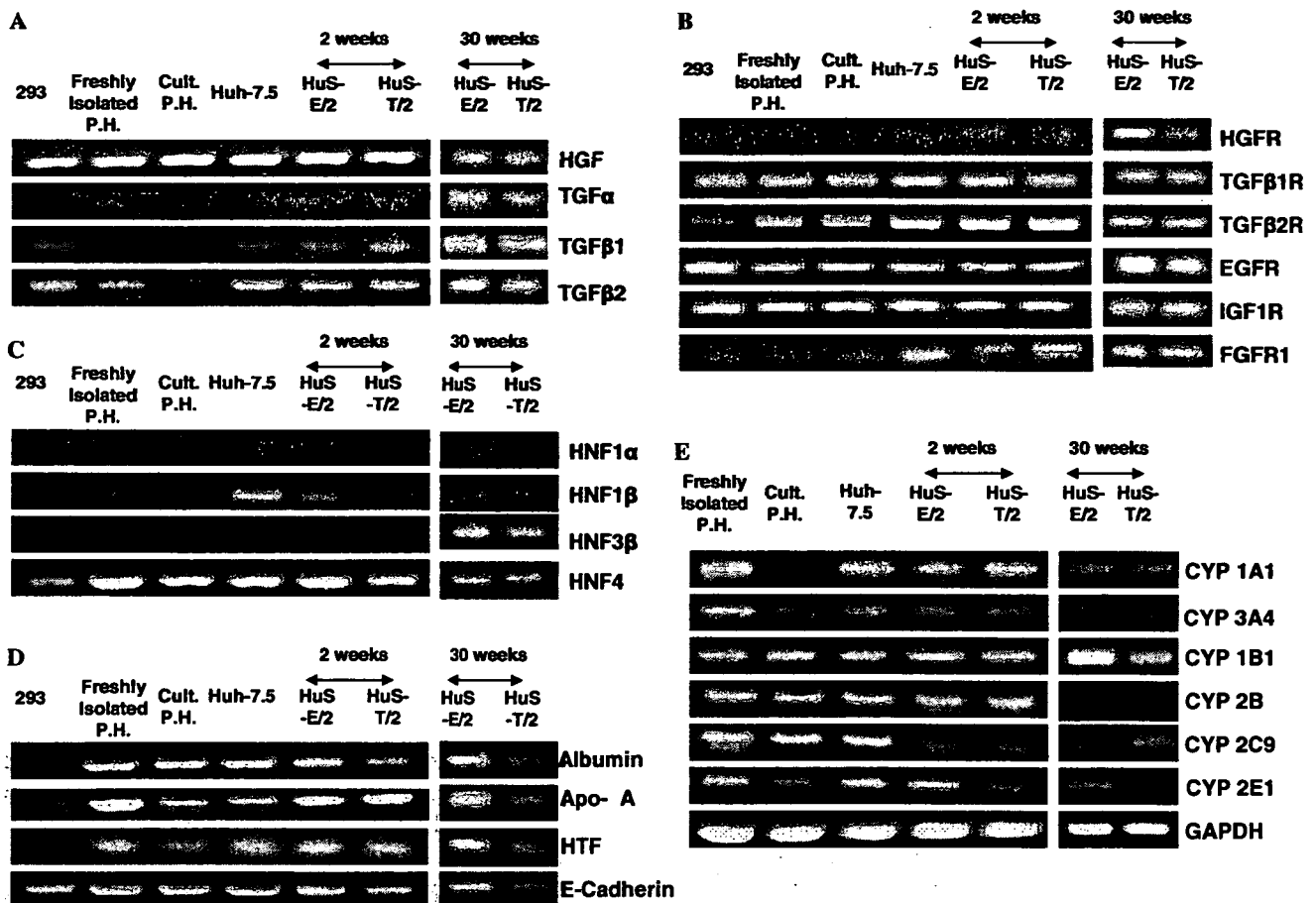
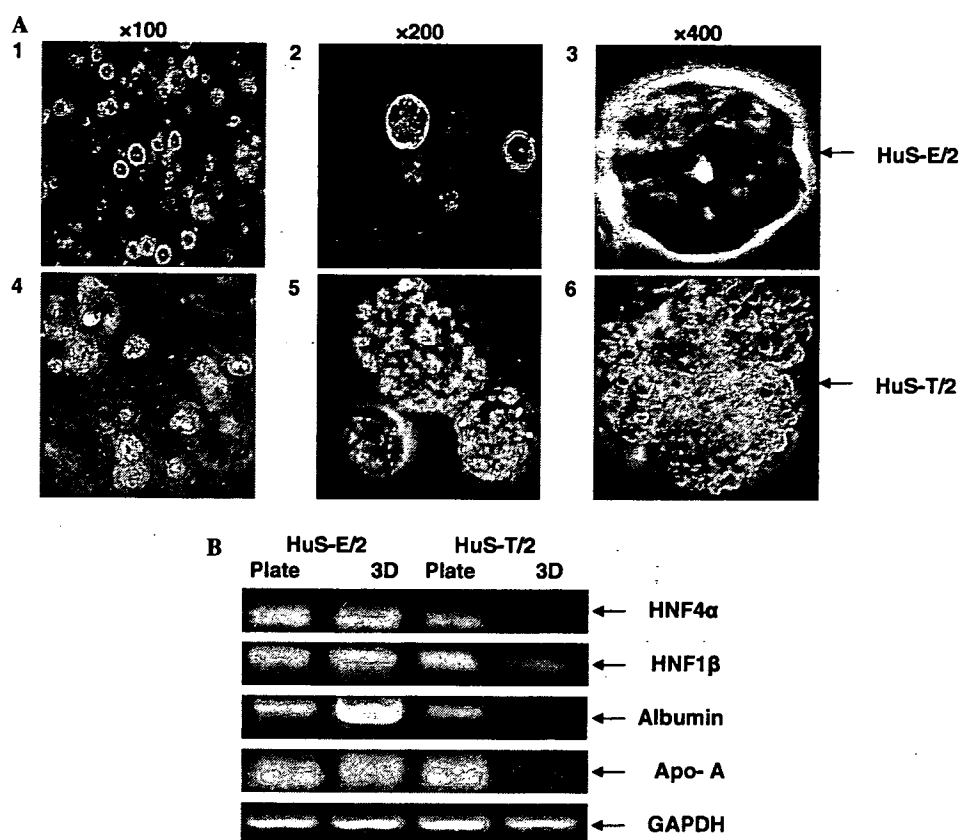


Fig. 2. Expression of the genes encoding growth factors (A), growth factor receptors (B), hepatocyte-specific nuclear factors (C), hepatocyte differentiation and functional markers (D), and CYP enzymes (E) in 293 cells, freshly isolated primary hepatocytes (P.H.), primary hepatocytes cultured for two 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. HuS-E/2 and HuS-T/2 cells were cultured in Mebiol Gel in 12-well plates at a concentration of  $5 \times 10^5$  cells/well. The microscopic characteristics of these cells after one week of 3D culture are shown. (B) The expressions of HNF4 $\alpha$ , HNF1 $\beta$ , 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 HNF4 $\alpha$ , HNF1 $\beta$ , 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-depleted 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, HNF1 $\beta$ , 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-in-

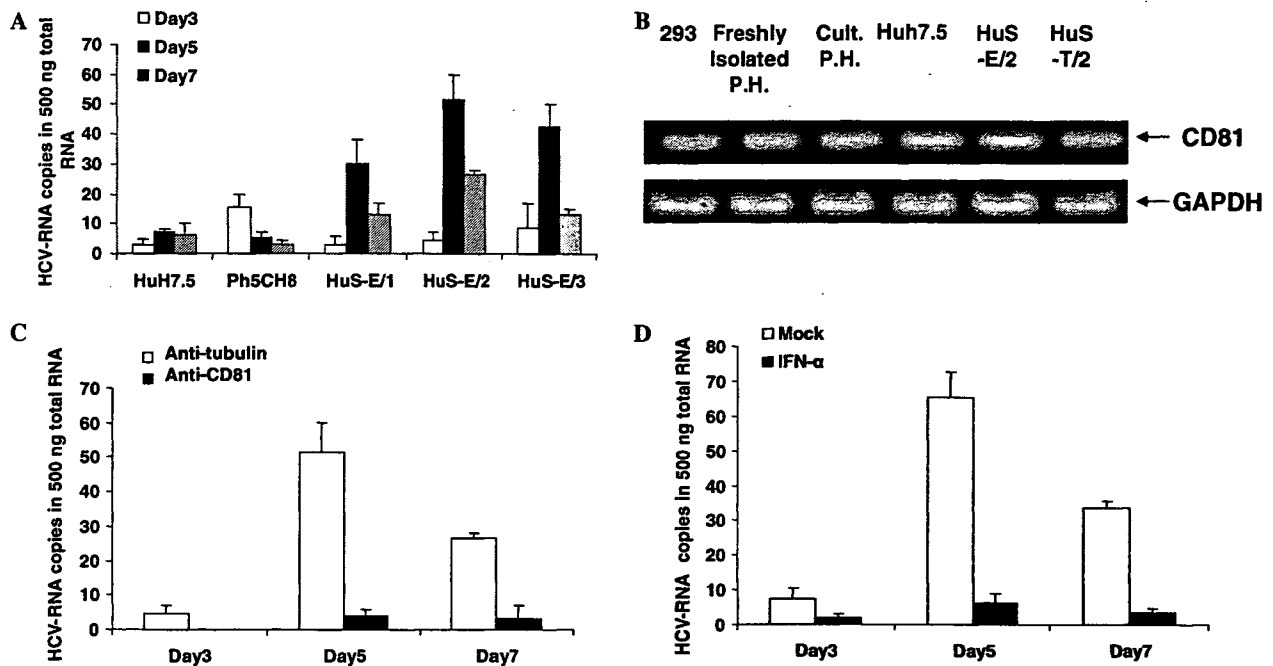
fecting serum. Of the three HuS-E clones examined, HuS-E/2 clone demonstrated the highest infectability with HCV genotype 1b in comparison to Huh-7.5, PH5CH8 (Fig. 4A), and HuS-T cells (data not shown), which were excluded from further experiments.

### 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. IFN $\alpha$ 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) IFN $\alpha$  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 IFN $\alpha$ .

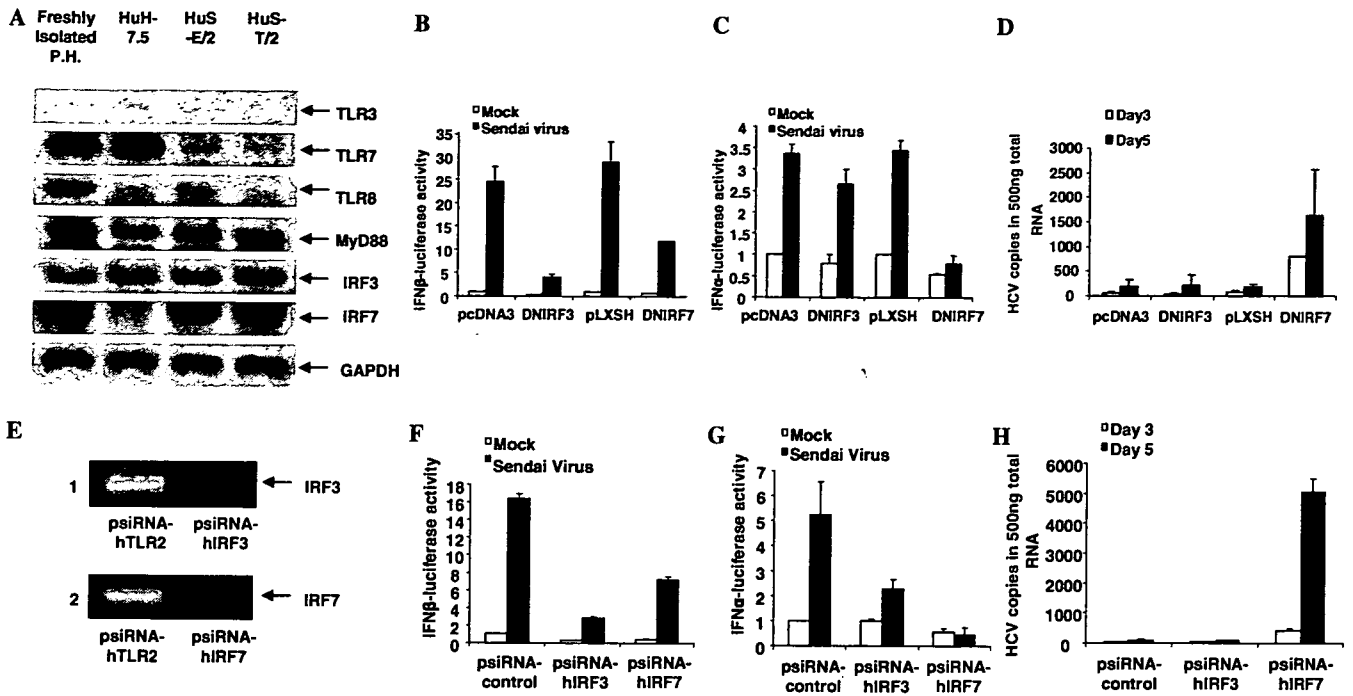
without or with 100 U/ml IFN $\alpha$ . The enhancement of the HCV-RNA genome titers on the fifth day (about 10-fold) was not observed in cells treated continuously with IFN $\alpha$  (Fig. 4D). This result suggests that IFN $\alpha$  inhibited HCV replication in infected HuS-E/2 cells.

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

Production of interferon-alpha (IFN $\alpha$ ) and interferon-beta (IFN $\beta$ ) 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- $\alpha/\beta$  production [25]. To determine 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 IFN $\beta$  and IFN $\alpha$  production by HuS-E/2 cells infected with Sendai virus were confirmed using assays of IFN $\beta$  or IFN $\alpha$  promoter-driven luciferase reporters. DNIRF-3 exhibited strong inhibition of IFN $\beta$  production (Fig. 5B) and weaker inhibition of IFN $\alpha$  transcription (Fig. 5C), while DNIRF-7 strongly inhibited IFN $\alpha$  production (Fig. 5C) and only weakly inhibited IFN $\beta$  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 IFN $\beta$  and IFN $\alpha$  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 pIFN $\beta$ -luc (B) or pIFN $\alpha$ -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  $\mu$ g/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  $\mu$ g/ml. Two weeks later, cells were cotransfected with pIFN $\beta$ -luc (F) or pIFN $\alpha$ -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 IFN $\alpha$  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 IFN $\alpha$ -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

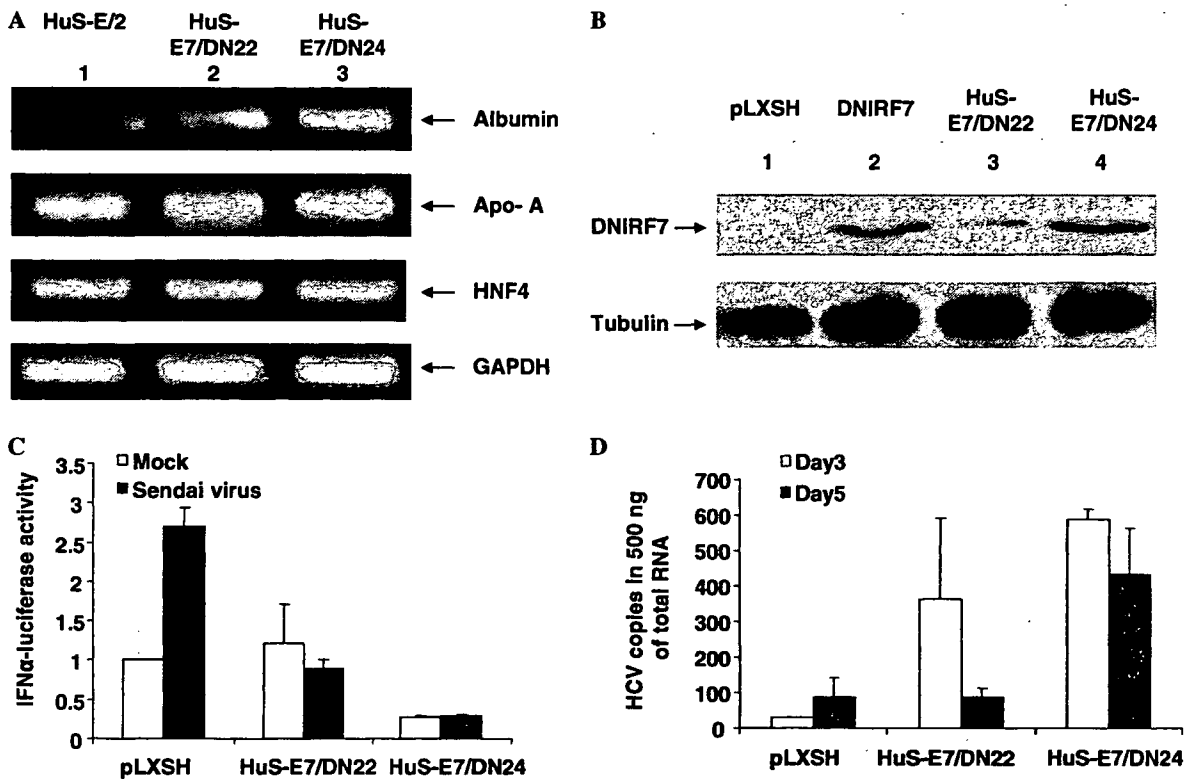


Fig. 6. (A) The pLXSH-HA-DNIRF-7 plasmid was transfected into HuS-E/2 cells, followed by selection in 100  $\mu$ g/ml Hygromycin B. Two clones, HuS-E7/DN22 (lane 2) and HuS-E7/DN24 (lane 3), were obtained. We investigated the expression of albumin, apo-A, HNF4, and GAPDH as an internal control in parental 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 IFN $\alpha$ -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.

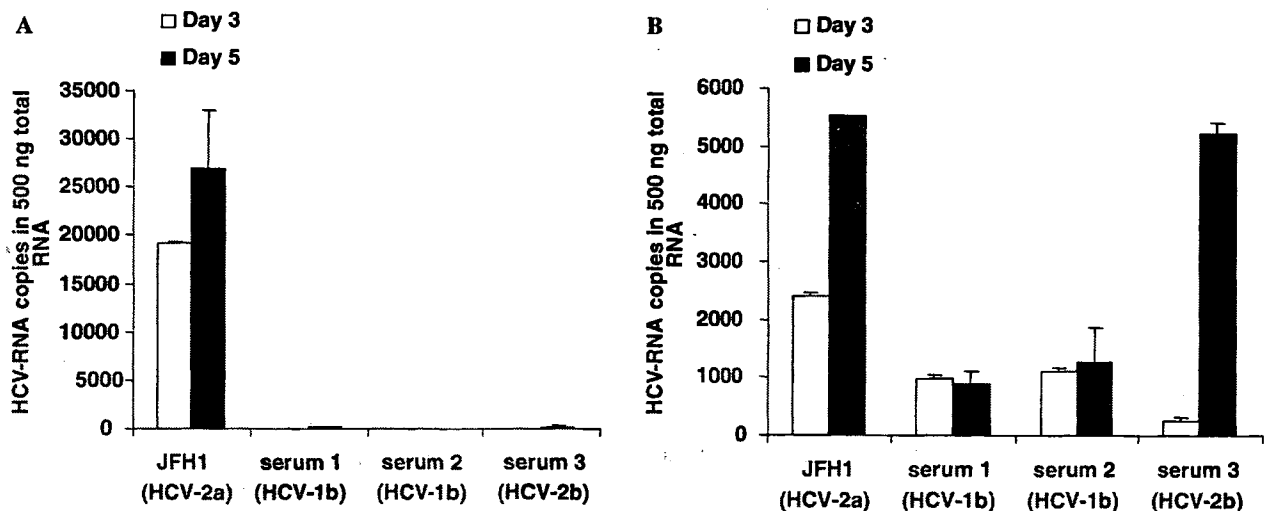


Fig. 7. The infectability of Huh-7.5 and HuS-E7/DN24 cells to different HCV genotypes. Huh-7.5 (A) and HuS-E7/DN24 (B) cells were infected with same titer of JFH1 (HCV-2a), two different HCV-1b serums and one HCV-2b serum. After removing the infected medium, the cells were washed in PBS and recultured 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 HNF1 $\alpha$  [27]. HuS-E/2 cells continued to express HNF1 $\alpha$  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 IFN $\alpha$  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 IFN $\alpha/\beta$  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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## Editor-Communicated Paper

***In Vitro* Infection of Immortalized Primary Hepatocytes by HCV Genotype 4a and Inhibition of Virus Replication by Cyclosporin**Mohamed A. El-Farrash<sup>1,†</sup>, Hussein H. Aly<sup>2,3,4,†</sup>, Koichi Watashi<sup>3</sup>, Makoto Hijikata<sup>3</sup>, Hiroto Egawa<sup>2</sup>, and Kunitada Shimotohno<sup>\*,3</sup><sup>1</sup>Department of Medical Microbiology and Immunology, Faculty of Medicine, Mansoura University, Mansoura, Egypt,<sup>2</sup>Graduate School of Medicine, Department of Transplant Surgery, Kyoto University Hospital, Kyoto, Kyoto 606–8507, Japan, <sup>3</sup>Laboratory for Human Tumor Viruses, Institute for Virus Research, Kyoto University, Kyoto, Kyoto 606–8507, Japan, <sup>4</sup>Hepatology Department, National Hepatology and Tropical Medicine Research Institute, Cairo, Egypt

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**Abstract:** Hepatitis C virus (HCV) is a major cause of liver cirrhosis and hepatocellular carcinoma worldwide. We previously reported that cyclosporin A (CsA) inhibits HCV-1b replication. However, its inhibition of JFH-1 (HCV-2a) was much less. Since HCV genotype clearly affects the *in vitro* and *in vivo* response to anti-viral therapy, we wished to examine the effect of CsA and its non-immunosuppressive derivative NIM811 on HCV genotype 4a replication. We first established an *in vitro* system supporting HCV-4a infection and replication using immortalized human hepatocytes, HuS-E7/DN24 (HuS) cells, and these cells were infected with sera obtained from Egyptian patients with chronic HCV-4a infection. HuS cells supported more robust HCV-4a replication than both HuH-7.5 and PH5CH8 cells, and HCV-4a infection and replication were completely inhibited by 3 µg/ml CsA and 0.5 µg/ml NIM811. Thus, HuS cells are a good model system supporting the infection and high-level replication of HCV-4a, and both CsA and NIM811 effectively inhibit HCV-4a replication in this system.

**Key words:** HCV-4a, Hepatitis, NIM811, HuS

Hepatitis C virus (HCV) is an enveloped, positive-stranded RNA virus of the genus *Hepacivirus* and family *Flaviviridae* (14). HCV is an important cause of morbidity and mortality worldwide. A high proportion of individuals infected with HCV develop chronic hepatitis, and this may progress to cirrhosis and hepatocellular carcinoma (2). The WHO estimates that there are at least 21.3 million HCV carriers in the Eastern Mediterranean countries, and this number approaches the combined estimated number of HCV carriers in the Americas (13.1 million) and Europe (8.9 million). Indeed, the prevalence of HCV infection in Egypt (15–25%) is amongst the highest in the world (18).

The genomes of a number of different HCV strains have been cloned, and the divergence of these

sequences indicates the existence of at least six HCV genotypes with a number of subtypes (12). In North America and Northern Europe, HCV subtype 1a is the most common followed by 2b and 3a. In Japan, subtype 1b is responsible for up to 73% of cases of HCV infection (13). However, genotypes other than 1, 2 or 3 represent most HCV cases in the remaining countries. HCV genotype 5 has been isolated almost exclusively from patients in South Africa, and genotype 6 is primarily found in Hong Kong, Vietnam and throughout South East Asia (13). However, it is now clear that genotype 4 is largely confined to Central Africa and the Middle East, a region containing approximately one-fifth of all HCV positive individuals worldwide (10).

*Abbreviations:* CsA, cyclosporin A; CyPB, cyclophilin B; HCV, hepatitis C virus; HCVpp, hepatitis C virus pseudoparticles; hTERT, human telomerase reverse transcriptase; HuS, HuS-E7/DN24; IFN-α, interferon-alpha; IRF-7, interferon regulatory factor-7; MAb, monoclonal antibody; Sv40-Lt, Simian virus 40-large T antigen.

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Our understanding of HCV was dramatically impaired by the lack of an effective virus culture system, and the establishment of self replicating full-length HCV genomic replicon systems for genotypes 1a and 1b in human hepatoma (HuH-7) cells provided valuable insight into the mechanisms of HCV replication (6). Recently, several groups reported the production of infectious virus following transfection of HCV genotype 2a genomic RNA into HuH-7 cells (1). However, immortalized, tumor derived cell lines may obscure some aspects of HCV biology, and human primary hepatocytes are the ideal cell to examine HCV replication under more physiologic conditions. Liver epithelial cells, hepatocytes and biliary cells proliferate *in vivo* in response to regenerative stimuli, but they do not proliferate under standard culture conditions *in vitro*. Recently, Aly et al. established a cell line derived from primary hepatocytes immortalized by the expression of the E6 and E7 genes of the human papillomavirus type 18 and human telomerase reverse transcriptase gene (hTERT) (1). This cell line, HuS-E7/DN24 (HuS), also lacks interferon regulatory factor-7 (IRF-7) (1), and it maintains a phenotype consistent with primary hepatocytes such as the continuous expression of albumin, apolipoprotein A, transferrin and E-cadherin without evidence of transformation even after prolonged culture (1).

At present, the only approved therapies for chronic HCV infection are interferon- $\alpha$  (IFN- $\alpha$ ) with or without ribavirin, but these drugs fail to clear HCV from a significant number of patients (5). Recently our group discovered that cyclosporin A (CsA), and its non-immunosuppressive analogue, NIM811 could suppress HCV genome replication in a cell culture system (4, 16). The anti-HCV effects of CsA correlated with cyclophilin B inhibition (CyPB). CyPB, a cellular target of CsA, regulates HCV replication through its interaction with the viral RNA-dependent RNA polymerase NS5B, and CsA suppresses HCV replication by disrupting the association of CyPB with NS5B (17). CsA strongly suppressed HCV-1b replication, but its effects on HCV-2a (JFH-1) replication were less profound, indicating that different HCV genotypes may be differentially susceptible to CsA (17). However, the ability of CsA to inhibit the replication of HCV-4a, one of the most common genotypes worldwide, has not been reported.

We infected HuS immortalized primary hepatocytes with serum samples obtained from Egyptian patients with chronic HCV-4a infection and examined the ability of CsA and NIM811 to suppress viral replication. Our data indicate that HCV-4a is highly susceptible to CsA and NIM811 treatment, suggesting that these may rep-

resent new treatment options to explore in HCV-4a infected individuals.

## Materials and Methods

**Cells.** The immortalized primary hepatocyte cell line HuS-E7/DN24 (HuS) was cultured as reported (1). The hepatoma cell line HuH-7.5 that supports infection and replication of the recombinant HCV strain JFH-1 (HCV-2a) (15) was cultured as previously described. Simian virus 40-large T antigen (Sv40-Lt) immortalized primary hepatocytes (PH5CH8) supporting infection and replication of HCV-1b genotype samples were cultured as reported (16).

**Serum samples.** Serum samples from Egyptian patients with chronic HCV hepatitis genotype 4a were collected in Egypt (after approval of the Medical Research Ethics Committee in Mansoura University). The virus titer in sera was determined using real-time PCR for the detection of HCV as described (8).

**Real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) analysis.** Five-hundred nanograms of total RNA isolated from cells was reverse transcribed as previously described (1), and the 5'-untranslated region of HCV genomic RNA was quantitated using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, Calif., U.S.A.) as described previously (8). The forward and reverse primers used in this experiment were 5'-CGGGA-GAGCCATAGTGG-3' and 5'-AGTACCACAAGGC-CTTTCG-3', respectively. The fluorogenic probe was 5'-CTGCGGAACCGGTGAGTACAC-3'. As an internal control, ribosomal RNA was also quantified using *TaqMan* ribosomal RNA control reagents (Applied Biosystems).

**Chemicals.** CsA and IFN- $\alpha$  were purchased from Sigma and Otsuka Pharmaceutical Co., respectively. NIM811 was generously provided by Novartis (Basel, Switzerland).

**In vitro infection experiments.** HuS, PH5CH8 and HuH-7.5 cells were harvested, washed, adjusted to  $5 \times 10^4$  cells/ml in growth medium and cultured in 12-well plates with 1 ml media per well. After 24 hr, the culture medium was replaced with 1 ml of complete medium containing HCV. Plates were incubated at 37 C for 24 hr, and the virus-containing media was removed. The cells were then washed twice with PBS, and 1 ml of complete culture medium was added to each well. Plates were re-incubated and the cells were harvested at the indicated times for evaluation. Total RNA was extracted from infected or non-infected cells, and HCV-RNA was measured by real time RT-PCR as described (8).



**HCV infection neutralization experiment.** HCV infection of HuS cells was inhibited using anti-CD81 (BD-Bioscience) or anti-HCV-E2 (AP33) as previously reported (7). The mouse monoclonal antibody (MAb) AP33 recognizes the E2 glycoprotein of HCV-1A, and it neutralizes retroviral pseudoparticles (HCVpp) carrying genetically diverse HCV envelope glycoproteins, including HCV-4a-glycoproteins (9). Anti-tubulin (Sigma, St. Louis, Mo., U.S.A.) was used as a control.

**Inhibition of HCV replication.** HuS cells were cultured at a concentration of  $5 \times 10^4$  cells/well in 12-well plates. After 24 hr, cells were infected by adding growth medium containing HCV-4a serum (adjusted to a final virus titer of  $5 \times 10^4$  copies of RNA/ml) and the

indicated concentrations of CsA, NIM811, or IFN- $\alpha$ . Plates were incubated at 37 C for 24 hr, and the medium was then removed, the cells were washed twice with PBS, and 1 ml of fresh medium containing CsA, NIM811, or IFN- $\alpha$  was added to the cells. On day 5 post-infection, the medium was removed, and the cells were washed, trypsinized and collected. Total RNA was extracted from infected or non-infected cells, and HCV-RNA was measured by real-time RT-PCR.

**CsA and NIM811 toxicity test.** HuS cells were cultured at a concentration of  $5 \times 10^4$  cells/well in 12-well plates. Cells were untreated, or treated with 3  $\mu$ g/ml CsA or 1  $\mu$ g/ml NIM811. Every 2 days, the medium was replaced with fresh, drug-containing medium.

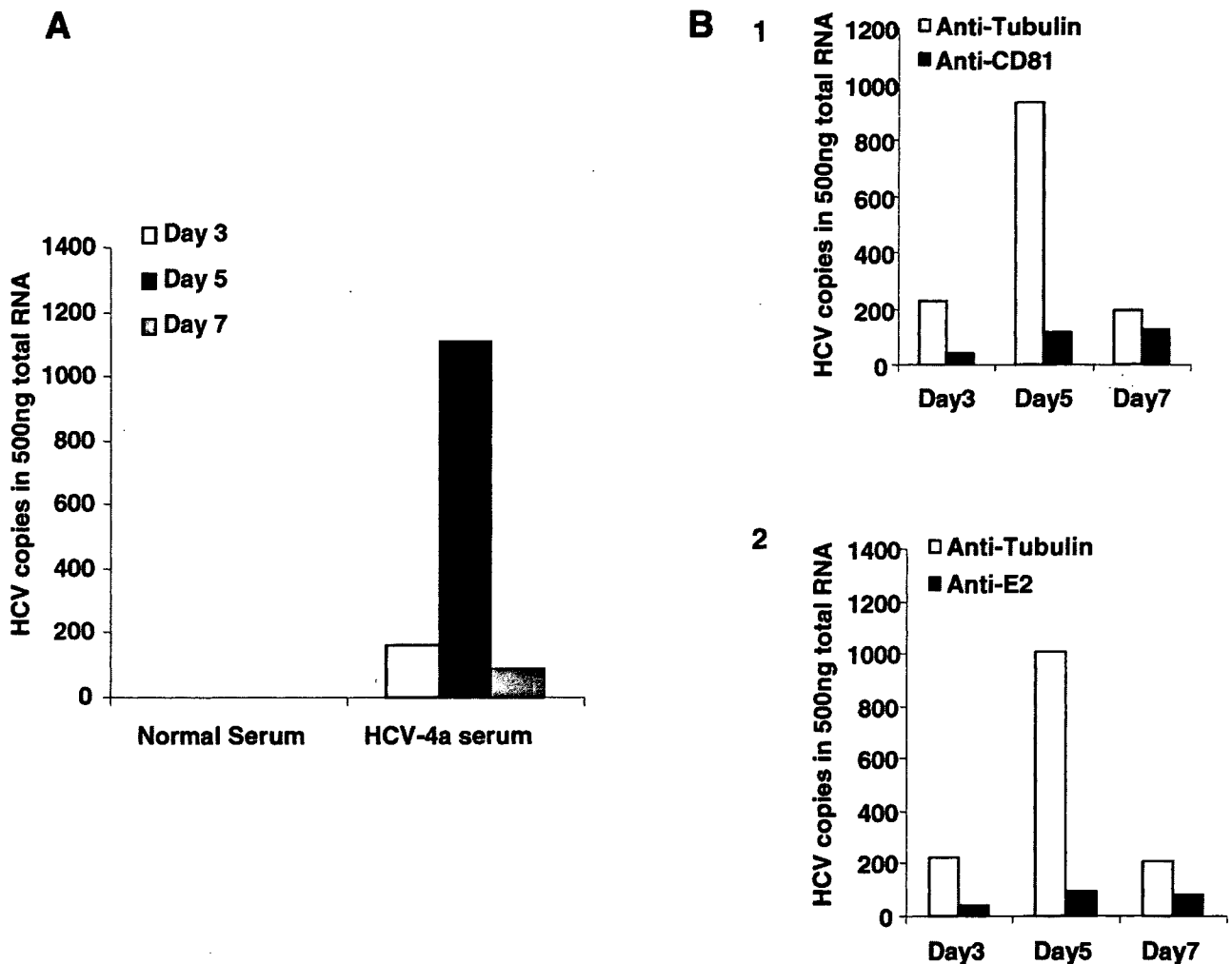


Fig. 1. Kinetics of HCV-4a replication in HuS-E7/DN24 (HuS) cells. (A) Cells were incubated with a serum sample containing HCV-4a virus for 24 hr, and the virus-containing medium was then removed, the cells were washed twice and incubated for the indicated periods of time. At 3, 5 and 7 days after infection, cells were washed again, and total RNA was extracted and used to determine the number of HCV-RNA copies after reverse transcription and real-time PCR. The results shown are the average of two independent experiments. (B) Cells were treated with anti-CD81 or anti-tubulin antibody (1), or HCV containing serum was pre-incubated with anti-E2 or anti-tubulin antibody (2). HCV-4a infection was performed and analyzed as above. The results shown are the average of two independent experiments.

Wells from each group were collected on days 1, 2, 3, 4, and 5 to examine the number of viable cells in each well after staining of the cells with trypan blue.

## Results

### *HCV-4a Infection and Kinetics in HuS Cells in Vitro*

We previously established the HuS cell line and showed that these cells were efficiently infected with HCV-1b and HCV-2b containing serum and JFH-1 (HCV-2a) concentrated medium (1). To determine whether HuS cells could support HCV-4a infection, HCV-4a containing serum was incubated with HuS cells for 24 hr, and, following washing and a change of media, the cells were harvested 3, 5, and 7 days after infection. Total RNA was extracted from the infected cells, and the amount of HCV-RNA in 500 ng total RNA was measured (Fig. 1A). As expected, after 3 days in culture the RNA extracted from non-infected HuS cells did not contain any HCV copies. However, viral RNA was detected in cells incubated with HCV-4a containing serum. The number of viral genomes detected was  $164.5 \pm 52$  copies/500 ng of total RNA at day 3, and this peaked at  $1,111 \pm 176$  copies/500 ng of total RNA at day 5. The increase from day 3 to day 5 after infection clearly indicates viral replication occurring in these cells rather than increased viral entry without replication. The number of viral genomes decreased dramatically on day 7 to  $80 \pm 18$  copies/500

ng of total RNA. Identical results were obtained using HCV-4a genotype samples isolated from two other individuals (data not shown).

### *HCV-4a Infection Is Neutralized by Anti-CD81 and Anti-E2*

CD81 is involved in the entry of HCV pseudoparticles and *in vitro*-synthesized JFH-1 (15). Similarly, HCV envelope protein E2 is essential for HCV cell entry and infection (7). Blocking antibodies against both CD81 and E2 inhibit viral infection in *in vitro* models. To determine if HCV-4a infection of HuS cells is analogous to previously described systems, we treated cells with either anti-CD81 (Fig. 1B-1) or anti-HCV-E2 (Fig. 1B-2) blocking antibodies. Consistent with results obtained using other HCV genotypes, antibodies against CD81 and HCV-E2 effectively reduced HCV-4a infectivity of HuS cells compared with a non-blocking anti-tubulin antibody.

### *HuS Cells Are More Permissive to Infection by HCV-4a than PH5CH8 and HuH-7.5 Cells*

HuS cells clearly support HCV-4a infection and replication. We wished to compare the ability of different cell lines to support infection and replication of HCV-4a. HuS, PH5CH8 and HuH-7.5 cells were infected with HCV-4a serum containing  $5.0 \times 10^4$  copies/ml, and the number of viral copies at 3, 5, and 7 days after infection was examined. PH5CH8 and HuH-

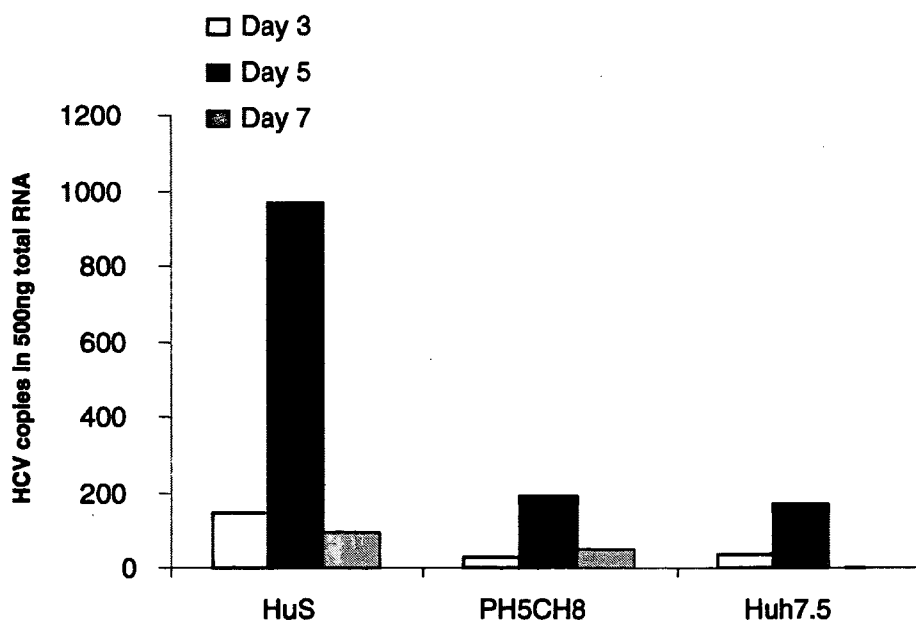


Fig. 2. HuS cells are more permissive to infection by HCV-4a than PH5CH8 and HuH-7.5 cells. HCV-4a replication was examined in HuS cells, PH5CH8 and HuH-7.5 cells. HuS, PH5CH8, and HuH-7.5 cells were infected with the same serum sample under the same conditions, and the extent of HCV genome replication was measured by real-time RT-PCR on days 3, 5, and 7 after infection. The results shown are the average of two independent experiments.

7.5 cells supported HCV-4a infection to some extent, but much less viral replication occurred in these cells (Fig. 2). Indeed, we would not detect any HCV RNA in HuH-7.5 cells 7 days after infection. Identical results were obtained using HCV-4a genotype virus isolated from two other individuals (data not shown). These data are consistent with a previous report showing increased viral infection and/or replication in HuS cells incubated with HCV genotypes 1b and 2b derived from patients' sera (1).

#### *Inhibition of HCV-4a Replication in HuS Cells by CsA and NIM811*

Having established that HuS cells support HCV-4a infection and replication similar to other HCV genotypes examined, we wished to determine if HCV-4a

was sensitive to treatment with CsA or its non-immunosuppressive derivative NIM811. Serum isolated from two different individuals was used as a source of HCV-4a for all experiments. For both samples, treatment of infected HuS cells with 1  $\mu\text{g/ml}$  of CsA reduced the number of recovered viral copies by 75% at 5 days compared with untreated cells, and increasing the concentration of CsA to 3  $\mu\text{g/ml}$  almost completely inhibited HCV-4a replication in HuS cells (Fig. 3A). Furthermore, we were unable to detect any viral RNA in HCV-4a infected HuS cells treated with either 0.5 or 1  $\mu\text{g/ml}$  NIM811 (Fig. 3B). For comparison, we also treated infected cells with 50 IU/ml IFN- $\alpha$ , and this reduced viral replication by 80–90% in HuS cells (Fig. 3C). The inhibition of HCV-4a replication by CsA or NIM811 could be due to a general cytotoxic effect, and,

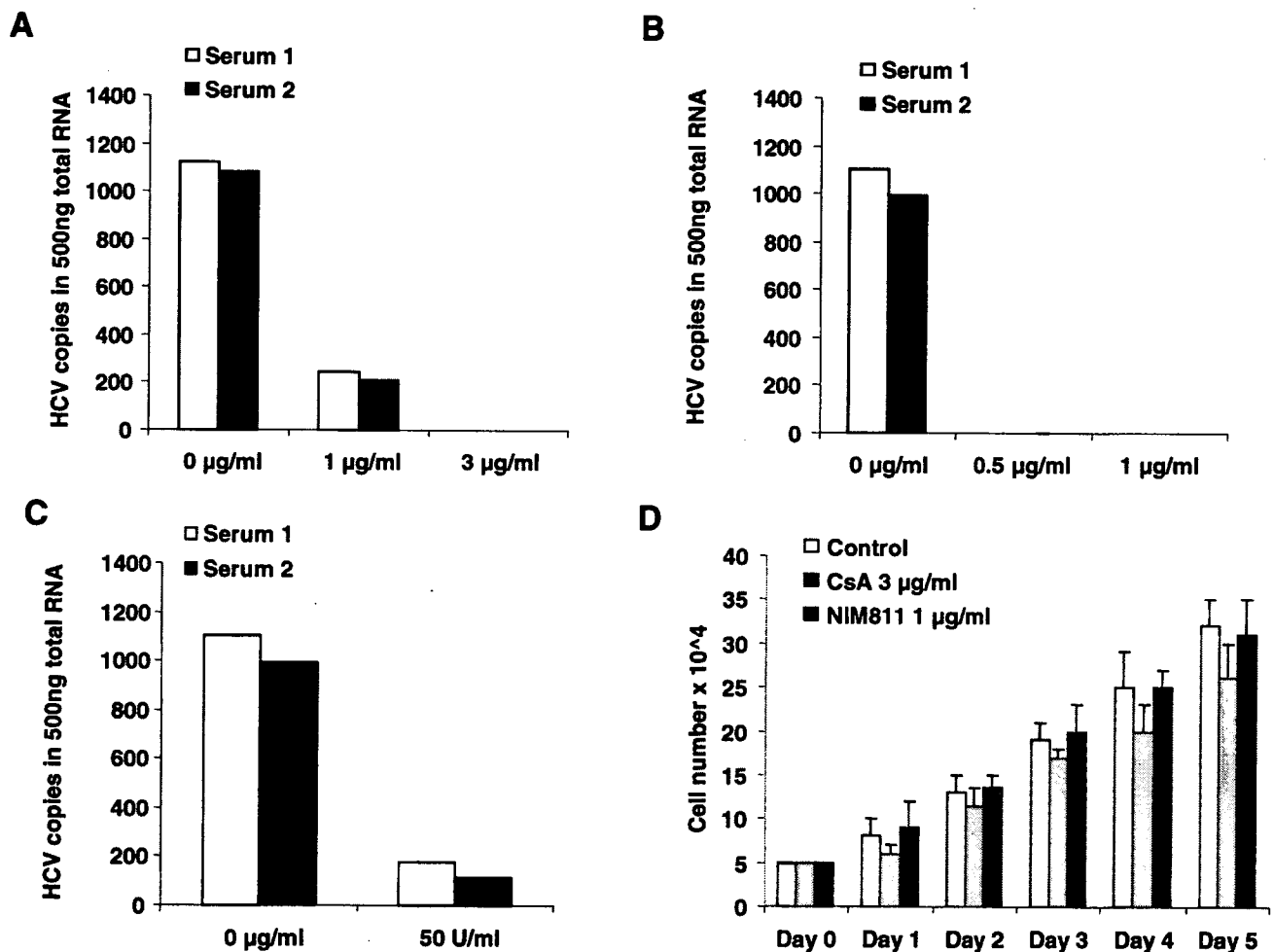


Fig. 3. Inhibition of HCV-4a replication in HuS cells by cyclosporin and NIM811. Two different serum samples containing HCV-4a were used to infect HuS cells in presence of CsA 0, 1 or 3  $\mu\text{g/ml}$  (A), NIM811 0, 0.5 or 1  $\mu\text{g/ml}$  (B) and IFN- $\alpha$  0 or 50 IU/ml (C). Cells were incubated for 5 days and the level of HCV replication was measured by real-time RT-PCR as described in Fig. 1. The results represent the average of two independent experiments. (D) HuS cells were left untreated or treated with either 3  $\mu\text{g/ml}$  CsA or 1  $\mu\text{g/ml}$  NIM811 for 5 days. Cells were harvested at the indicated times, and the number of viable cells in each well was determined. The results shown are the average of 3 independent experiments.

to exclude this possibility, we cultured HuS cells with or without CsA or NIM811 for 5 days. Although HuS cell proliferation was slightly reduced by the inclusion 3 µg/ml of CsA in the culture media, it was not affected by the addition of 1 µg/ml of NIM811 compared to control untreated cells (Fig. 3D). Thus, the inhibition of HCV-4a replication by NIM811 and CsA in HuS cells appear to be due to direct anti-viral effects.

## Discussion

There are six major HCV genotypes with a number of different subtypes that vary by geographic distribution and mode of transmission (12). Subtypes 1a, 1b, 2a, 2b, and 3a are distributed globally and account for the majority of HCV infections worldwide (13). HCV-4a is the predominant genotype seen in the Middle East, a region encompassing approximately 20% of the estimated 170 million HCV carriers in the world (11). HCV-4a responds poorly to interferon treatment, and individuals infected with HCV-4a are at higher risk of liver related death and the need for transplantation (19). Thus, the development of new treatments that are effective across a broader range of HCV genotypes is urgently needed.

The development of more effective antiviral therapies and an effective HCV vaccine remain the largest challenges for HCV research in the near future. However, because HCV genotype affects the outcome of antiviral therapy, it has become increasingly clear that an *in vitro* system supporting HCV-4a infection and replication is needed to develop anti-viral therapies effective against this important viral subtype. Recent studies have examined several different viral genotypes using the replicon system in HuH-7 cells, but this is the first report specifically designed to determine *in vitro* conditions compatible with HCV-4a infection and replication.

We recently established the HuS cell line, and this cell line is able to be infected *in vitro* by sera containing HCV-1b and -2b (1). In this study, we showed that HuS cells also clearly support the infection and replication of HCV-4a derived from patient serum samples. A high level of HCV-RNA was seen in these cells 5 days after infection, but this rapidly declined by day 7. The kinetics of infection observed for HCV-4a in HuS cells was similar to that observed when PH5CH8 cells were infected with HCV-1b containing plasma (16). Since the aim of this paper was to study the suppressive effect of CsA and NIM811 on HCV infection and replication, which was monitored clearly on days 3 and 5, we didn't culture the infected cells for more than 7 days. The extent of HCV-4a infection of HuS was much greater

than that for the PH5CH8 and HuH-7.5 cell lines, and these differences in permissiveness may arise from the relative amounts or activities of host cell factors required for RNA replication. Alternatively, HuS cells lack functioning IRF-7, and this likely impairs their ability to produce IFN-α. The absence IFN-α from the culture media following infection could clearly contribute to the increased replication seen in HuS cells.

There is an urgent need for improved HCV drug therapies. We previously reported that CsA suppresses HCV replication *in vitro* (16), and these effects are also seen using its non-immunosuppressive derivative NIM811. These compounds can induce multiple-log reductions in HCV-RNA levels in a replicon cell culture system using HCV-1b genotype virus (4). However, the ability of CsA to inhibit a HCV-2a genotype virus, JFH-1, was greatly reduced. In this study we examined the effects of CsA and NIM811 on HCV-4a infection and replication in HuS cells. As was seen with HCV-1b, both CsA and NIM811 strongly inhibited HCV-4a infection and replication in HuS cells. Furthermore, the inhibition by NIM811 was much greater at lower doses than that seen for CsA. These findings were similar to those previously reported for HuH-7 cells harboring a HCV-1b replicon (4).

The HuS cell line is the only system described available for the study of HCV-4a infection and replication. Using this cell line, we showed that HCV-4a replication is impaired by CsA and to a greater extent by its non-immunosuppressive derivative NIM811. Despite these findings, more studies are clearly needed to determine the activity of these drugs *in vivo* as well as characterize the possible differences in the HCV-4a life-cycle.

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