

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).

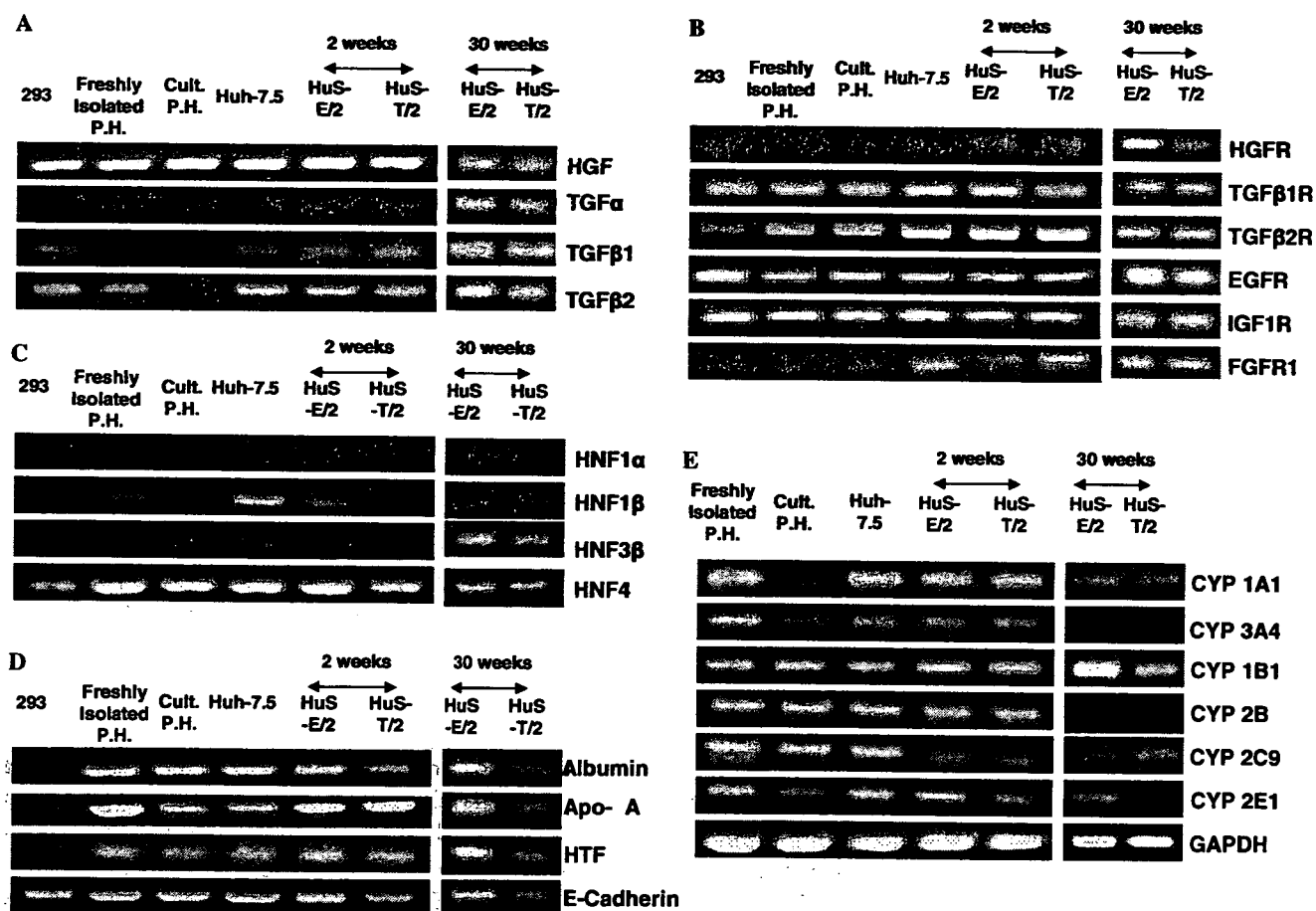
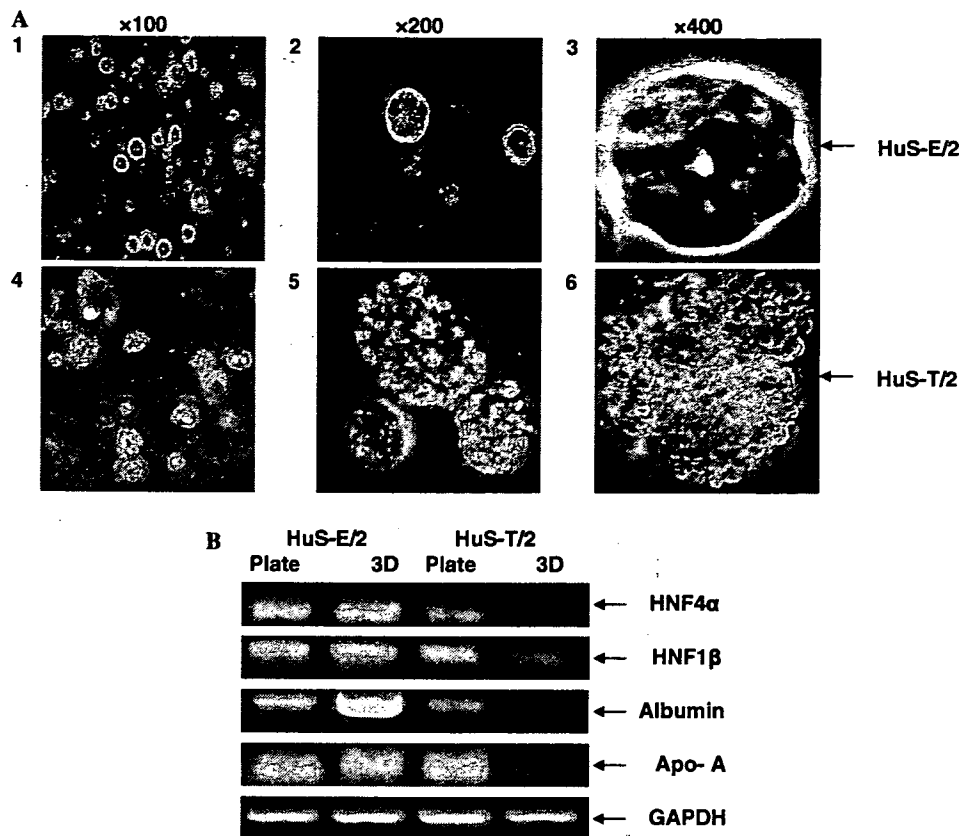


Fig. 2. Expression of the genes encoding growth factors (A), growth factor receptors (B), hepatocyte-specific nuclear factors (C), hepatocyte differentiation and functional markers (D), and CYP enzymes (E) in 293 cells, freshly isolated primary hepatocytes (P.H.), primary hepatocytes cultured for two 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-

fectured 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

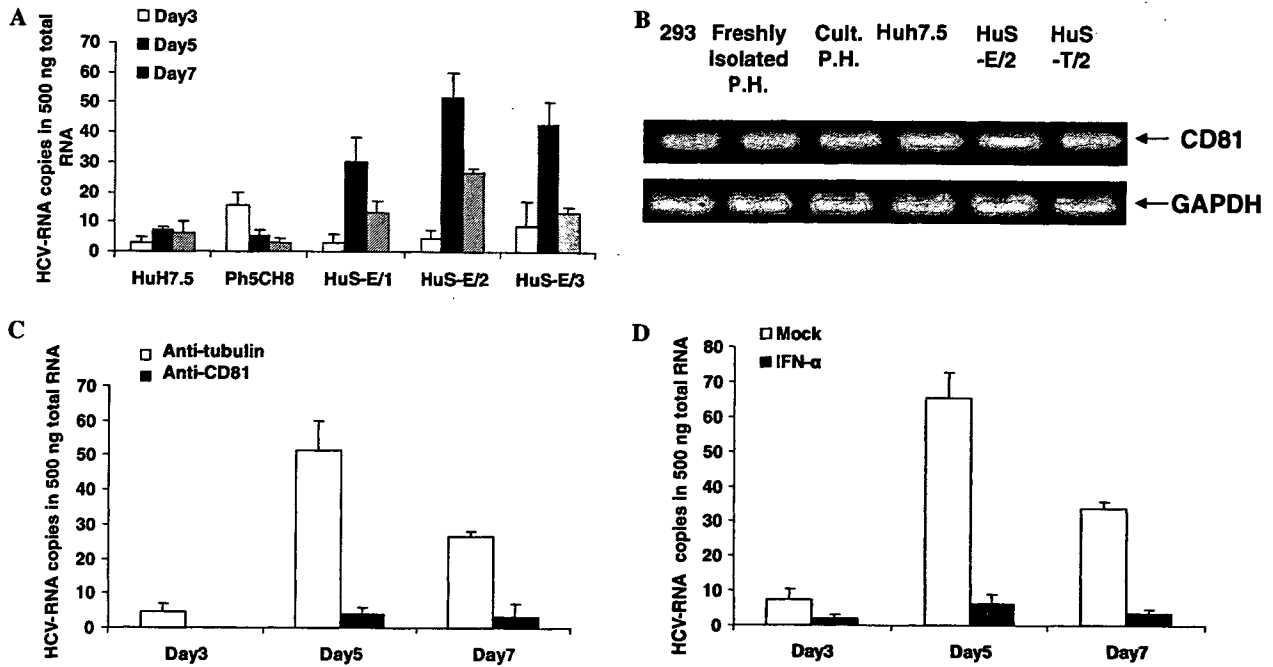


Fig. 4. (A) Serum from an HCV patient was used to infect Huh-7.5 cells, PH5CH8 cells, and three HPV E6E7-immortalized clones (HuS-E/1-3) for 24 h. After washing three times in phosphate-buffered saline (PBS), cells were cultured in fresh medium. Cells were then harvested and lysed at the indicated time 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 $\alpha$ .

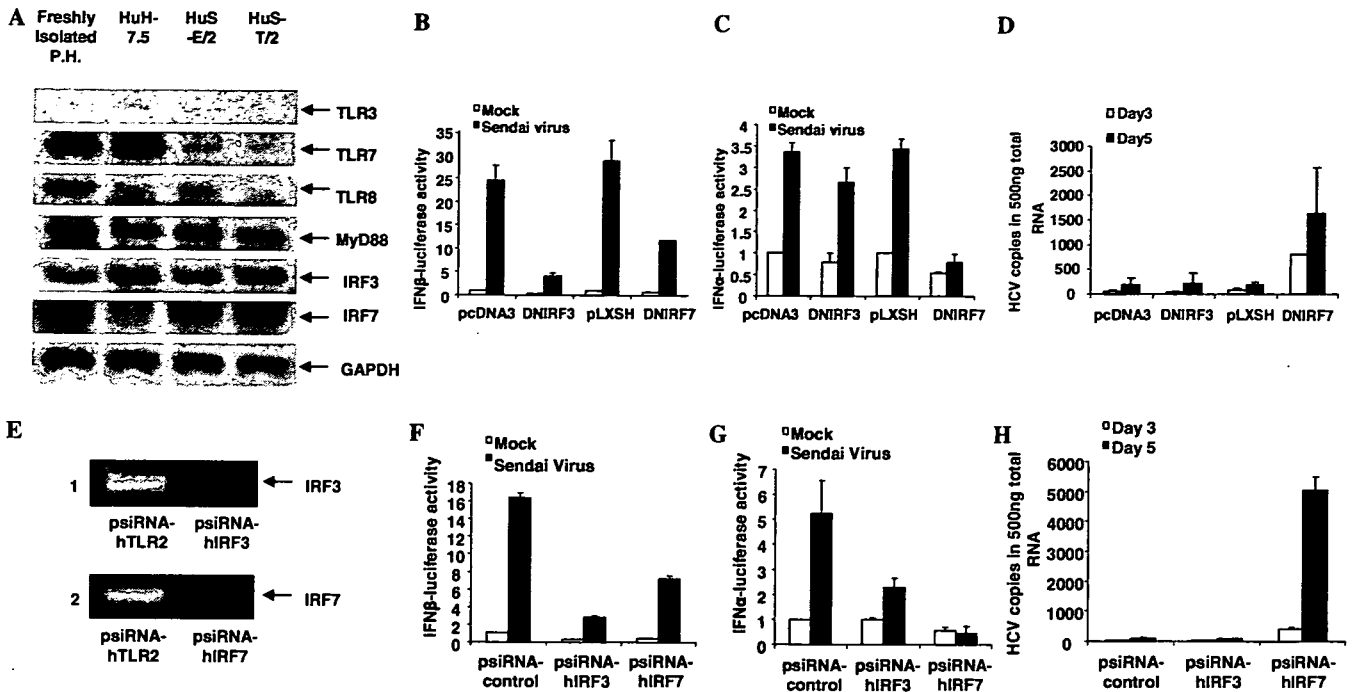
without or with 100 U/ml IFN $\alpha$ . The enhancement of the HCV-RNA genome titers on the fifth day (about 10-fold) was not observed in cells treated continuously with IFN $\alpha$  (Fig. 4D). This result suggests that IFN $\alpha$  inhibited HCV replication in infected HuS-E/2 cells.

### 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

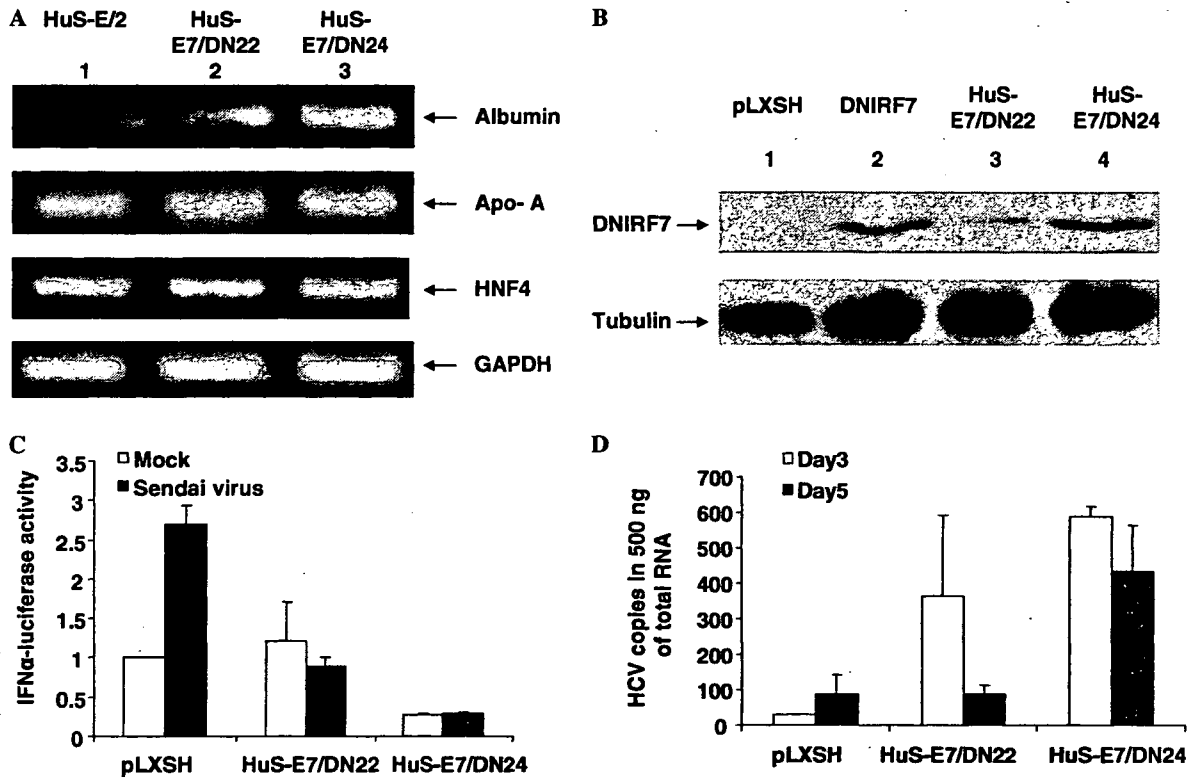


Fig. 6. (A) The pLXSH-HA-DNIRF-7 plasmid was transfected into HuS-E/2 cells, followed by selection in 100  $\mu$ g/ml Hygromycin B. Two clones, HuS-E7/DN22 (lane 2) and HuS-E7/DN24 (lane 3), were obtained. We investigated the expression of albumin, apo-A, HNF4, and GAPDH as an internal control in parental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

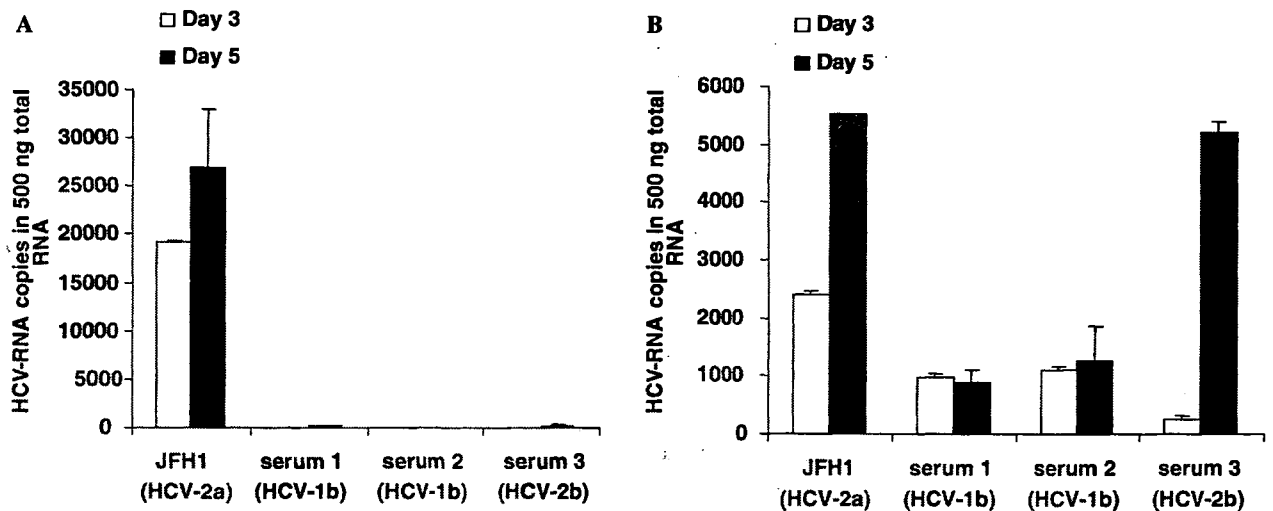


Fig. 7. The infectability of Huh-7.5 and HuS-E7/DN24 cells to different HCV genotypes. Huh-7.5 (A) and HuS-E7/DN24 (B) cells were infected with same titer of JFH1 (HCV-2a), two different HCV-1b serums and one HCV-2b serum. After removing the infected medium, the cells were washed in PBS and recultured 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 re-

present 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.

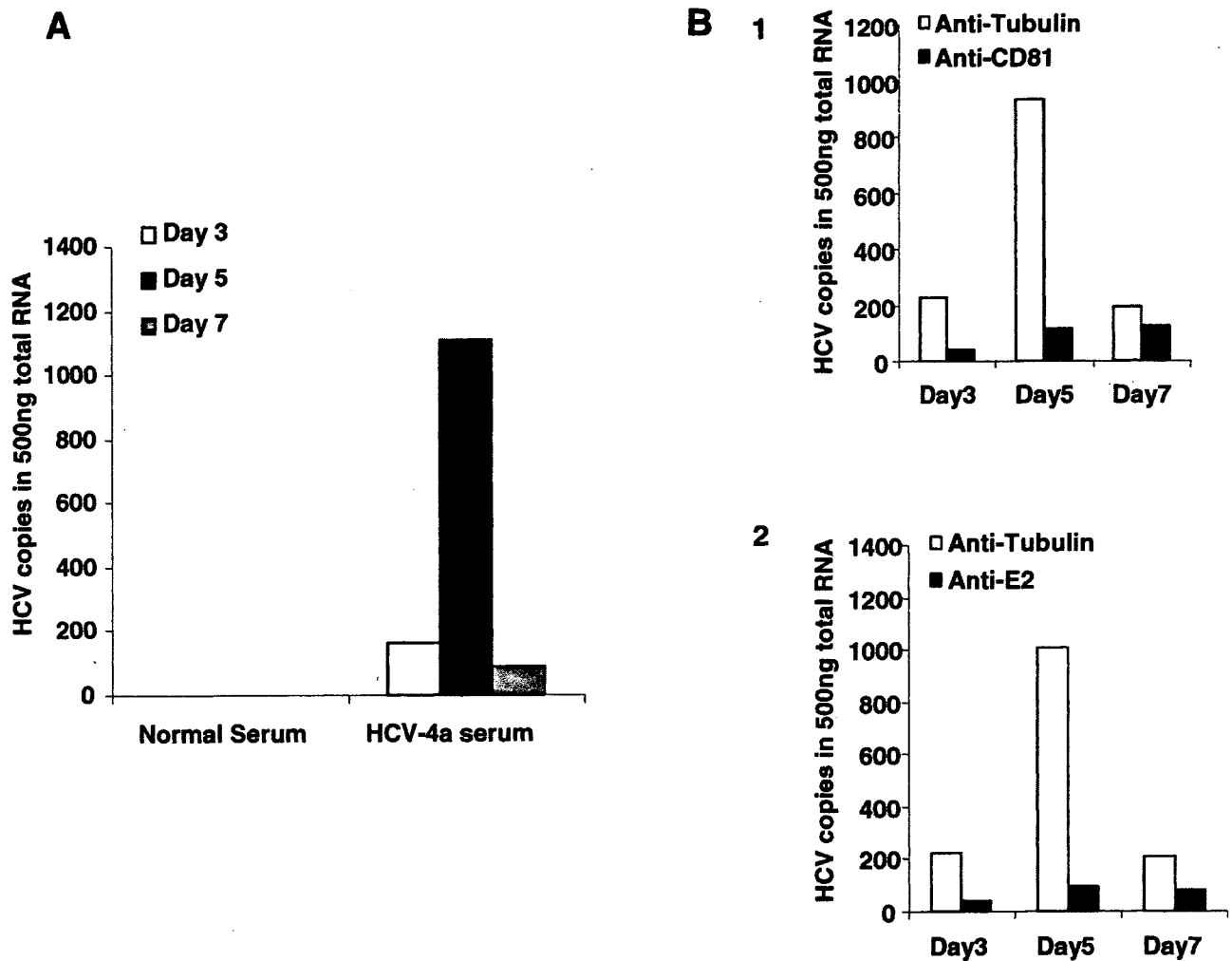


Fig. 1. Kinetics of HCV-4a replication in HuS-E7/DN24 (HuS) cells. (A) Cells were incubated with a serum sample containing HCV-4a virus for 24 hr, and the virus-containing medium was then removed, the cells were washed twice and incubated for the indicated periods of time. At 3, 5 and 7 days after infection, 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-

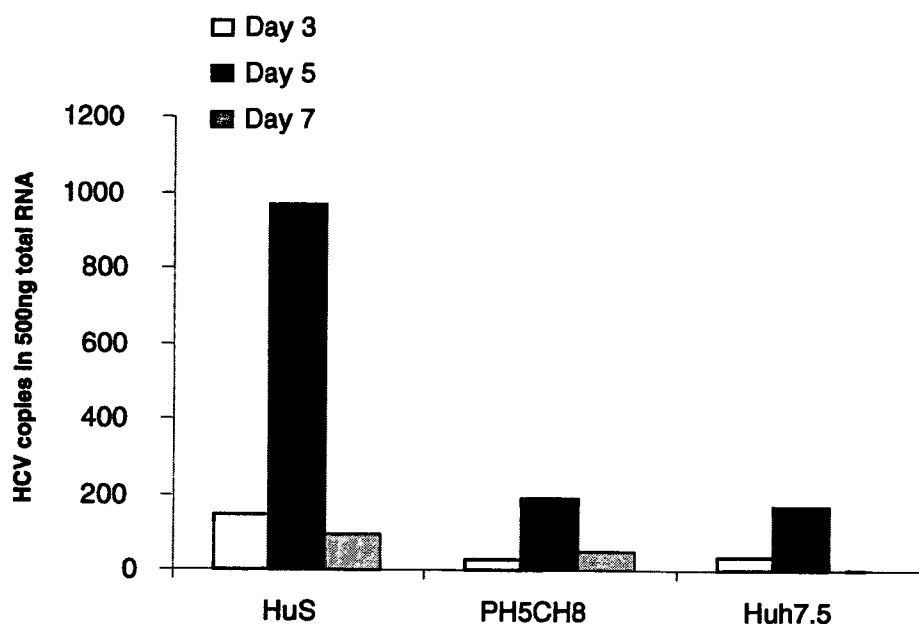


Fig. 2. HuS cells are more permissive to infection by HCV-4a than PH5CH8 and HuH-7.5 cells. HCV-4a replication was examined in HuS cells, PH5CH8 and HuH-7.5 cells. HuS, PH5CH8, and HuH-7.5 cells were infected with the same serum sample under the same conditions, and the extent of HCV genome replication 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,

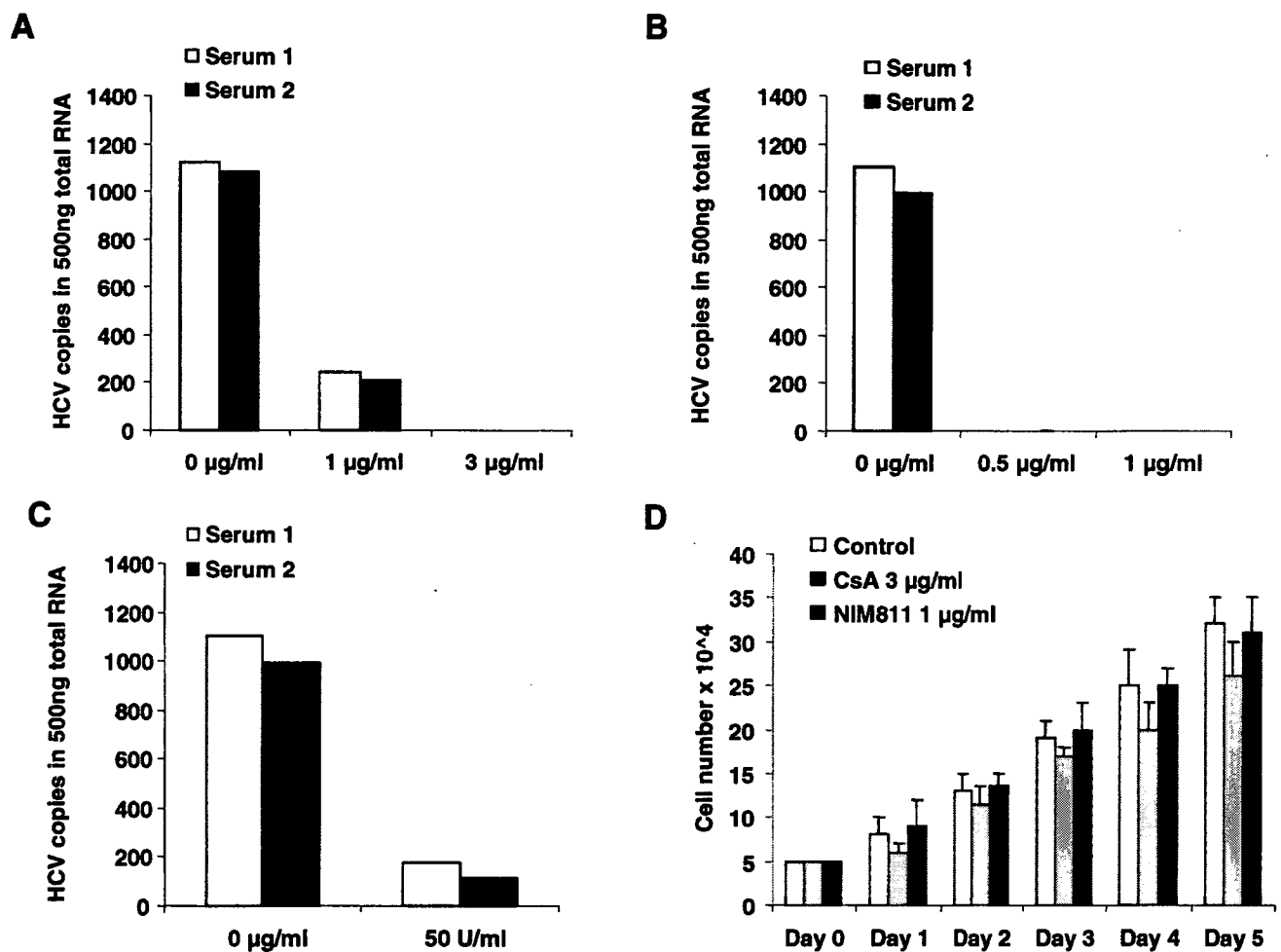


Fig. 3. Inhibition of HCV-4a replication in HuS cells by cyclosporin and NIM811. Two different serum samples containing HCV-4a were used to infect HuS cells in presence of CsA 0, 1 or 3  $\mu\text{g/ml}$  (A), NIM811 0, 0.5 or 1  $\mu\text{g/ml}$  (B) and IFN- $\alpha$  0 or 50 IU/ml (C). Cells were 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  $\mu\text{g/ml}$  of CsA in the culture media, it was not affected by the addition of 1  $\mu\text{g/ml}$  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- $\alpha$ . The absence IFN- $\alpha$  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.

The CsA derivative, NIM811 was kindly provided by Novartis (Basel, Switzerland). This work was supported by Grants-in-Aid for cancer research and for the second-term comprehensive 10-year strategies for cancer control from the Ministry of Health, Labour and Welfare, Japan, by Grants-in-Aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, by Grants-in-Aid for the research for the future program from the Japan Society for the Promotion of Science. Dr. M.A. El-Farrash (Ph.D.) was a recipient of the JSPS Invitation Fellowship Programs for Research in Japan. Dr. Hussein H. Aly is a recipient of the Japanese Gakushu Shoreihi scholarship and was partly supported by the Prof. Yassin A. El Ghaffar memorial scholarship for the improvement of liver research in Egypt.

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The background of the cover is a fluorescence microscopy image showing several cells. Each cell contains multiple bright spots of different colors: green, red, and blue. The spots are distributed throughout the cells, with some appearing more concentrated in certain areas. The overall image has a dark, almost black background, making the colorful spots stand out prominently.

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**Focus on  
Development and Disease**

**Hepatitis C infection  
A role for lipid droplets**

**TLRs balance neurogenesis  
Apoptotic mitochondrial breakdown**

# The lipid droplet is an important organelle for hepatitis C virus production

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**The lipid droplet (LD) is an organelle that is used for the storage of neutral lipids. It dynamically moves through the cytoplasm, interacting with other organelles, including the endoplasmic reticulum (ER)<sup>1–3</sup>. These interactions are thought to facilitate the transport of lipids and proteins to other organelles. The hepatitis C virus (HCV) is a causative agent of chronic liver diseases<sup>4</sup>. HCV capsid protein (Core) associates with the LD<sup>5</sup>, envelope proteins E1 and E2 reside in the ER lumen<sup>6</sup>, and the viral replicase is assumed to localize on ER-derived membranes. How and where HCV particles are assembled, however, is poorly understood. Here, we show that the LD is involved in the production of infectious virus particles. We demonstrate that Core recruits nonstructural (NS) proteins and replication complexes to LD-associated membranes, and that this recruitment is critical for producing infectious viruses. Furthermore, virus particles were observed in close proximity to LDs, indicating that some steps of virus assembly take place around LDs. This study reveals a novel function of LDs in the assembly of infectious HCV and provides a new perspective on how viruses usurp cellular functions.**

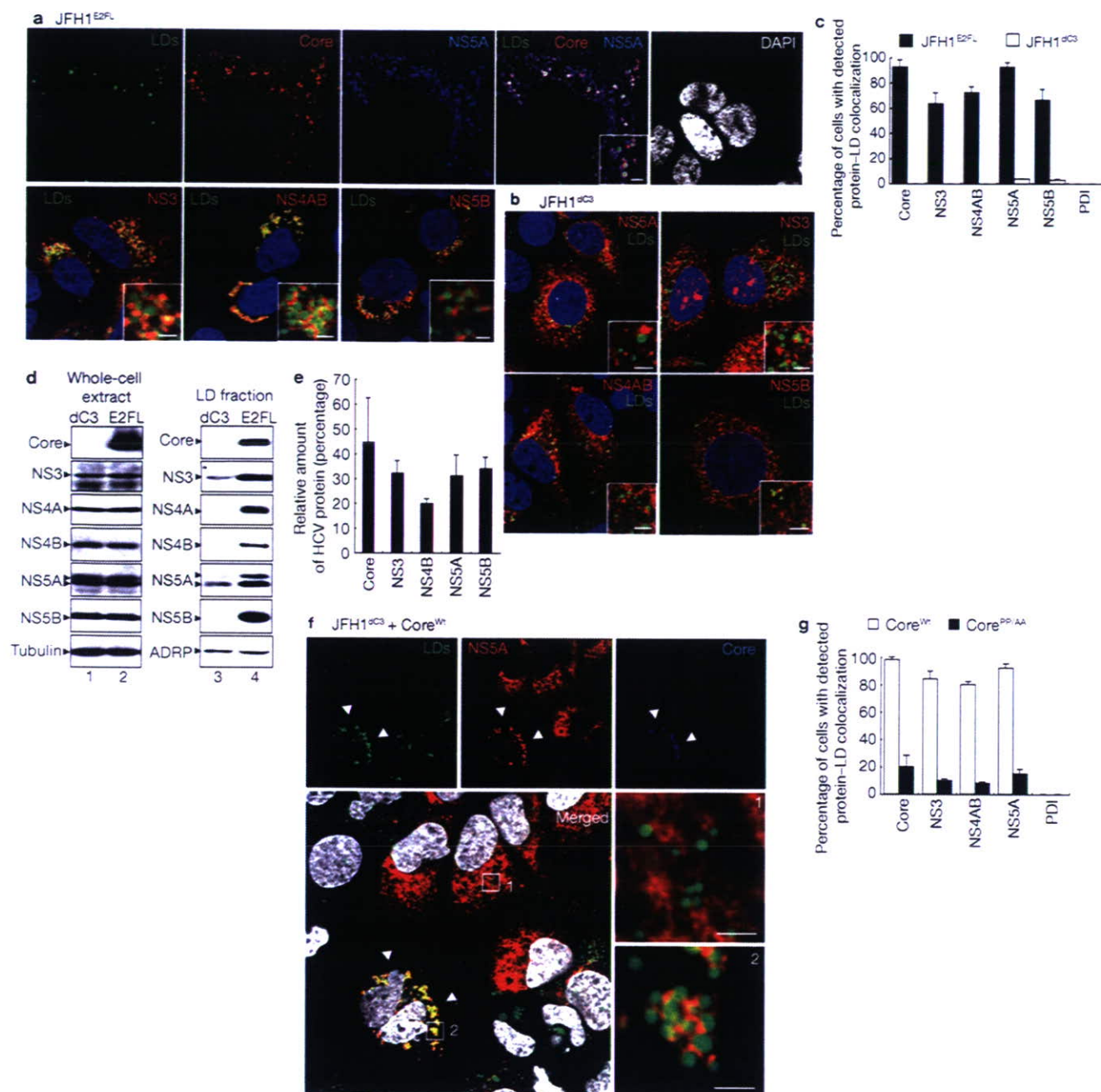
Hepatitis C virus (HCV) has a plus-strand RNA genome that encodes the viral structural proteins Core, E1 and E2, the p7, and the nonstructural (NS) proteins 2, 3, 4A, 4B, 5A and 5B (refs 7, 8). NS proteins are reported to localize on the cytoplasmic side of endoplasmic reticulum (ER) membranes<sup>9</sup>. To elucidate the mechanisms of virus production, we used a HCV strain, JFH1, which can produce infectious viruses<sup>10–12</sup>. We first investigated the subcellular localization of the HCV proteins in cells that had been transfected with JFH1<sup>E271</sup> RNA, in which a part of the hypervariable region 1 of E2 was replaced by the FLAG epitope tag (see Supplementary Information, Fig. S1, S2a–d). Core localized to the lipid droplets (LDs; Fig. 1a), as previously reported<sup>5</sup>. Interestingly, NS proteins were also detected around LDs in 60–90% of JFH1<sup>E271</sup>-replicating cells (Fig. 1a, c). Similar levels of colocalization of LDs with viral proteins were observed in cells that had been transfected with chimeric HCV genomes

expressing structural proteins, p7 and part of NS2 of the genotype 1b (Con1) or the genotype 1a (H77) isolate (see Supplementary Information, Fig. S1, S2e)<sup>13</sup>. In contrast, there was no close association between the LDs and NS proteins in cells that had been transfected with JFH1<sup>ΔC3</sup> RNA (Fig. 1b, c), which lacked the coding region of Core (Supplementary Information, Fig. S1). NS proteins were diffusely present on the ER, suggesting that NS proteins are translocated from the ER to LDs in JFH1<sup>E271</sup>-replicating cells in a Core-dependent manner. Importantly, there was no association between LDs and PDI, an ER marker protein, indicating that either ER membranes were absent in close proximity to LDs or that PDI was excluded from such membranes (Fig. 1c). These results were supported by western blot analysis of the LD fraction (Fig. 1d). The LD fraction contained ADRP, an LD marker, but not the ER markers Calnexin and Grp78 (data not shown), indicating that there was no ER contamination in the LD fraction. However, the LD fraction from JFH1<sup>E271</sup>-replicating cells contained high levels of viral proteins in contrast to the LD fraction from JFH1<sup>ΔC3</sup>-replicating cells (in which HCV proteins were virtually absent (Fig. 1d, LD fraction)), even though the expression levels of the NS proteins in whole-cell extracts were similar (Fig. 1d, whole-cell extract). About 20–45% of the total HCV proteins associated with the LDs in JFH1<sup>E271</sup>-replicating cells (Fig. 1e). Consistent with previous reports that Core enhances the formation of LDs<sup>14</sup>, overproduction of LDs was observed in JFH1<sup>E271</sup>-, but not JFH1<sup>ΔC3</sup>-replicating cells (Supplementary Information, Fig. S3a–l). Treatment of the cells with oleic acid, which enhanced the formation of LDs, did not affect either HCV protein levels or the recruitment of viral proteins to LDs in JFH1<sup>ΔC3</sup>-replicating cells (Supplementary Information, Fig. S3m–p). Thus, the overproduction of LDs is insufficient for the recruitment of HCV proteins to LDs. To examine the ability of Core to recruit NS proteins to LDs, JFH1<sup>ΔC3</sup>-replicating cells were transfected with a plasmid-expressing Core (Core<sup>Wt</sup>) (Fig. 1f, g). NSSA accumulated around LDs (Fig. 1f, arrowheads and panel 2), as did NS3 and NS4AB (Fig. 1g), in cells expressing Core<sup>Wt</sup>. The translocation of NS proteins to LDs was, however, not observed in JFH1<sup>ΔC3</sup>-replicating cells expressing Core<sup>pp-AA</sup> (Fig. 1g and Supplementary Information, Fig. S2f–h),

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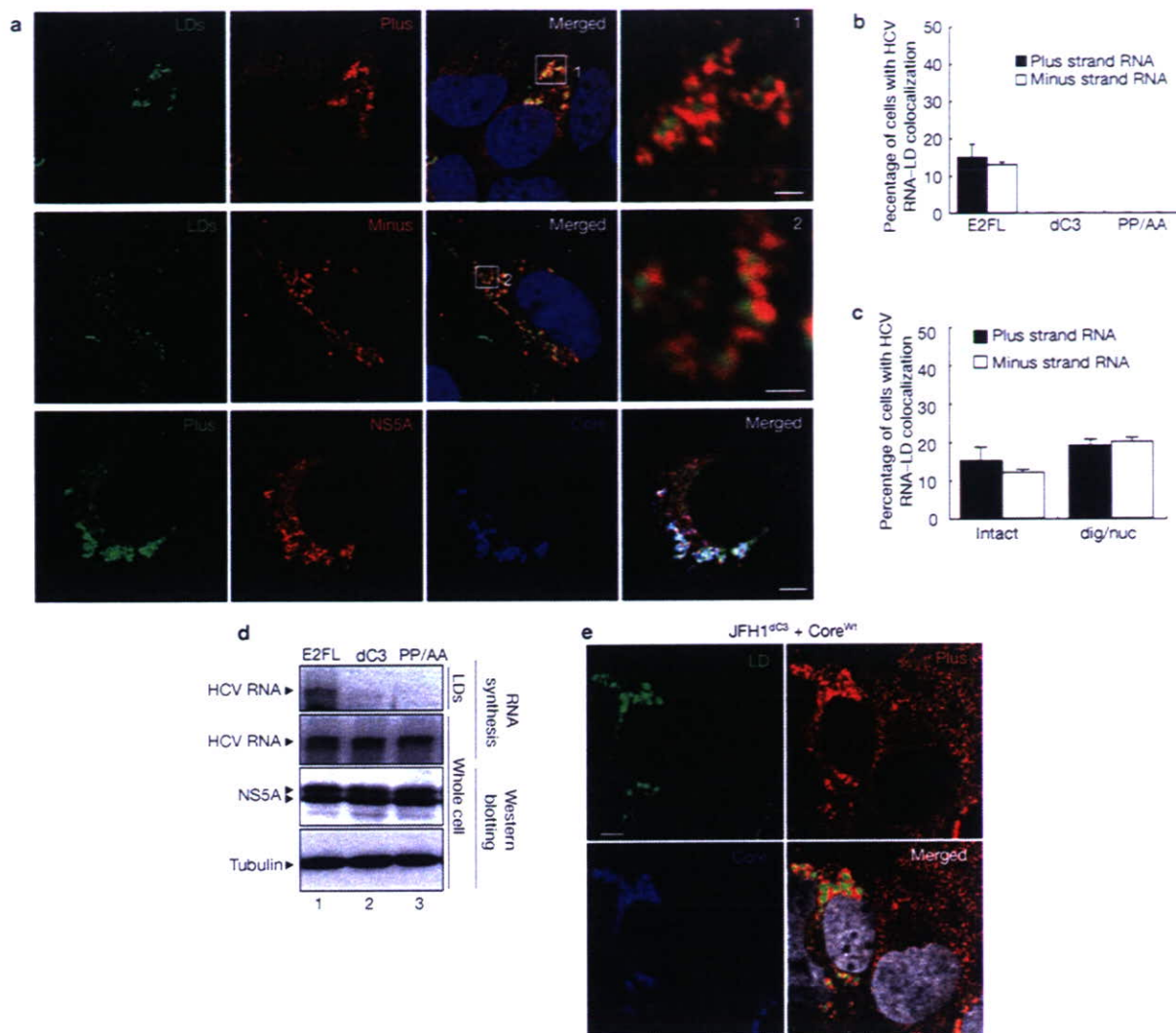


**Figure 1** Core recruits NS proteins to LDs. (a) Huh-7 cells transfected with JFH1<sup>E2FL</sup> RNA were labelled with antibodies against Core (red), NS5A (blue), NS3 (red), NS4AB (red) or NS5B (red). Lipid droplets (LDs) and nuclei were stained with BODIPY 493/503 (green) and DAPI (white in upper panel, blue in lower panels), respectively. Insets are high magnification images of areas in the respective panel. (b) JFH1<sup>ΔC3</sup> replicon-bearing cells were labelled with DAPI (blue), BODIPY 493/503 (green) and indicated antibodies (red). The insets are high magnifications of the corresponding panel. (c) Percentages of JFH1<sup>E2FL</sup>- or JFH1<sup>ΔC3</sup>-bearing cells in which hepatitis C virus (HCV) proteins or PDI colocalize with LDs ( $n > 200$ ). (d) Western blot analysis of HCV proteins and marker proteins in whole-cell extracts and the LD fractions from cells transfected with JFH1<sup>E2FL</sup> (E2FL) or JFH1<sup>ΔC3</sup> (ΔC3) RNA. (e) HCV proteins were quantified by using

western blotting data of the purified LD fraction and whole-cell extracts of JFH1<sup>E2FL</sup>-replicating cells. Results are shown as relative amounts of HCV proteins co-fractionated with LDs. This results correspond well with results obtained by quantitative immunofluorescence staining (data not shown). (f) Trans-complementation with Core<sup>Wt</sup> relocates NS proteins to LDs. JFH1<sup>ΔC3</sup> replicon-bearing cells were transfected with pcDNA3-Core<sup>Wt</sup> and labelled with BODIPY 493/503 (green), DAPI (white) and antibodies against NS5A (red) and Core (blue). Arrowheads indicate Core<sup>Wt</sup>-expressing cells. Higher-magnification images of area 1 and area 2 are shown in panels 1 and 2, respectively. Scale bars, 2  $\mu$ m. (g) The percentages of cells in which HCV proteins colocalize with LDs in the presence of Core<sup>Wt</sup> or Core<sup>PP-AA</sup> ( $n > 200$ ). Uncropped images of gels are shown in Supplementary Information Fig. S6. All error bars are derived from s.d.

a variant of Core containing two alanine substitutions at amino-acid positions 138 and 143 that fails to associate with LDs<sup>15</sup>. These results show that LD-associated Core recruits NS proteins from the ER to LDs.

Next, we investigated whether Core also recruited HCV RNA to LDs. *In situ* hybridization analysis showed that in more than 80% of JFH1<sup>E2FL</sup>-replicating cells, both plus- and minus-strand RNAs were diffusely

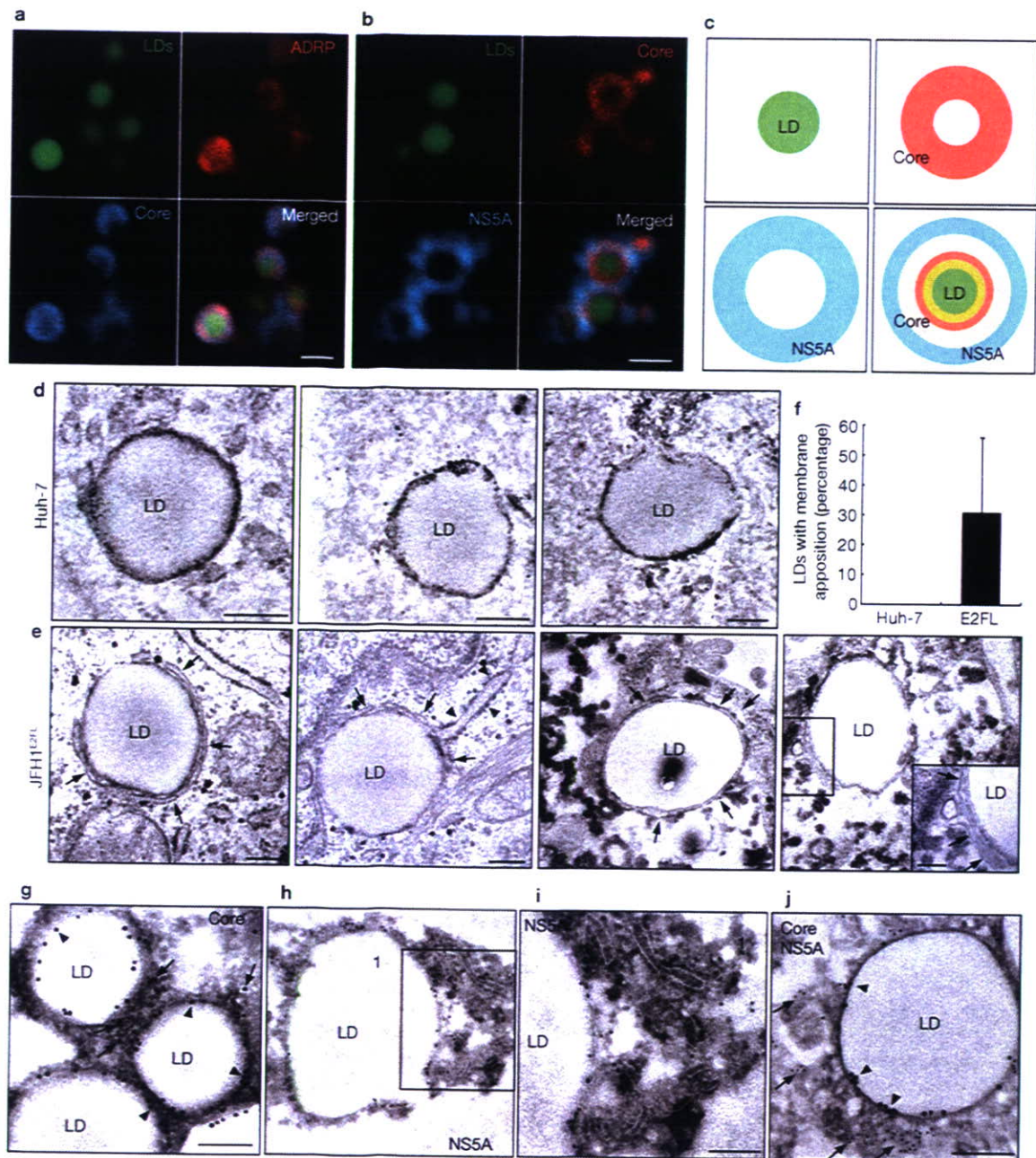


**Figure 2** Core-dependent recruitment of active HCV replication complexes to the LD. (a) Huh-7 cells transfected with JFH1<sup>E2FL</sup> RNA were analysed by *in situ* hybridization with strand-specific probes (plus or minus). The cells were labelled to simultaneously visualize lipid droplets (LDs), NS5A and Core (lower panels). Higher-magnification images of area 1 and area 2 are shown in the upper and middle right panels 1 and 2, respectively. Scale bars: 2  $\mu$ m (panels 1, 2); 10  $\mu$ m (lower right panel). (b) The percentages of JFH1<sup>E2FL</sup>-, JFH1<sup>dC3</sup>- and JFH1<sup>PP/AA</sup>-expressing cells positive for overlapping signals for LDs and plus- or minus-strand hepatitis C virus (HCV) RNA ( $n > 200$ ). (c) Intact or digitonin and nuclease-treated (dig/nuc) JFH1<sup>E2FL</sup> replicon-bearing cells were analysed

by *in situ* hybridization. The percentages of cells with overlapping signals for LD and plus- or minus-strand HCV RNA are shown ( $n > 200$ ). (d) RNA-synthesizing activity in the LD fractions purified from cells transfected with JFH1<sup>E2FL</sup>, JFH1<sup>dC3</sup> or JFH1<sup>PP/AA</sup> RNA (top panel). As a control, HCV RNA synthesis activity in digitonin-permeabilized cells was analysed (second panel from the top). HCV protein levels represented by NS5A are shown, together with the level of tubulin (bottom two panels). (e) Localization of plus-strand HCV RNA and Core in JFH1<sup>dC3</sup> replicon-bearing cells transfected with pcDNA3-Core<sup>WT</sup> (Scale bar, 10  $\mu$ m). Uncropped images of gels are shown in Supplementary Information Fig. S6. All error bars are derived from s.d.

located in the perinuclear region (see Supplementary Information, Fig. S4a). More importantly, in about 20% of these cells, plus- and minus-strand RNAs accumulated around LDs (Fig. 2a, upper and middle panels; 2b) and colocalized with HCV proteins such as Core and NS5A (Fig. 2a, lower panels). No association between HCV RNA and LDs was detected in JFH1<sup>dC3</sup>- or JFH1<sup>PP/AA</sup>-replicating cells (Fig. 2b). Northern blot analysis revealed that 4.8% and 5.4% of total plus- and minus-strand HCV RNA, respectively, were detected in purified LD fractions of JFH1<sup>E2FL</sup>-replicating cells (data not shown). Induction of LD formation with oleic acid did not affect HCV RNA accumulation around LDs (data not shown). These results provide strong evidence that Core recruits HCV RNA as well as NS proteins to LDs.

The HCV replication complex is compartmentalized by lipid bilayer membranes<sup>16–18</sup>. Therefore, HCV RNA in the complex is resistant to nuclease treatment in digitonin-permeabilized cells<sup>17</sup> (Supplementary Information, Fig. S4b–d). *In situ* hybridization analysis did not reveal a significant difference in the number of cells containing LD-associated HCV RNA before and after nuclease treatment (Fig. 2c), indicating that HCV RNA around LDs is part of the replication complex. An RNA synthesis assay showed that the purified LD fraction from JFH1<sup>E2FL</sup>-, but not JFH1<sup>dC3</sup>- or JFH1<sup>PP/AA</sup>-replicating cells, possessed HCV RNA synthesis activity, even though the expression levels of viral proteins and RNA-synthesizing activities in total cell lysates were similar (Fig. 2d). Moreover, the addition of Core<sup>WT</sup> rescued the localization of plus- and minus-strand



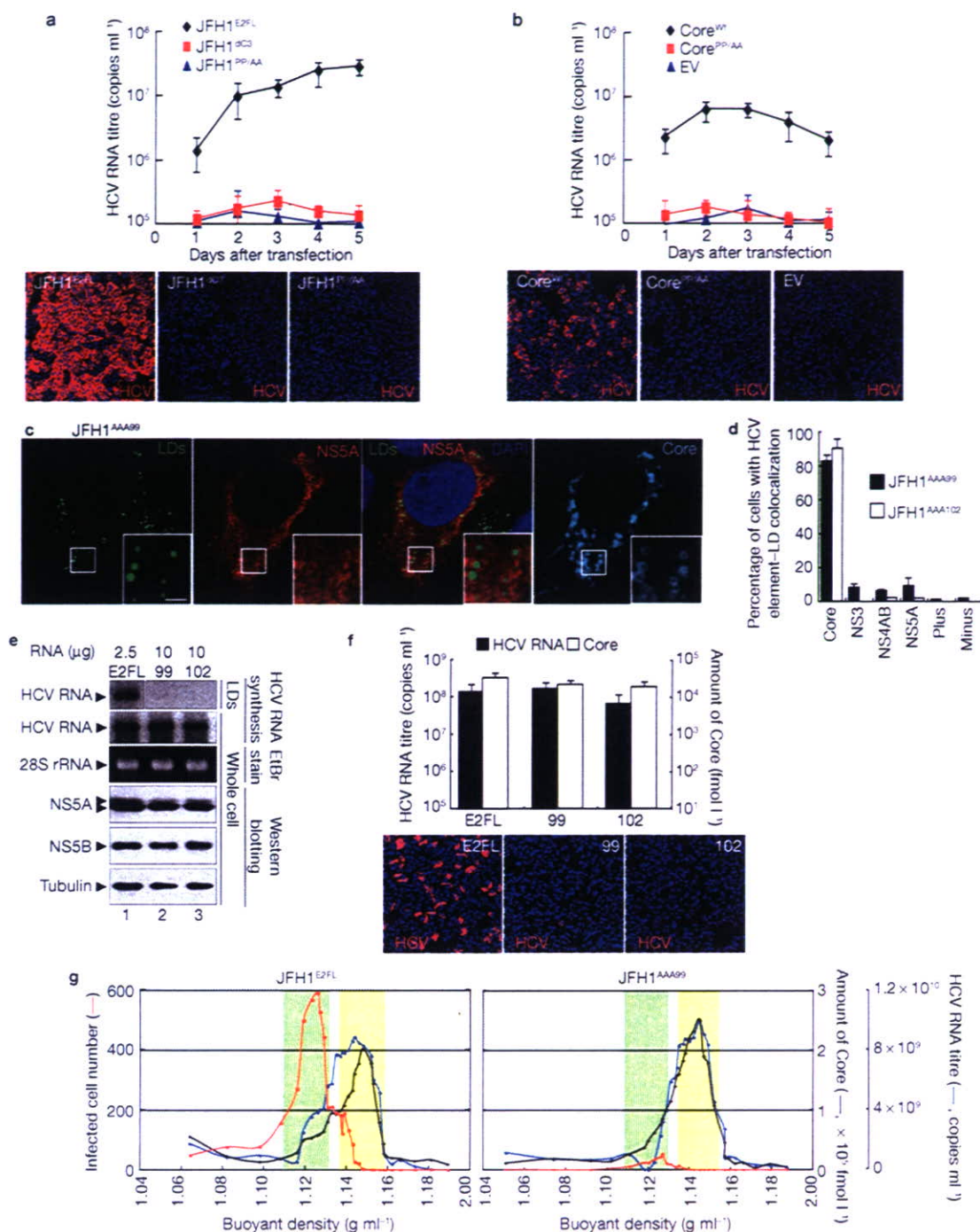
**Figure 3** Spatial distribution of Core and NS5A relative to the LD. (a, b) The localizations of Core, NS5A and ADRP around the lipid droplets (LDs) in JFH1<sup>E2FL</sup> replicon-bearing cells were analysed using immunofluorescence microscopy. Scale bars, 1  $\mu$ m. (c) Typical images of the localization of LDs, Core, NS5A and merged images are shown with the relative scale of each image. (d, e) Transmission electron micrographs of LDs in naïve Huh-7 cells and JFH1<sup>E2FL</sup>-expressing cells. Arrows and arrowheads indicate LD-associated membranes and rough ER membranes, respectively. (f) Frequency of LDs with close appositions

of membrane cisternae. About 100 Huh-7 cells or JFH1<sup>E2FL</sup>-expressing cells, respectively, were chosen randomly. LDs with apposed membrane cisternae, as exemplified in panel e, were counted as positive. The LDs judged as positive were divided by the total number of LDs. (g–j) Immunoelectron micrographs of LDs labelled with antibodies against Core (g), NS5A (h, i) or both (j) are shown. Panel i is a higher magnification of area 1 in panel h. In panel j, Core and NS5A are labelled with 15 nm and 10 nm gold particles, respectively. Scale bars, 200 nm. All error bars are derived from s.d.

HCV RNA around LDs in JFH1<sup>E2FL</sup>-replicating cells (Fig. 2e and data not shown). Both plus- and minus-strand RNA associated with LDs were nuclease resistant (data not shown). These results demonstrate that Core recruits biologically active replication complexes to LDs.

The LD is surrounded by a phospholipid monolayer<sup>48</sup>, whereas HCV replication complexes are likely to be surrounded by lipid bilayer membranes<sup>46,47</sup>. Therefore, the replication complexes might not be directly

associated with the membranes of LDs. To characterize the colocalization of LDs, viral proteins and replication complexes more precisely, we analysed the localization of NS5A with high-resolution immunofluorescence microscopy. Core was completely colocalized with ADRP, residing on the surface of LDs<sup>20</sup> (Fig. 3a), thus indicating that Core also directly associates with the surface of LDs. More importantly, NS5A mainly localized around the Core-positive area, resulting in a doughnut-shaped signal with a diameter slightly



**Figure 4** LD associations of Core and NS proteins are necessary for the production of infectious HCV particles. (a) The culture medium from JFH1<sup>E2FL</sup>, JFH1<sup>ΔC3</sup>- or JFH1<sup>ΔP</sup>-replicating cells was collected at the indicated time points and the titre of hepatitis C virus (HCV) RNA was measured by real-time RT-PCR (upper panel,  $n = 3$ ). The culture medium was added to naïve Huh7.5 cells and 24 h after inoculation, and cells were labelled with anti-HCV antibodies (lower panels, red). (b) JFH1<sup>ΔC3</sup> replicon-bearing cells were transfected with pcDNA3 (EV), pcDNA3–Core<sup>WT</sup> (Core<sup>WT</sup>) or pcDNA3–Core<sup>ΔP</sup> (Core<sup>ΔP</sup>). The level of HCV RNA and the infectivity of the culture medium were examined as described above ( $n = 3$ ). (c) Subcellular localization of NS5A and Core in cells expressing JFH1<sup>AAA99</sup>. The insets are high magnifications of the area of the corresponding panel. Scale bar, 2 μm. (d) Percentages of cells in which the signals for given HCV proteins, and plus- and minus-strand HCV RNA, overlapped with those for LDs ( $n > 200$ ). (e) Different amounts of JFH1<sup>E2FL</sup> (E2FL), JFH1<sup>AAA99</sup> (99) or JFH1<sup>AAA102</sup> (102) RNAs, respectively, were transfected into the same number of

Huh-7 cells. HCV RNA synthesis activity in purified LD fractions (LD) and whole-cell lysates (whole cell) was analysed (HCV RNA synthesis). 28S rRNA was used as a control. Western blot analysis of NS5A, NS5B and tubulin in cells is also shown. All the RNA samples in the top panel were run on the same gel. (f) Analysis of HCV released from cells expressing JFH1<sup>E2FL</sup>, JFH1<sup>AAA99</sup> or JFH1<sup>AAA102</sup>. HCV RNA titres (black bars) and amounts of Core (white bars) accumulated in the culture medium at 5 d after RNA transfection were measured (upper panel,  $n = 3$ ). Infectivity of the culture medium for naïve Huh-7.5 cells was analysed as described above (lower panels). (g) Concentrated culture medium from JFH1<sup>E2FL</sup>- and JFH1<sup>AAA99</sup>-replicating cells was fractionated using 20–50% sucrose density-gradient centrifugation at 100,000 g for 16 h. For each fraction, the amounts of Core (black line), HCV RNA (blue line) and infectivity (represented by infected cell numbers in a well; red line) are plotted against the buoyant density (x-axis) ( $n = 3$ ). Uncropped images of gels are shown in Supplementary Information Fig. S6. All error bars are derived from s.d.