

Figure 4 LD associations of Core and NS proteins are necessary for the production of infectious HCV particles. (a) The culture medium from JFH1^{E2FL}, JFH1^{ΔC3} or JFH1^{PP/AA}-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^{ΔC3} replicon-bearing cells were transfected with pcDNA3 (EV), pcDNA3-Core^{WT} (Core^{WT}) or pcDNA3-Core^{PP/AA} (Core^{PP/AA}). 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^{AAA99}. 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^{E2FL} (E2FL), JFH1^{AAA99} (99) or JFH1^{AAA102} (102) RNAs, respectively, were transfected into the same number of

Huh-7 cells. HCV RNA 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^{E2FL}, JFH1^{AAA99} or JFH1^{AAA102}. 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^{E2FL}- and JFH1^{AAA99}-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.

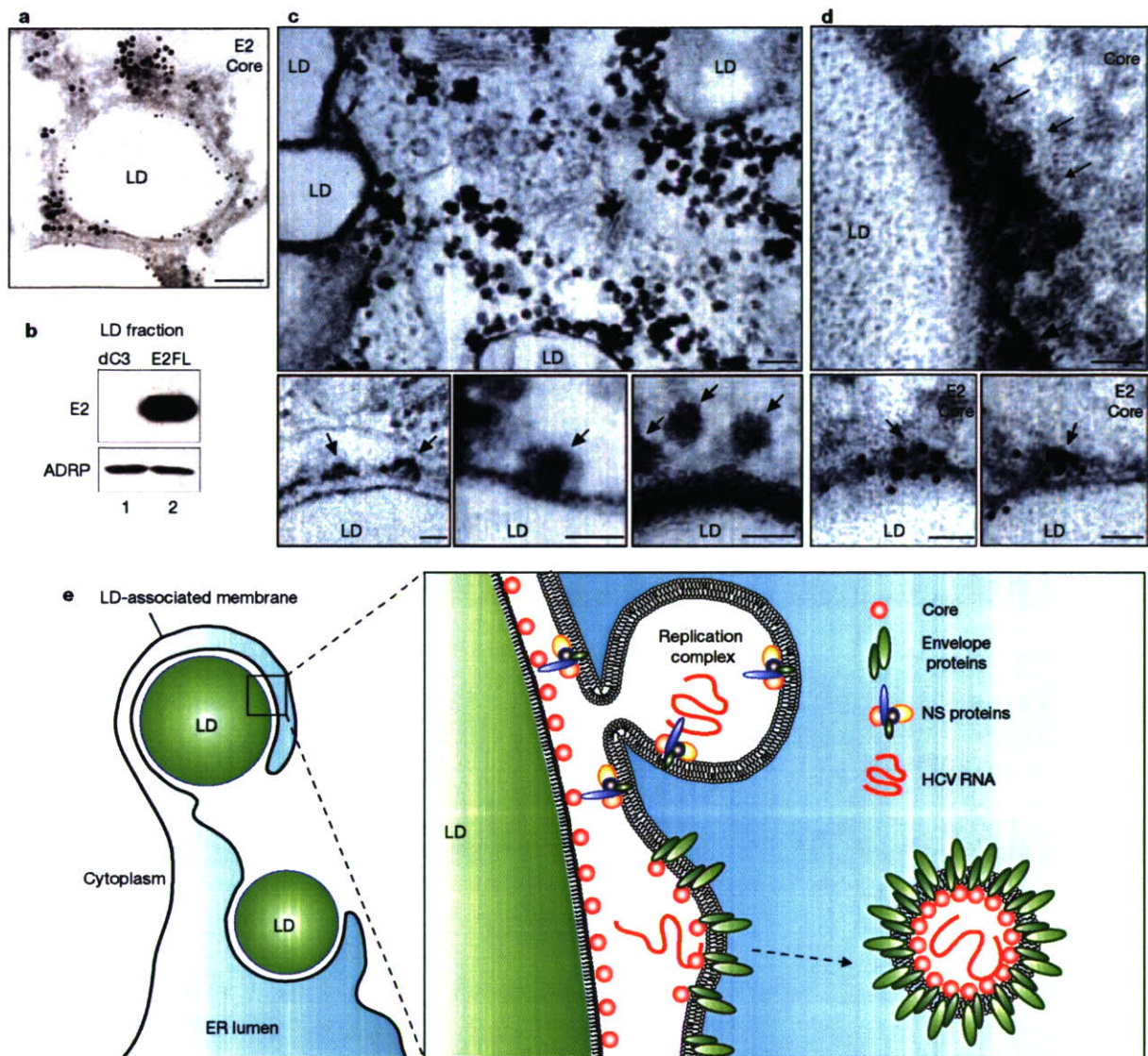


Figure 5 Virus-assembly takes place around the LDs. (a) Immunoelectron microscopic detection of E2 and Core in JFH1^{E2FL}-replicating cells. E2 and Core are labelled with 15 nm and 10 nm gold particles, respectively. (b) Western blot analysis of the lipid droplet (LD) fraction from JFH1^{E2FL} and JFH1^{dC3} replicon-bearing cells with anti-E2 and anti-ADRP antibodies. (c) Transmission electron micrographs of JFH1^{E2FL}-replicating cells. Arrows indicate virus-like particles. (d) Immunoelectron micrographs of LDs labelled with antibodies against Core (10 nm) and E2 (15 nm) are shown. Arrows show Core in electron-dense granules. Scale bar: a and upper panel of c: 100 nm;

in d and lower panels of c: 50 nm. (e) A model for the production of infectious hepatitis C virus (HCV). Core mainly localizes on the monolayer membrane that surrounds the LD. HCV induces the apposition of the LD to the endoplasmic reticulum (ER)-derived bilayer membranes (LD-associated membrane). Core recruits NS proteins, as well as replication complexes, to the LD-associated membrane. NS proteins around the LD can then participate in infectious virus production. E2 also localizes around the LD. Through these associations, virion assembly proceeds in this local environment. Uncropped images of gels are shown in Supplementary Information Fig. S6.

larger than that of Core (Fig. 3b). The LD-proximal NS5A signal partially overlapped with the Core signal (Fig. 3b, c, grey). This concentric staining pattern was also observed with the other NS proteins (Supplementary Information, Fig. S5a), indicating that NS proteins associate with Core on the surface of LDs. Electron microscopic analysis only rarely revealed a close association of LDs with other organelles in naive Huh-7 cells (Fig. 3d, f). However, in the case of JFH1^{E2FL}-replicating cells, about 30% of the LDs were in close proximity to membrane cisternae (Fig. 3e, arrows; 3f), arguing for a HCV-induced membrane rearrangement around LDs. Core was mainly located on the periphery of LDs, and occasionally signals were

observed in more distal areas of the LDs (Fig. 3g, arrowheads and arrows, respectively). Although some NS5A signals were observed on the surface of the LD, the majority of NS5A signals were detected more distal of LDs (Fig. 3h, i). Furthermore, we often observed membrane cisternae as white lines in the same area as NS5A signals (Fig. 3i, arrows). When the same section was labelled with anti-Core and anti-NS5A antibodies, Core was detected on the surface of the LDs, whereas NS5A was mainly observed in the peripheral area of the LDs (Fig. 3j, arrowheads and arrows, respectively). In summary, these results show that Core recruits NS proteins, as well as HCV replication complexes, to the LD-associated membranes.

The above results prompted us to ask whether Core-LD colocalization is important for the production of infectious virus particles. JFH1^{E2FL}-replicating cells released virions into the culture medium and these viruses were highly infectious for naïve Huh-7.5 cells^{11,21}, although culture medium from JFH1^{PP/AA}- or JFH1^{dC3}-replicating cells did not contain significant levels of HCV RNA and infectious virus (Fig. 4a). However, following trans-complementation with Core^{wt}, a high titre of HCV RNA and infectious virus could be rescued from JFH1^{dC3}-replicating cells (Fig. 4b; and see Supplementary Information, Fig. S5b, c). In contrast, the production of infectious viruses was not rescued by trans-complementation with Core^{PP/AA} (Fig. 4b). RNA-binding properties and oligomerization of Core^{wt} and Core^{PP/AA}, which are both necessary for virus assembly, were similar (Supplementary Information, Fig. S5d; ref. 22), arguing that the primary defect of this mutant in preventing infectious virus production is the inability to associate with LDs.

To investigate the contribution of NS proteins around LDs to infectious virus production, we used variants of NS5A, which were not recruited to LDs even in the presence of Core. We assumed that NS5A was crucial for recruiting other NS proteins to LDs, because the level of NS5A recruited to LDs via Core was higher than the levels of the other recruited NS proteins (Fig. 1c, JFH1^{E2FL}). Using alanine-scanning mutagenesis within the NS5A coding region of JFH1^{E2FL}, we generated two mutants, JFH1^{AAA99} and JFH1^{AAA102}, in which the amino-acid sequence APK (aa 99–101 of NS5A) or PPT (aa 102–104 of NS5A) was replaced by AAA (Supplementary Information, Fig. S1). In JFH1^{AAA99}- and JFH1^{AAA102}-replicating cells, NS5A was rarely detected around LDs, whereas Core was still localized to LDs (Fig. 4c, d). Importantly, these mutations impaired not only the NS5A association with LDs, but also the recruitment of other NS proteins and viral RNAs to LDs (Fig. 4d). These results indicate that NS5A is a key protein that recruits replication complexes to LDs. Importantly, HCV RNA synthesis activity in the LD fractions from these mutant JFH1-replicating cells was also severely impaired (Fig. 4e), corroborating the lack of association of HCV replication complexes with LDs.

To investigate the infectious virus production of these NS5A mutants, we prepared cells expressing similar levels of HCV proteins and RNA by adjusting the amount of transfected HCV RNA (Fig. 4e). This was necessary, because replication activities of these mutants were lower compared with JFH1^{E2FL}. Under these conditions, the amounts of Core and HCV RNA that were released into the culture medium from cells transfected with the mutants were comparable to JFH1^{E2FL} (Fig. 4f, upper graph). However, infectivity titres of the mutants were severely reduced (Fig. 4f, lower panels). In sucrose density-gradient centrifugation of culture medium from JFH1^{E2FL}-bearing cells, two types of HCV particles were detected: low-density particles (about 1.12 g ml⁻¹) with high infectivity (Fig. 4g, green area of JFH1^{E2FL}), and high-density particles (about 1.15 g ml⁻¹) without infectivity (yellow area). This result indicates that only a minor portion of released HCV particles is infectious, whereas the majority of released particles lack infectivity. In contrast, cells bearing the JFH1^{AAA99} mutant almost exclusively released non-infectious particles of around 1.15 g ml⁻¹, whereas infectious particles were barely detectable (Fig. 4g, JFH1^{AAA99}). Taken together, these results provide convincing evidence that the association of NS proteins and replication complexes around LDs is critical for producing infectious viruses, whereas production of non-infectious viruses seems to follow a different pathway.

The results described so far imply that some step(s) of HCV assembly take place around LDs. To explore this possibility, we analysed the distribution of the major envelope protein E2 around the LD. Electron microscopic analysis revealed that, in about 90% of JFH1^{E2FL}-replicating cells, E2 was localized in the peripheral area of the LDs (Fig. 5a, large grains). This labelling pattern was similar to the one observed for NS5A (Fig. 3j), indicating that E2 also localizes on the LD-associated membranes. Western blot analysis of the LD fraction supported this conclusion, because the LD fraction that was purified from JFH1^{E2FL}-replicating cells, but not from JFH1^{dC3}-replicating cells, contained E2 (Fig. 5b). Furthermore, spherical virus-like particles with an average diameter of about 50 nm were observed around LDs in JFH1^{E2FL}-replicating cells (Fig. 5c, upper panel). These particles were never observed in naïve Huh-7 cells. A more refined analysis indicates that these particles are closely associated with membranes in close proximity to LDs (Fig. 5c, lower panels, arrows). Finally, these particles around the LDs reacted with Core- and E2-specific antibodies, arguing that the particles represent true HCV virions (Fig. 5d). These results suggest that infectious HCV particles are generated from the LD-associated membranous environment.

In this study, we have demonstrated that Core recruits NS proteins, HCV RNAs and the replication complex to LD-associated membranes. Mutations of Core and NS5A (Fig. 4), which failed to associate with LDs, impaired the production of infectious virus. We note that the mutant Core retains the ability to interact with RNA (Supplementary Information, Fig. S5b) and to assemble into nucleocapsid²². Similarly, the NS5A mutant still supports viral genome replication and the formation of capsids or virus-like particles, arguing that the introduced mutations in Core and NS5A do not affect overall protein folding, stability or function (Fig. 4). Taken together, the data show that the association of HCV proteins with LDs is important for the production of infectious viral particles (Fig. 5e).

Our results also indicate that NS proteins around the LDs participate in the assembly of infectious virus particles. In one scenario, NS proteins may indirectly contribute to the different steps of virus production — for example, by establishing the microenvironment around the LDs that is required for infectious virus production. Alternatively, NS proteins around the LDs may directly participate in virus production — for example, as components of the replication complex that provide the RNA genome to the assembling nucleocapsid.

In support of the role of LDs in virus formation, we observed that colocalization of HCV protein with LDs was low in cases of the chimera Jc1, supporting up to 1,000-fold higher infectivity titres compared with JFH1 (ref. 13). In a Jc1-infected cell, only about 20% of LDs demonstrated detectable colocalization with Core, but this value increased to 80% in the case of a Jc1 mutant lacking most of the envelope glycoprotein genes and thus being unable to produce infectious virus particles (data not shown). This inverse correlation between the efficiency of virus production and Core protein accumulation on LDs indicates that rapid assembly and virus release results in the rapid liberation of HCV proteins from the LDs.

Steatosis and abnormal lipid metabolism caused by chronic HCV infection may be linked to enhanced LD formation¹⁴. In fact, the overproduction of LDs is induced by Core (Supplementary Information, Fig. S3) and HCV also induces membrane rearrangements around LDs (Fig. 3d–f). Our findings suggest that excessive Core-dependent formation of LDs

LETTERS

and membrane rearrangements are required to supply the necessary microenvironment for virus production. NS proteins and HCV RNA seem to be translocated from the ER to the LD-associated membranes. Interestingly, the LD-associated membranes were occasionally found in continuity with ribosome-studded rough ER (Fig. 3e, arrowheads). Thus, at least parts of the LD-associated membranes are likely to be derived from ER membranes. ER marker proteins, however, were not detected in the LD fraction, suggesting that the LD-associated membrane is characteristically distinct from that of ER membranes.

To our knowledge, this is the first report showing that LDs are required for the formation of infectious virus particles. The fact that capsid protein of the hepatitis G virus also localizes to LDs¹⁵ indicates that LDs might be important for the production of other viruses as well. Our findings demonstrate a novel function of LDs, provide an important step towards elucidating the mechanism of HCV virion production and open new avenues for novel antiviral intervention. □

METHODS

Antibodies. The antibodies used for immunoblotting and immunolabelling were specific for Core (#32-1 and RR8); E2 (AP-33 (ref. 23); 3/11, CBH5 and Flag M2 (Sigma-Aldrich, St Louis, MO); NS3 (R212)¹⁷; NS4A and 4B (PR12); NS5A (NS5ACL1); NS5B (NS5B-6 and JFH1-1)²⁴; ADRP (Progen Biotechnik, Heidelberg, Germany); tubulin (Oncogene Research Products, MA, USA); Grp78 (StressGen, Victoria, Canada); PDI (StressGen); and Calnexin-NT (StressGen). Antibodies specific for Core (#32-1 and RR8), NS3 (R212) and NS4AB (PR12) were gifts from Dr Kohara (The Tokyo Metropolitan Institute of Medical Science, Japan). Anti-E2 antibody (AP-33) was provided by Dr Patel (MRC Virology Unit, UK). Anti-NS5B (NS5B-6) antibody was kindly provided by Dr Fukuya (Osaka University, Japan). Rabbit polyclonal antibodies specific for NS5A were raised against a bacterially expressed GST-NS5A (1–406 aa) fusion protein. In the case of the HCV chimeras Con1/C3 and H77/C3, immunofluorescence analyses were performed by using the following antibodies: Core (C7/50)⁵, a JFH1 NS3-specific rabbit polyclonal antiserum; NS4B (#86)²⁵; and NS5A (Austral Biologicals, San Ramon, CA).

Indirect immunofluorescence analysis. Indirect immunofluorescence analysis was performed essentially as described previously¹⁷, with slight modifications. Cells transfected with JFH1 RNA were seeded onto a collagen-coated Labtech II 8-well chamber (Nunc, NY, USA). The coating with collagen was performed using rat-tail collagen type I (BD Bioscience, Palo Alto, CA) according to manufacturer's instructions. Three days after seeding, the cells were washed twice with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ and 1.4 mM KH₂PO₄) and fixed with fixation solution (4% paraformaldehyde and 0.15 M sodium cacodylate at pH 7.4) for 15 min at room temperature. After washing with PBS, the cells were permeabilized with 0.05% Triton X-100 in PBS for 15 min at room temperature. For the precise localization of the proteins, the cells were permeabilized with 50 µg ml⁻¹ of digitonin in PBS for 5 min at room temperature²⁶. After incubating the cells with blocking solution (10% fetal bovine serum and 5% bovine serum albumin (BSA) in PBS) for 30 min, the cells were incubated with the primary antibodies. The fluorescent secondary antibodies were Alexa 568- or Alexa 647-conjugated anti-mouse or anti-rabbit IgG antibodies (Invitrogen, Carlsbad, CA). Nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI). LDs were visualized with BODIPY 493/503 (Invitrogen). Analyses of JFH1 were performed on a Leica SP2 confocal microscope (Leica, Heidelberg, Germany). Analysis of the Con1/C3 and the H77/C3 chimeras was performed in the same way, except that imaging was performed on a Nikon C1 confocal microscope (Nikon, Tokyo, Japan).

Electron microscopy. For conventional electron microscopy, cells cultured in plastic Petri dishes were processed *in situ*. The cells were fixed in 2.5% glutaraldehyde and 0.1 M sodium phosphate (pH 7.4), and then in OsO₄ and 0.1 M sodium phosphate (pH 7.4). The cells were then dehydrated in a graded ethanol series and embedded in an epoxy resin. Ultrathin sections were cut perpendicular to the base of the dish. For immuno-electron microscopy, cells were detached

from the dish with a cell scraper after fixation in 4% paraformaldehyde, 0.1% glutaraldehyde and 0.1 M sodium phosphate (pH 7.4) for 24 h, and washed in 0.1 M lysine, 0.1 M sodium phosphate (pH 7.4) and 0.15 M sodium chloride. After dehydrating the cells in a graded series of cold ethanol, they were embedded in Lowicryl K4M at -20 °C. Ultrathin sections were labelled with primary antibodies and colloidal gold particles (15 nm) conjugated to anti-mouse IgG or anti-rabbit IgG antibodies. For double labelling, colloidal gold particles with different diameters (10 nm and 15 nm) conjugated to anti-mouse IgG or anti-rabbit antibodies were used. Samples were observed after staining with uranyl acetate and lead citrate with a JEM 1010 electron microscope at the accelerating voltage of 80 kV. Anti-Core (#32-1 and RR8), anti-NS5A (NS5ACL1) and anti-E2 (Flag M2) antibodies were used.

Preparation of the lipid droplets. Cells at a confluency of ~80% on a dish with a diameter of 14 cm were scraped in PBS. The cells were pelleted by centrifugation at 1,500 rpm. The pellet was resuspended in 500 µl of hypotonic buffer (50 mM HEPES, 1 mM EDTA and 2 mM MgCl₂ at pH 7.4) supplemented with protease inhibitors (Roche Diagnostics, Basel, Switzerland) and was incubated for 10 min at 4 °C. The suspension was homogenized with 30 strokes of a glass Dounce homogenizer using a tight-fitting pestle. Then, 50 µl of 10× sucrose buffer (0.2 M HEPES, 1.2 M KoAc, 40 mM Mg(oAc)₂ and 50 mM DTT at pH 7.4) was added to the homogenate. The nuclei were removed by centrifugation at 2,000 rpm for 10 min at 4 °C. The supernatant was collected and centrifuged at 16,000 g for 10 min at 4 °C. The supernatant (S16) was mixed with an equal volume of 1.04 M sucrose in isotonic buffer (50 mM HEPES, 100 mM KCl, 2 mM MgCl₂ and protease inhibitors). The solution was set at the bottom of 2.2-ml ultracentrifuge tube (Hitachi Koki, Tokyo, Japan). One milliliter of isotonic buffer was loaded onto the sucrose mixture. The tube was centrifuged at 100,000 g in an S55S rotor (Hitachi Koki) for 30 min at 4 °C. After the centrifugation, the LD fraction on the top of the gradient solution was recovered in isotonic buffer. The suspension was mixed with 1.04 M sucrose and centrifuged again at 100,000 g, as described above, to eliminate possible contamination with other organelles. The collected LD fraction was used for western blotting or the HCV RNA synthesis assay.

HCV RNA synthesis assay. An assay of HCV RNA synthesis using digitonin-permeabilized cells was performed as described previously¹⁷. For RNA synthesis assays using the LD fraction, the LD fraction collected by sucrose-gradient sedimentation was suspended in buffer B, which contained 2 mM manganese (II) chloride, 1 mg ml⁻¹ acetylated BSA (Nacalai Tesque, Kyoto, Japan), 5 mM phosphocreatine (Sigma), 20 units/ml creatine phosphokinase (Sigma), 50 µg ml⁻¹ actinomycin D, 500 µM ATP, 500 µM CTP, 500 µM GTP (Roche Diagnostics) and 1.85 MBq of [α -³²P] UTP (GE Healthcare, Little Chalfont, UK), and incubated at 27 °C for 4 h. The reaction products were analysed by gel electrophoresis followed by autoradiography.

Note: Supplementary Information is available on the Nature Cell Biology website.

ACKNOWLEDGEMENTS

We thank T. Fujimoto and Y. Ohsaki at Nagoya University for helpful discussions and technical assistance. Y.M. is a recipient of a JSPS fellowship. K.S. is supported by Grants-in-Aid for cancer research and for the second-term comprehensive 10-year strategy for cancer control from the Ministry of Health, Labour and Welfare, as well as by a Grant-in-Aid for Scientific Research on Priority Areas "Integrative Research Toward the Conquest of Cancer" from the Ministry of Education, Culture, Sports, Science and Technology of Japan. T.W. is also supported, in part, by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science; and by the Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation. R.B. is supported by the Sonderforschungsbereich 638 (Teilprojekt A5) and the Deutsche Forschungsgemeinschaft (BA1505/2-1). M.Z. and R.B. thank the Nikon Imaging Center at the University of Heidelberg for providing access to their confocal fluorescence microscopes and Ulrike Engel for the excellent support.

AUTHOR CONTRIBUTIONS

Y.M. and K.S. planned experiments and analyses. Y.M. was responsible for experiments for Figs 1, 2, 3a–c, 4a–e and 5b. K.A., N.U., electron microscopy; T.H., Fig. 1e; M.Z., R.B., Fig. S2e; and K.S. and K.W., Fig. 4f–g. T.W. provided JFH1 strain. Y.M. and K.S. wrote the manuscript. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at <http://www.nature.com/naturecellbiology/>
Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>

- Martin, S. & Parton, R. G. Lipid droplets: a unified view of a dynamic organelle. *Nature Rev. Mol. Cell Biol.* **7**, 373–378 (2006).
- Blanchette-Mackie, E. J. *et al.* Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes. *J. Lipid Res.* **36**, 1211–1226 (1995).
- Vock, R. *et al.* Design of the oxygen and substrate pathways. VI. structural basis of intracellular substrate supply to mitochondria in muscle cells. *J. Exp. Biol.* **199**, 1689–1697 (1996).
- Liang, T. J. *et al.* Viral pathogenesis of hepatocellular carcinoma in the United States. *Hepatology* **18**, 1326–1333 (1993).
- Moradpour, D., Englert, C., Wakita, T. & Wands, J. R. Characterization of cell lines allowing tightly regulated expression of hepatitis C virus core protein. *Virology* **222**, 51–63 (1996).
- Deleersnyder, V. *et al.* Formation of native hepatitis C virus glycoprotein complexes. *J. Virol.* **71**, 697–704 (1997).
- Kato, N. *et al.* Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc. Natl Acad. Sci. USA* **87**, 9524–9528 (1990).
- Hijikata, M. & Shimotohno, K. [Mechanisms of hepatitis C viral polyprotein processing]. *Virusu* **43**, 293–298 (1993).
- Dubuisson, J., Penin, F. & Moradpour, D. Interaction of hepatitis C virus proteins with host cell membranes and lipids. *Trends Cell Biol.* **12**, 517–523 (2002).
- Wakita, T. *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nature Med.* **11**, 791–796 (2005).
- Lindenbach, B. D. *et al.* Complete replication of hepatitis C virus in cell culture. *Science* **309**, 623–626 (2005).
- Zhong, J. *et al.* Robust hepatitis C virus infection in vitro. *Proc. Natl Acad. Sci. USA* **102**, 9294–9299 (2005).
- Pietschmann, T. *et al.* Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc. Natl Acad. Sci. USA* **103**, 7408–7413 (2006).
- Moriya, K. *et al.* Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J. Gen. Virol.* **78**, 1527–1531 (1997).
- Hope, R. G., Murphy, D. J. & McLauchlan, J. The domains required to direct core proteins of hepatitis C virus and GB virus-B to lipid droplets share common features with plant oleosin proteins. *J. Biol. Chem.* **277**, 4261–4270 (2002).
- Egger, D. *et al.* Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.* **76**, 5974–5984 (2002).
- Miyazari, Y. *et al.* Hepatitis C virus non-structural proteins in the probable membranous compartment function in viral genome replication. *J. Biol. Chem.* **278**, 50301–50308 (2003).
- Quinkert, D., Bartenschlager, R. & Lohmann, V. Quantitative analysis of the hepatitis C virus replication complex. *J. Virol.* **79**, 13594–13605 (2005).
- Tauchi-Sato, K., Ozeki, S., Houjou, T., Taguchi, R. & Fujimoto, T. The surface of lipid droplets is a phospholipid monolayer with a unique fatty acid composition. *J. Biol. Chem.* **277**, 44507–44512 (2002).
- Londos, C., Brasaemle, D. L., Schultz, C. J., Segrest, J. P. & Kimmel, A. R. Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. *Semin. Cell Dev. Biol.* **10**, 51–58 (1999).
- Blight, K. J., McKeating, J. A. & Rice, C. M. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J. Virol.* **76**, 13001–13014 (2002).
- Klein, K. C., Dellos, S. R. & Lingappa, J. R. Identification of residues in the hepatitis C virus core protein that are critical for capsid assembly in a cell-free system. *J. Virol.* **79**, 6814–6826 (2005).
- Owsianka, A. *et al.* Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein. *J. Virol.* **79**, 11095–11104 (2005).
- Ishii, N. *et al.* Diverse effects of cyclosporine on hepatitis C virus strain replication. *J. Virol.* **80**, 4510–4520 (2006).
- Lohmann, V., Korner, F., Herian, U. & Bartenschlager, R. Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. *J. Virol.* **71**, 8416–8428 (1997).
- Ohsaki, Y., Maeda, T. & Fujimoto, T. Fixation and permeabilization protocol is critical for the immunolabeling of lipid droplet proteins. *Histochem. Cell Biol.* **124**, 445–452 (2005).

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

Hussein H. Aly^{1,2,3}, Koichi Watashi², Makoto Hijikata², Hiroyasu Kaneko², Yasutugu Takada¹, Hiroto Egawa¹, Shinji Uemoto¹, Kunitada Shimotohno^{2,*}

¹Graduate School of Medicine, Department of Transplant Surgery, Kyoto University Hospital, Kyoto, Japan

²Laboratory of Human Tumor Viruses, Institute of Virus Research, Kyoto University, Japan

³Hepatology Department, National Hepatology and Tropical Medicine Research Institute, Cairo, Egypt

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.

© 2006 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

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- α and ribavirin, which results in a sustained response in only 30–60% of patients [2–5]. Many patients, however, do not qualify for or tolerate standard therapy [6]. Thus, it is important to develop an efficient *in vitro* infection system for HCV to facilitate the discovery of new anti-HCV strategies. Only Huh7 cell line is permissive for replication, infection and release of the fulminant hepatitis-derived HCV-2a (JFH-1) strain and its chimeric derivatives [7–9]. No other hepatocyte cell lines are able to support HCV replication efficiently.

Received 5 June 2006; received in revised form 24 July 2006; accepted 1 August 2006; available online 30 October 2006

* Corresponding author. Tel.: +81 75 751 4000; fax: +81 75 751 3998.

E-mail address: kshimoto@virus.kyoto-u.ac.jp (K. Shimotohno).

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 pIFN β promoter-luc and pIFN α promoter-luc plasmids were gifts from Dr. Taniguchi of the Tokyo University. The psiRNA-hIRF-3 and psiRNA-hIRF-7 plasmids were purchased from InvivoGen (USA).

2.3. Immunoblot analysis

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

2.4. Transfection, small interfering RNA silencing and luciferase assays

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

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

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

2.6. HCV infection experiment

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

2.7. Blocking of HCV infectivity by anti-CD81

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

3. Results

3.1. Establishment of immortalized primary human hepatocytes

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

Table 1
Primer sequences and RT-PCR parameters

Genes	Primer sequence 5'–3'	PCR parameters ^a
HGF	F: AGGAGCCAGCCTGAATGATGA R: CCCTCTGATGTCCCAAGATTAGC	95, 56, 72 1 min, 45 s, 1 min
TGF α	F: ATGGTCCCCTCGGCTGGA R: GGCCTGCTTCTTCTGGCTGGCA	95, 59, 72 45 s, 30 s, 1 min
TGF β 1	F: GCCCTGGACACCAACTATTGCT R: AGGCTCCAAATGTAGGGGCAGG	95, 58, 72 45 s, 30 s, 1 min
TGF β 2	F: GATTTCCATCTACAAGACCACGAGGGACTTGC R: CAGCATCAGTTACATCGAAGGAGAGCCATTCG	95, 58, 72 45 s, 30 s, 1 min
HGFR	F: TGGTCCTTGGCGTCGCTCTC 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 β 1R	F: CGTGCTGACATCTATGCAAT R: AGCTGCTCCATTGGCATAAC	95 s, 54, 72 30 s, 45 s, 1 min
TGF β 2R	F: TGCACATCGTCTGTGGAC R: GTCTCAAACGCTCTGAAGTGTTTC	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 α	F: GTGTCTACAACCTGGTTTGCC R: TGTAGACACTGTCACTAAGG	95, 52, 72 45 s, 30 s, 1 min
HNF1 β	F: GAAACAATGAGATCACTTCTCTCC R: CTTTGTGCAATTGCCATGACTCC	95, 52, 72 1 m, 45 s, 1 min
HNF3 β	F: CACCCTACGCCTTAACCAC R: GGTAGTAGGAGGTATCTGCGG	95, 56, 72 1 m, 45 s, 1 min
HNF4	F: CTGCTCGGAGCCACAAAGAGATCCATG R: ATCATCTGCCACGTGATGCTCTGCA	95, 58, 72 45 s, 30 s, 1 min
Albumin	F: AGTTTGCAGAAGTTTCCAAGTTAGTG R: AGGTCCGCCCTGTCATCAG	95, 55, 72 45 s, 30 s, 1 min
Apolipoprotein-a	F: AGGCTCGGCATTTCTGGCAG R: TATCCCAGAACTCTGGGGT	95, 55, 72 45 s, 30 s, 1 min
HTF	F: TCGCTACAGCCTTTGCAATG R: TTGAGGGTACGGAGGAGTTCC	95, 55, 72 45 s, 30 s, 1 min
E-cadherin	F: TCCATTTCTTGGTCTACGCC R: TTTGTCCTACCGACTTCCAC	95, 55, 72 45 s, 30 s, 1 min
CYP 1B1	F: CACCAAGGCTGAGACAGTGA R: GCCAGGTAAACTCCAAGCAC	94, 57, 72 30 s, 30 s, 1 min
CYP 2C9	F: GGACAGAGACGACAAGCACA R: TGGTGGGGAGAAGGTCAAT	94, 57, 72 30 s, 30 s, 1 min
CYP 2B	F: GGCACACAGCCAAGTTTACA R: CCAGCAAAGAAGAGCGAGAG	94, 57, 72 30 s, 30 s, 1 min
CYP 3A4	F: TGTGCCTGAGAACACCAGAG R: GCAGAGGAGCCAAATCTACC	94, 57, 72 30 s, 30 s, 1 min
CYP 2E1	F: CCGCAAGCATTTTGACTACA R: 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 ^a
CD81	F: CTCAACTGTTGTGGCTCCAAC R: CCAATGAGGTACAGCTTCCC	95, 55, 72 45 s, 30 s, 1 min
TLR3	F: GATCTGTCTCATAATGGCTTG R: GACAGATTCCGAATGCTTGTG	95, 55, 72 45 s, 30 s, 1 min
TLR7	F: CCAGACATCTCCCCAGCGTC R: GGCAAAAACAGTAGGGACGGC	95, 55, 72 45 s, 30 s, 1 min
TLR8	F: CTGTGAGTTATGCGCCGAAG R: CGGGATTTCCGTTCTGGTGC	95, 55, 72 45 s, 30 s, 1 min
Myd88	F: GGTCTCCTCCACATCCTCCC R: CCAGCTTGGTAAGCAGCTCG	95, 55, 72 45 s, 30 s, 1 min
IRF3	F: GAACCCCAAAGCCACGGATC R: CCTCCCGGGAACATATGCAC	95, 55, 72 45 s, 30 s, 1 min
IRF7	F: 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.

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

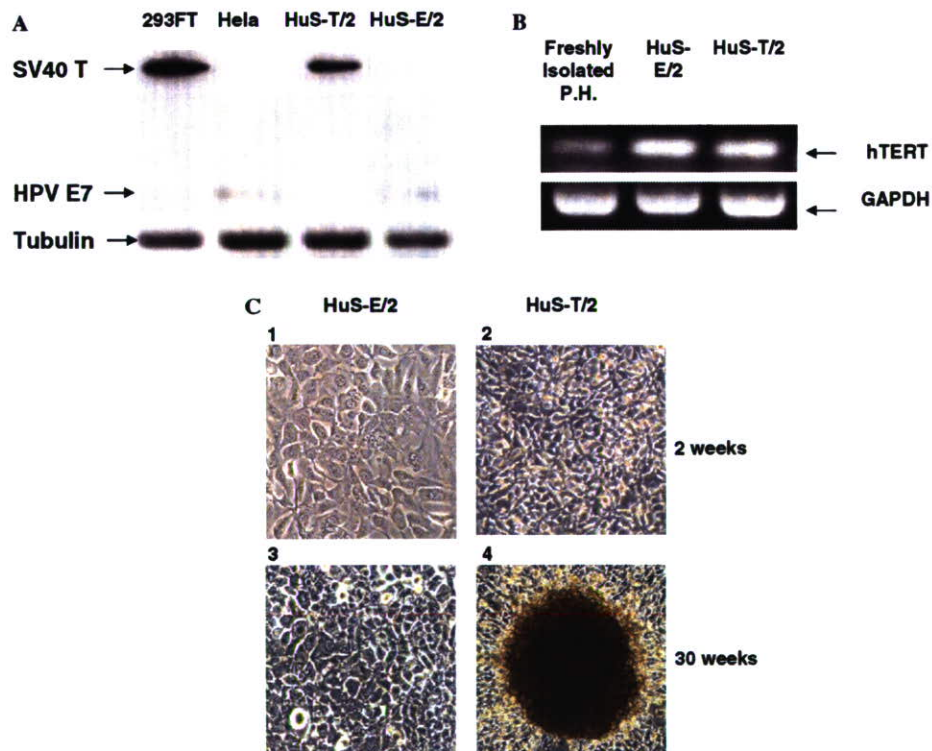


Fig. 1. (A) Immunoblot detection of SV40 T and HPV E7 expression in HuS-T/2 and HuS-E/2 cells, respectively. 293-FT and HeLa cells were used as positive controls for SV40 T and HPV E7 expression, respectively. The specific bands representing the targets are indicated. Detection of tubulin expression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

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

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

The HuS-E/2 and HuS-T/2 clones demonstrated the highest expression of hepatocyte-specific markers and transcription factors by RT-PCR (data not shown); these cells were used as representative for each group in this study. To address if HuS-E/2 and HuS-T/2 maintained similar characteristics as primary hepatocytes, they were both cultured continuously for 30 weeks and the expression profiles of a variety of growth factors (Fig. 2A),

growth factor receptors (Fig. 2B), hepatocyte-specific nuclear factors (Fig. 2C), albumin, apolipoprotein-A1, transferrin (Fig. 2D), cytochrome p450 (CYP) genes (Fig. 2E), and GAPDH were compared with freshly isolated primary hepatocytes after isolation or two weeks of culture, Huh-7.5 cells, and 293 cells. After two weeks in culture, the expression of nearly all examined genes was similar between freshly isolated hepatocytes and the HuS-E/2 cell line. HuS-E/2 cells, however, exhibited higher expression of TGF β 2 (Fig. 2A), TGF β 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 β 1 and TGF β 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 α (Fig. 2C), and CYPs 2B, 2E1 (Fig. 2E).

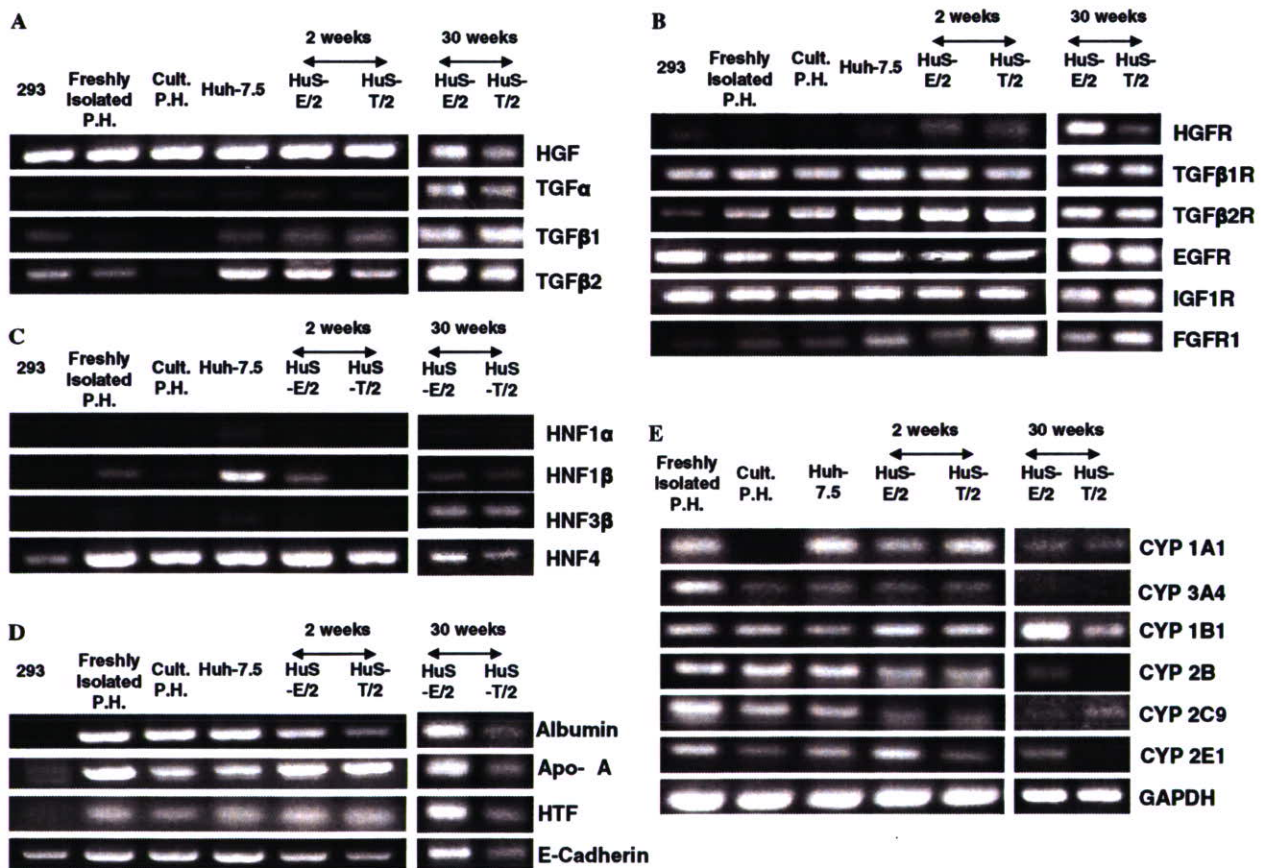


Fig. 2. Expression of the genes encoding growth factors (A), growth factor receptors (B), hepatocyte-specific nuclear factors (C), hepatocyte differentiation and functional markers (D), and CYP enzymes (E) in 293 cells, freshly isolated primary hepatocytes (P.H.), primary hepatocytes cultured for two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

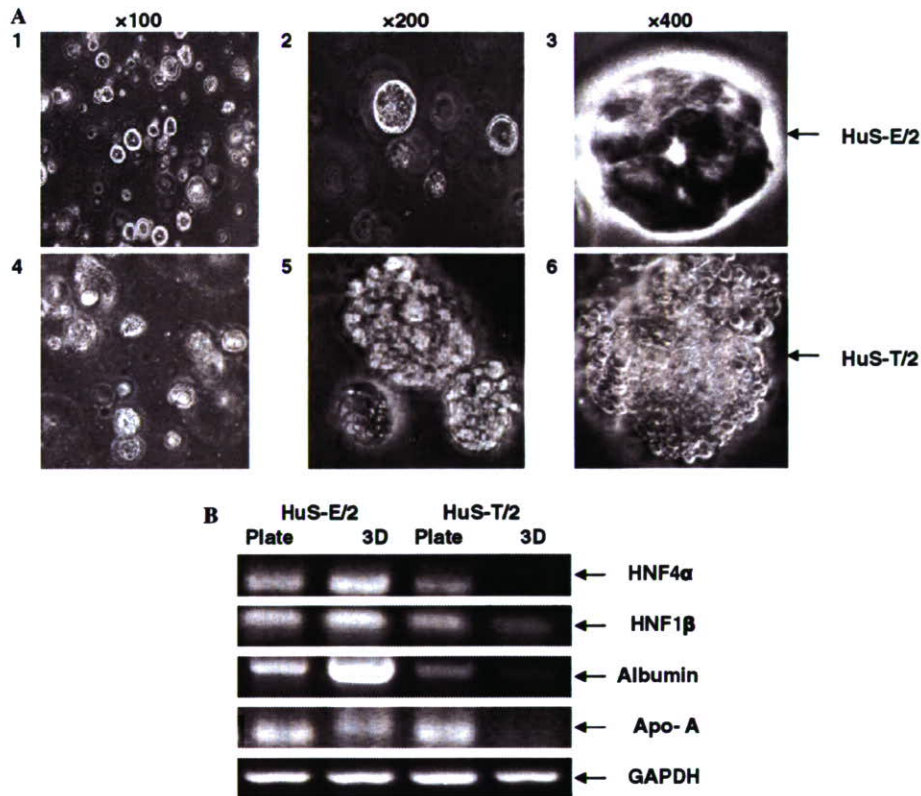


Fig. 3. (A) The morphology of HuS-E/2 and HuS-T/2 cells in 3D culture. HuS-E/2 and HuS-T/2 cells were cultured in Mebiol Gel in 12-well plates at a concentration of 5×10^5 cells/well. The microscopic characteristics of these cells after one week of 3D culture are shown. (B) The expressions of HNF4 α , HNF1 β , 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 α , HNF1 β , 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 β , 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 α blocked HCV infectivity

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

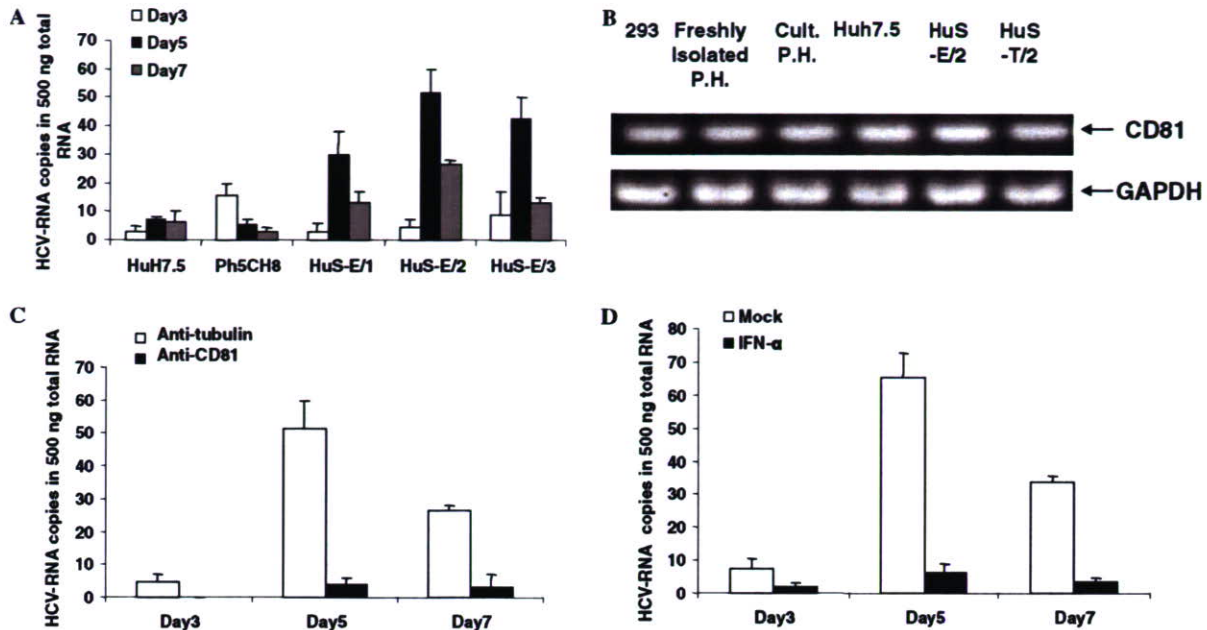


Fig. 4. (A) Serum from an HCV patient was used to infect Huh-7.5 cells, PH5CH8 cells, and three HPV E6E7-immortalized clones (HuS-E/1-3) for 24 h. After washing three times in phosphate-buffered saline (PBS), cells were cultured in fresh medium. Cells were then harvested and lysed at the indicated time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

without or with 100 U/ml IFN α . The enhancement of the HCV-RNA genome titers on the fifth day (about 10-fold) was not observed in cells treated continuously with IFN α (Fig. 4D). This result suggests that IFN α 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 α) and interferon-beta (IFN β) limits viral replication and spread, providing one of the most effective innate antiviral responses [25]. Signaling through IRF-3 and IRF-7 plays important roles in the stimulation of IFN- α/β production [25]. To 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 β and IFN α production by HuS-E/2 cells infected with Sendai virus were confirmed using assays of IFN β or IFN α promoter-driven luciferase reporters. DNIRF-3 exhibited strong inhibition of IFN β production (Fig. 5B) and weaker inhibition of IFN α transcription (Fig. 5C), while DNIRF-7 strongly inhibited IFN α production (Fig. 5C) and only weakly inhibited IFN β 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 β and IFN α production

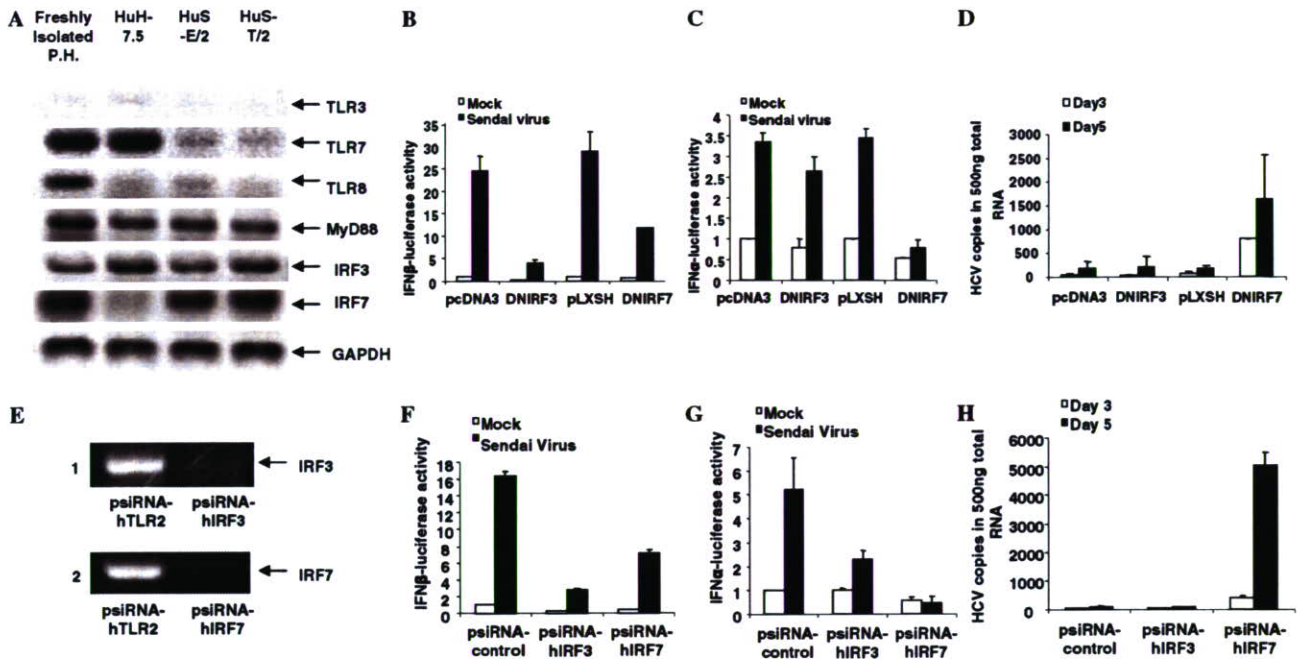


Fig. 5. (A) We examined the expression of TLR3, TLR7, TLR8, MyD88, IRF-3, and IRF-7, as well as GAPDH as an internal control in freshly isolated primary hepatocytes and Huh-7.5, HuS-E/2, and HuS-T/2 cells was investigated by RT-PCR. (B and C) HuS-E/2 cells were cotransfected with pIFN β -luc (B) or pIFN α -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 μ 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 μ g/ml. Two weeks later, cells were cotransfected with pIFN β -luc (F) or pIFN α -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 α 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 α -luciferase reporter assay (Fig. 6C). HuS-E7/DN24 also exhibited a higher HCV infectability in comparison to parental HuS-E/2 cells and the HuS-E7/DN22 clone (Fig. 6D).

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

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

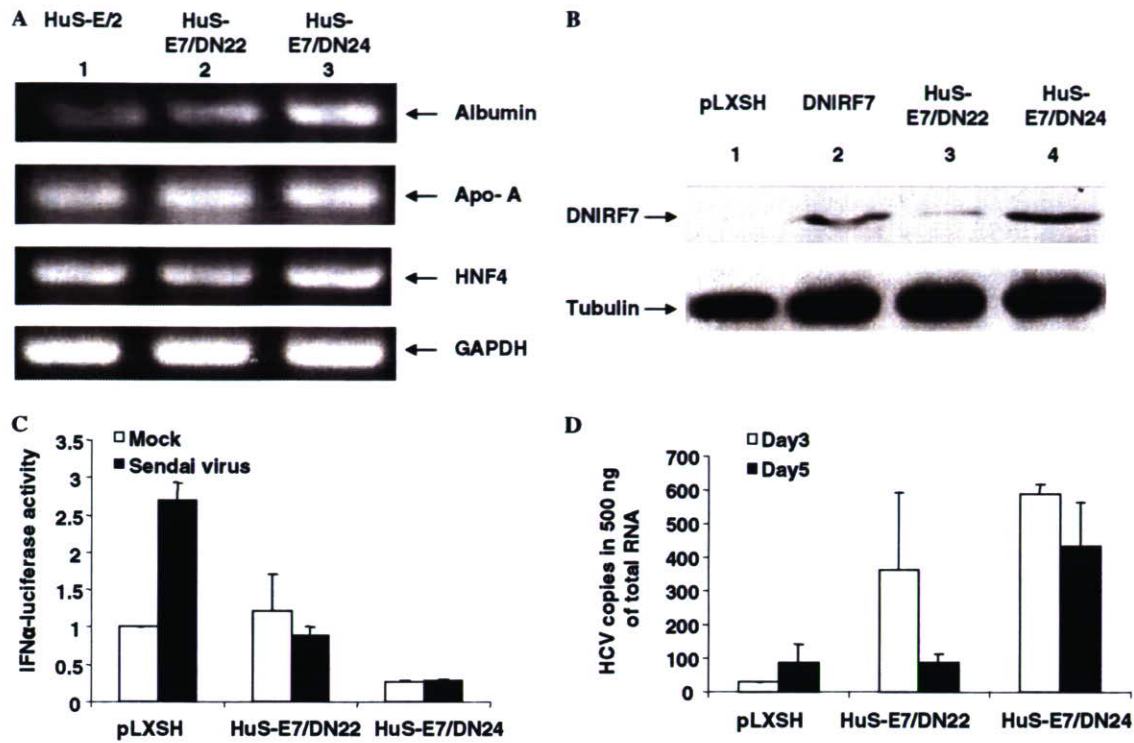


Fig. 6. (A) The pLXSH-HA-DNIRF-7 plasmid was transfected into HuS-E/2 cells, followed by selection in 100 µg/ml Hygromycin B. Two clones, HuS-E7/DN22 (lane 2) and HuS-E7/DN24 (lane 3), were obtained. We investigated the expression of albumin, apo-A, HNF4, and GAPDH as an internal control in parental HuS-E/2, HuS-E7/DN22, and HuS-E7/DN24 hepatocytes cultured for two weeks by RT-PCR. (B) Expression of HA-tagged DNIRF-7 (upper panel) and tubulin (control, lower panel) was detected by immunoblotting analysis. HuS-E/2 cells transiently transfected with either empty pLXSH vector (lane 1) or pLXSH-HA-DNIRF-7 (lane 2) were used as negative and positive controls, respectively, after 48 h. (C) HuS-E/2, HuS-E7/DN24, and HuS-E7/DN22 cells were transfected with IFN α -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.

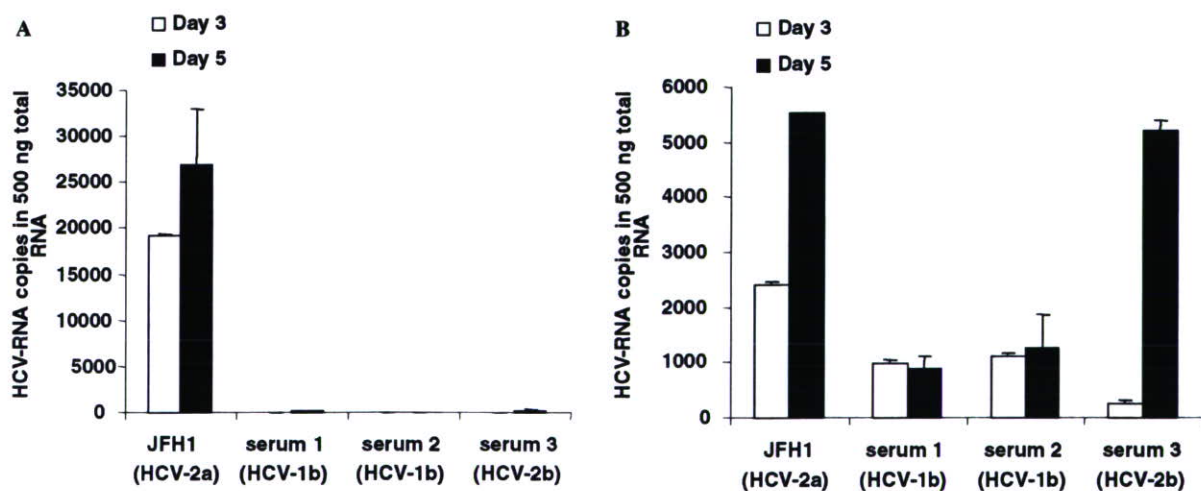


Fig. 7. The infectability of Huh-7.5 and HuS-E7/DN24 cells to different HCV genotypes. Huh-7.5 (A) and HuS-E7/DN24 (B) cells were infected with same titer of JFH1 (HCV-2a), two different HCV-1b serums and one HCV-2b serum. After removing the infected medium, the cells were washed in PBS and recultured 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 α [27]. HuS-E/2 cells continued to express HNF1 α 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 α 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 α/β genes. We established a clone stably expressing DNIRF-7 (HuS-7E/DN24), which demonstrated higher infectivity with different HCV strains than the parental HuS-E/2 clone.

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

Acknowledgements

We thank Dr. Akagi at Osaka Bioscience Institute for providing hTERT expressing vector, Dr. Sakai at the Institute for Virus Research, Kyoto University, for providing HPV18/E6E7 expressing plasmid, and Dr. Taniguchi of the University of Tokyo for providing IFN β , and IFN α promoters' reporter plasmids. 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, Labor and Welfare, by Grants-in-Aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, by Grants-in-Aid for the research for the future program from the Japanese society for the Promotion of Science. Dr. Hussein H. Aly is a receiver of the Japanese *Gakushu Shorei* scholarship and was partly supported by Prof. Yassin A. El Ghaffar memorial scholarship for the Improvement of Liver research in Egypt.

References

- [1] Wasley A, Alter MJ. Epidemiology of hepatitis C: geographic differences and temporal trends. *Semin Liver Dis* 2000;20:1–16.
- [2] Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001;358:958–965.
- [3] Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales Jr FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002;347:975–982.
- [4] Hadziyannis SJ, Sette Jr H, Morgan TR, Balan V, Diago M, Marcellin P, et al. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004;140:346–355.
- [5] Muir AJ, Bornstein JD, Killenberg PG. Peginterferon alfa-2b and ribavirin for the treatment of chronic hepatitis C in blacks and non-Hispanic whites. *N Engl J Med* 2004;350:2265–2271.
- [6] Falck-Ytter Y, Kale H, Mullen KD, Sarbah SA, Sorescu L, McCullough AJ. Surprisingly small effect of antiviral treatment in patients with hepatitis C. *Ann Intern Med* 2002;136:288–292.
- [7] Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005;11:791–796.
- [8] Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, et al. Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci USA* 2005;102:9294–9299.
- [9] Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. *Science* 2005;309:623–626.
- [10] Delgado JP, Parouchev A, Allain JE, Pennarun G, Gauthier LR, Dutrillaux AM, et al. Long-term controlled immortalization of a primate hepatic progenitor cell line after Simian virus 40 T-Antigen gene transfer. *Oncogene* 2005;24:541–551.
- [11] Mizuguchi T, Mitaka T, Katsuramaki T, Hirata K. Hepatocyte transplantation for total liver repopulation. *J Hepatobiliary Pancreat Surg* 2005;12:378–385.
- [12] Isom HC, Tevethia MJ, Kreider JW. Tumorigenicity of simian virus 40-transformed rat hepatocytes. *Cancer Res* 1981;41:2126–2134.
- [13] Ray FA, Waltman MJ, Lehman JM, Little JB, Nickoloff JA, Kraemer PM. Identification of SV40 T-antigen mutants that alter T-antigen-induced chromosome damage in human fibroblasts. *Cytometry* 1998;31:242–250.
- [14] Chen WH, Lai WF, Deng WP, Yang WK, Lo WC, Wu CC, et al. Tissue engineered cartilage using human articular chondrocytes immortalized by HPV-16 E6 and E7 genes. *J Biomed Mater Res A* 2006;76:512–520.
- [15] Dimri G, Band H, Band V. Mammary epithelial cell transformation: insights from cell culture and mouse models. *Breast Cancer Res* 2005;7:171–179.
- [16] Harms W, Rothamel T, Miller K, Harste G, Grassmann M, Heim A. Characterization of human myocardial fibroblasts immortalized by HPV16 E6–E7 genes. *Exp Cell Res* 2001;268:252–261.
- [17] Shiga T, Shirasawa H, Shimizu K, Dezawa M, Masuda Y, Simizu B. Normal human fibroblasts immortalized by introduction of human papillomavirus type 16 (HPV-16) E6–E7 genes. *Microbiol Immunol* 1997;41:313–319.
- [18] Akimov SS, Ramezani A, Hawley TS, Hawley RG. Bypass of senescence, immortalization, and transformation of human hematopoietic progenitor cells. *Stem Cells* 2005;23:1423–1433.
- [19] Hung SC, Yang DM, Chang CF, Lin RJ, Wang JS, Low-Tone Ho L, et al. Immortalization without neoplastic transformation of human mesenchymal stem cells by transduction with HPV16 E6E7 genes. *Int J Cancer* 2004;110:313–319.
- [20] Wang G, Johnson GA, Spencer TE, Bazer FW. Isolation, immortalization, and initial characterization of uterine cell lines: an in vitro model system for the porcine uterus. *In vitro Cell Dev Biol Anim* 2000;36:650–656.
- [21] Hino H, Tateno C, Sato H, Yamasaki C, Katayama S, Kohashi T, et al. A long-term culture of human hepatocytes which show a high growth potential and express their differentiated phenotypes. *Biochem Biophys Res Commun* 1999;256:184–191.
- [22] Watashi K, Hijikata M, Hosaka M, Yamaji M, Shimotohno K. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. *Hepatology* 2003;38:1282–1288.
- [23] Murata T, Ohshima T, Yamaji M, Hosaka M, Miyanari Y, Hijikata M, et al. Suppression of hepatitis C virus replicon by TGF-beta. *Virology* 2005;331:407–417.
- [24] Dasgupta A, Hughey R, Lancin P, Larue L, Moghe PV. E-cadherin synergistically induces hepatospecific phenotype and maturation of embryonic stem cells in conjunction with hepatotrophic factors. *Biotechnol Bioeng* 2005;92:257–266.
- [25] Civas A, Island ML, Genin P, Morin P, Navarro S. Regulation of virus-induced interferon-A genes. *Biochimie* 2002;84:643–654.
- [26] Ishiyama T, Kano J, Minami Y, Iijima T, Morishita Y, Noguchi M. Expression of HNFs and C/EBP alpha is correlated with immunocytochemical differentiation of cell lines derived from human hepatocellular carcinomas, hepatoblastomas and immortalized hepatocytes. *Cancer Sci* 2003;94:757–763.
- [27] Wege H, Le HT, Chui MS, Liu L, Wu J, Giri R, et al. Telomerase reconstitution immortalizes human fetal hepatocytes without disrupting their differentiation potential. *Gastroenterology* 2003;124:432–444.
- [28] Mamane Y, Heylbroeck C, Genin P, Algarte M, Servant MJ, LePage C, et al. Interferon regulatory factors: the next generation. *Gene* 1999;237:1–14.
- [29] Foy E, Li K, Wang C, Sumpter Jr R, Ikeda M, Lemon SM, et al. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science* 2003;300:1145–1148.
- [30] Sumpter Jr R, Loo YM, Foy E, Li K, Yoneyama M, Fujita T, et al. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J Virol* 2005;79:2689–2699.
- [31] Breiman A, Grandvaux N, Lin R, Ottone C, Akira S, Yoneyama M, et al. Inhibition of RIG-I-dependent signaling to the interferon pathway during hepatitis C virus expression and restoration of signaling by IKKepsilon. *J Virol* 2005;79:3969–3978.
- [32] Li K, Foy E, Ferreon JC, Nakamura M, Ferreon AC, Ikeda M, et al. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc Natl Acad Sci USA* 2005;102:2992–2997.
- [33] Foy E, Li K, Sumpter Jr R, Loo YM, Johnson CL, Wang C, et al. Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. *Proc Natl Acad Sci USA* 2005;102:2986–2991.
- [34] Zhang T, Lin RT, Li Y, Douglas SD, Maxcey C, Ho C, et al. Hepatitis C virus inhibits intracellular interferon alpha expression in human hepatic cell lines. *Hepatology* 2005;42:819–827.
- [35] Lee J, Wu CC, Lee KJ, Chuang TH, Katakura K, Liu YT, et al. Activation of anti-hepatitis C virus responses via Toll-like receptor 7. *Proc Natl Acad Sci USA* 2006;103:1828–1833.
- [36] Honda K, Yanai H, Negishi H, Asagiri M, Sato M, Mizutani T, et al. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* 2005;434:772–777.

Is hepatitis C virus NS3 protease quasispecies heterogeneity predictive of progression from cirrhosis to hepatocellular carcinoma?

S. Vallet,^{1,2} S. Gouriou,² G. Nkontchou,³ H. Hotta,⁷ M. Vilerio,² M. C. Legrand-Quillien,¹ M. Beaugrand,³ J. C. Trinchet,³ J. B. Nousbaum,⁴ P. Dény,⁵ C. Gaudy,⁶ A. Goudeau,⁶ B. Picard⁵ and C. Payan^{1,2} ¹Microbiologie, CHU Morvan, Brest, France; ²Bactériologie-Virologie EA3882, Faculté de Médecine, Brest, France; ³Hépatogastroentérologie, CH J. Verdier, Bondy, France; ⁴Hépatogastroentérologie, CHU La Cavale Blanche, Brest, France; ⁵Bactériologie-Virologie-Hygiène, CH Avicenne, Bobigny, France; ⁶Virologie, CHU Bretonneau, Tours, France; ⁷Divisions of Microbiology, Kobe University Graduate School of Medicine, Kobe, Japan

Received December 2005; accepted for publication February 2006

SUMMARY. We investigated whether an HCV NS3 protease quasispecies heterogeneity was associated with progression from viral cirrhosis to hepatocellular carcinoma (HCC). The NS3 protease quasispecies structure of 10 HCV-1b cirrhotic patients (controls) was compared with that of 10 paired HCV-1b cirrhotic patients who displayed progression to HCC (cases). NS3 protease genetic complexity and diversity did not differ significantly between cases and controls. Amino acid substitutions were detected at 20 (11%) and 25 (14%) sites in at least two variants of the NS3 protease in cases and controls, respectively. Significant differences in the percentage of substituted clones were observed for 10 NS3 sites. Mutations Y56F, I71V, T72I, Q86P, P89S, S101G/D, R117H, S122G/T/N, V132I and V170I were more frequently observed in the NS3 protease sequences of controls

than in those of cases. Residue V107 was substituted in NS3 cases but not in controls. However, these differences did not allow the definition of a specific NS3 profile related to HCC occurrence. The NS3 secondary structure B1-1 previously identified as potentially predictive of HCC was identified with a higher frequency in cases quasispecies (84.2%) than in controls (55.9%; $P < 0.05$). Our results suggest that there may be a relationship to fibrosis progression when diversity parameters are considered together with secondary structure profiles. Further investigations are required to determine the cellular interactions of HCV NS3 protease in the context of carcinogenesis.

Keywords: cirrhosis, hepatitis C virus, hepatocellular carcinoma, NS3 protease, polymorphism, quasispecies.

INTRODUCTION

Hepatitis C virus (HCV) infection is a major public health problem worldwide, with about 3% of the world's population being infected. Three to four million people become infected each year, and there are 170 million chronic carriers at risk of developing liver cirrhosis and, possibly, liver cancer. Hepatocellular carcinoma (HCC) mostly affects patients with liver cirrhosis and is currently the most common cause of death in these patients. The annual incidence of HCC varies between 1% and 4% of cirrhotic HCV-infected patients [1]. New HCV treatments are required, because bitherapy is only

partly effective, particularly for HCV genotype 1 infections, and limited by undesirable side effects.

The HCV genome encodes a polyprotein of about 3000 amino acids, which is co- and postranslationally cleaved by cellular and viral proteases. The N-terminal domain of HCV NS3, which contains a 181-amino acid chymotrypsin-like serine protease, is responsible for well-ordered proteolytic cleavage at the NS3/NS4A (in cis) and NS5A/NS5B, NS4A/NS4B and NS4B/NS5A junctions (in trans) of the viral polyprotein [2–5]. The NS3 protease is unique among serine proteases in requiring the action of a virus-encoded protein cofactor, NS4A, for cleavage at the NS3/NS4A and NS4B/NS5A junctions and for increasing cleavage efficiency at the NS4A/NS4B and NS5A/NS5B junctions [2,5]. The NS3 protease is essential for HCV replication and is therefore one of the most promising targets for specific anti-HCV therapy [6,7].

Various lines of evidence suggest that the HCV NS3 protease is involved in carcinogenesis. This protein is thought to

Abbreviations: HCC, hepatocellular carcinoma; HCV, Hepatitis C virus.

Correspondence: Dr Sophie Vallet, Département de Microbiologie, Centre Hospitalier Morvan, 2 avenue Foch, 29609 Brest Cedex, France. E-mail: sophie.vallet@univ-brest.fr

affect normal cellular functions, such as cell proliferation and cell death, and to be involved, either directly or indirectly, in HCV hepatocarcinogenesis. NS3 can induce the transformation of murine fibroblasts [8,9] and NS3 protease abolishes the actinomycin-D-induced apoptosis of NIH 3T3 cells [10]. NS3 protease interacts with the p53 tumour suppressor [11]; it may also specifically repress p21 promoter activity in a dose-dependent manner [12]. It was also recently suggested that analysis of the secondary structure of HCV NS3 protease might be useful for predicting the relative risk of developing HCC [13,14]. In these studies, a particular secondary conformation of the amino-terminal 120 residues of the protease was found to be significantly associated with HCC.

In the present work, we aimed to determine whether a particular nucleotide and amino acid signature pattern of the HCV-1b NS3 protease was predictive of progression from viral cirrhosis to HCC. HCV is never found as a homogeneous population of identical RNA genomes *in vivo*; instead, it exists as a mixture of closely related genomes defined as quasispecies [15–17]. We compared the NS3 protease quasispecies structure of two groups of patients as homogeneous as possible to eliminate exogenous factors that might affect cirrhosis progression: 10 HCV-1b-infected cirrhotic patients with that of 10 paired HCV-1b-infected cirrhotic patients displaying subsequent progression to HCC.

PATIENTS AND METHODS

Patients

Twenty women with histologically proved cirrhosis related to HCV-1b genotype, without detectable HCC and prospectively screened for HCC by US scan combined to serum dosage of α -foetoprotein every 4 months, were included in this case-control study. Patients were selected according to the following criteria: Caucasian origin; absence of comorbidities as alcohol abuse, HIV or HBV coinfection; no prior antiviral treatment. Ten patients (cases) who developed HCC during the follow-up were matched with 10 patients with the same age at diagnosis of cirrhosis and who had not developed HCC. These patients have been followed in a monitoring programme over a period of time equal or higher than that of the cases (controls) (Table 1). Period of follow-up corresponded to 1991–2004 between cirrhosis and HCC or non-HCC diagnoses. The diagnosis of HCC was made according to noninvasive criteria (four patients) [18] or on histology (six patients) [19]. In all cases, serums were collected at the time of the diagnosis of cirrhosis and kept at -20°C . A consent was obtained for all patients.

Genotyping and quantification

Hepatitis C virus genotyping was carried out by 5' noncoding region sequencing, using the Trugene HCV method

(TRUGENE 5'NC; Bayer HealthCare LLC, Berkeley, CA, USA), as previously described [20]. If HCV subtype had not been determined, we sequenced the NS5B region according to the French ANRS consensus protocol [21]. Serum HCV-RNA quantification was performed by the RT-PCR-based method (Monitor HCV RNA 2.0; Roche Diagnostics, Meylan, France) with a cutoff level at 600 IU/mL.

Hepatitis C virus NS3 RNA amplification and sequencing

RNA was extracted from 140 μL of serum (QIAmp Viral RNA Mini kit; Qiagen, Courtaboeuf, France) and eluted in 60 μL of molecular biology grade water. cDNA was synthesized at 42°C for 50 min with 200 IU of Superscript II Reverse Transcriptase (Invitrogen, Cergy Pontoise, France) and random hexamers (Applera, Courtaboeuf, France) in a final volume of 20 μL . The complete NS3 protease gene (543 nucleotides) was amplified using previously published primers G (sense: 5'-GAGCCCGTCGTCCTTC-3', nucleotides 3243–3259 according to HCV-J numbering) and J (anti-sense: 5'-AGGAACTTGCCGTAGGTGGAGTA-3', 4206–4228, HCV-J numbering) [22]. Introduction of artefactual mutations was minimized by carrying out the amplification reactions with *Pfu* DNA polymerase (Stratagene, Amsterdam, The Netherlands), a high-fidelity DNA polymerase. If amplicon yield was insufficient, a nested reaction was performed with the NS3-protease-specific primers previously described: P1b0 (sense: 5'-TCGTCITTTCTGACATGGAG-3', 3250–3259, HCV-J numbering) and P1b1 (anti-sense: 5'-TTGTACCCTTGGGCTGCATA-3', 4059–4078, HCV-J numbering) [23]. Amplicons were purified (High Pure PCR Product Purification Kit; Roche Molecular Biochemicals, Meylan, France). Amplicons with 3'-A overhangs were inserted into pCR 2.1TM-TOPO (TOPO TA Cloning Kit; Invitrogen). Plasmids containing protease NS3 inserts were purified and sequenced on both strands with the M13 universal and M13 reverse primers, by the dideoxy chain termination method, on a MegaBace DNA Analysis System (Amersham Biosciences, Orsay, France). Electrophoregram data were analysed with the Staden package program last updated in August 2002 [24]. Each of the 20 purified PCR products were also directly sequenced.

Sequence analysis of the protease NS3 region

Quasispecies sequences were aligned with the Clustal X program [25] and the final alignment was manually edited with the alignment editor GeneDoc [26]. A consensus sequence was determined for both the case and control groups. The number of viral variants and the genetic diversity of the HCV quasispecies were assessed. Genetic complexity, defined as the number of viral variants within HCV quasispecies, was evaluated by calculating the Shannon entropy S , according to the formula $S = -\sum_i (p_i \ln P_i)$, where P_i is the relative frequency of each sequence in the

Table 1 Patient characteristics. HCV infection with cirrhosis and subsequent HCC occurrence (cases, patients K1–K10) or absence of HCC (controls, patients C11–C20)

	Sex	Year of contamination	Age at the time of contamination (years)	Presumed mode of contamination	Age at the time of cirrhosis diagnosis	Child –Pugh	Viral load (IU/mL)*	ALT (IU/L)	Time between cirrhosis diagnosis and HCC or duration of HCC free follow-up (years)
Cases									
K1	F	1953	7	T	53.4	A	0.82×10^6	92	2.9
K2	F	1945	ND	ND	53.6	A	4.32×10^6	45	4.5
K3	F	1981	48	T	60.7	B	2.03×10^6	110	8.6
K4	F	1948	20	T	64.1	A	7.83×10^6	175	6
K5	F	1934	9	T	68.0	A	3.83×10^6	122	7.4
K6	F	1949	25	T	69.5	A	3.96×10^6	56	5.8
K7	F	1957	20	T	64.4	A	0.13×10^6	75	3.8
K8	F	1940	70	T	71.2	A	1.46×10^6	70	10.2
K9	F	1946	73	T	73.5	A	4.93×10^6	120	8.3
K10	F	ND	ND	T	79.3	A	1.95×10^6	146	4
Median			22.5		66.2		2.93×10^6	101	5.9
Controls									
C11	F	1955	19	SUR	58	A	0.8×10^6	94	10.5
C12	F	ND	ND	ND	49.5	A	0.07×10^6	157	7
C13	F	1971	38	T	60.5	A	1.03×10^6	80	11.1
C14	F	1970	40	ACP	63.1	A	2.15×10^6	94	10.9
C15	F	ND	ND	ND	67.1	A	6370	103	11.9
C16	F	1954	28	SUR	66.7	A	0.59×10^6	233	6.7
C17	F	1976	52	T	70.1	A	0.84×10^6	46	10.1
C18	F	1951	27	T	71.1	A	4.59×10^6	41	7.8
C19	F	ND	ND	ND	69.4	A	2.03×10^6	400	8.3
C20	F	1978	57	T	78.7	A	0.3×10^6	90	5
Median			38		66.9		0.94×10^6	94	9.2

T, transfusion; ACP, acupuncture; SUR, surgical intervention; ND, data not available; ALT, alanine aminotransferase.

*IU/mL (Cobas Amplicor Monitor; Roche Diagnostics, Meylan, France).

viral quasispecies. The normalized entropy S_n was calculated as $S_n = S / \ln N$, where N is the total number of sequences analysed [27]. S_n theoretically varies from 0 (all isolates identical) to 1 (all isolates different). The normalized Shannon entropy was calculated at both nucleotide and amino acid levels.

The diversity of the NS3 protease gene was evaluated using the Mega program to determine the type of substitution (version 3; Department of Biology, Arizona State University; <http://www.megasoftware.net>). The number of synonymous and nonsynonymous substitutions per synonymous and nonsynonymous site (d_S) and (d_N), respectively, was calculated by the Nei–Gobojori method, with Jukes–Cantor correction to account for multiple substitutions at the same site [28]. Genetic distance was estimated for the viral quasispecies of each patient, by means of pairwise comparisons, using Kimura's two-parameter gamma model of the Mega software with a gamma parameter of 0.29 [29].

Phylogenetic analysis was performed with MEGA3 software, using the neighbour-joining method, a Kimura two-parameter model and a gamma distribution ($\alpha = 0.29$). The reliability of the various inferred clades was estimated by bootstrapping (525 replicates).

Secondary structure prediction

The secondary structure of the amino-terminal 120 residues of NS3 protease was predicted by computer-assisted Robson analysis [30] with GENETIX-MAC software (version 10.1; Software Development Co., Ltd, Tokyo, Japan), as previously described by Ogata *et al.* [13,14].

Statistical analyses

Groups were compared by means of chi-squared tests for percentages and Mann–Whitney and Student's *t*-tests for quantitative variables. $P < 0.05$ was considered significant.

Nucleotide sequence GenBank accession numbers

All nucleotide sequence data have been deposited in the GenBank sequence database under accession numbers DQ338124–DQ338432.

RESULTS

Characteristics of the patients

All 20 patients were contaminated in Europe (mostly in France), via blood transfusion in most cases (Table 1). Median age at the time of cirrhosis diagnosis was 66.2 and 66.9 years for cases and controls, respectively. The duration of HCV disease progression was longer in cases than in controls (medians of 45 vs 23 years). All but one of the cases

had a Child–Pugh cirrhosis classification of A. Patient K3 has class B cirrhosis. Viral load was almost statistically different ($P = 0.051$) between the two groups of patients (2.93×10^6 and 0.94×10^6 IU/mL for cases and controls, respectively). Median serum alanine aminotransferase level was similar, with 2.2 and 2.1 times upper limit of normal in cases and controls, respectively. The median time between the diagnosis of cirrhosis and the diagnosis of HCC or non-HCC outcome was 5.9 and 9.2 years for cases and controls, respectively.

NS3 protease quasispecies features and subsequent progression or nonprogression to HCC

Hepatitis C virus NS3 protease region was analysed both at nucleotide and amino acid levels, corresponding to fragments of 543 bases and 181 residues, respectively. Inter- and intrasamples' variability of sequences was assessed. A median of 15 NS3 protease clones per sample was analysed in the two groups. A total of 289 NS3 protease sequences were obtained (146 sequences for cases and 143 for controls).

Consensus sequences were determined by aligning the NS3 variants in each group; 98.3% of nucleotide sites were identical when the consensus sequences of the two groups were compared. In variants from cases and controls, 206 and 205, respectively, of the 543 nucleotide sites showed at least one substitution. A stop codon was identified in two control clones from patients C12 and C16, at NS3 protease codons 85 and 86, respectively.

Complexity and diversity

The intra- and intersample complexity and diversity of NS3 protease were evaluated for both nucleotide and amino acid sequences (Table 2). Genetic complexity, estimated by Shannon entropy and defined as the number of different sequences or clusters of sequences appearing at a given time point, did not differ significantly between cases and controls. The diversity of the NS3 protease gene was evaluated as the mean genetic distance within quasispecies. The mean within-sample genetic distance calculated for the quasispecies in each of the 20 patients was 0.017. Genetic distances in the quasispecies present in samples from cases and controls varied from patient to patient but were similar between the two groups. The proportion of synonymous substitutions was significantly higher than the proportion of nonsynonymous substitutions ($P < 0.0001$), suggesting that NS3 protease mutations mostly resulted from random genetic drift rather than from positive selective pressure.

A phylogenetic tree built from an alignment of 289 nucleotide sequences showed no specific distribution of NS3 protease clones according to subsequent progression or nonprogression of cirrhosis to HCC (Fig. 1). It was actually not possible to identify a dominant intrasample