Complete replication of hepatitis B virus and hepatitis C virus in a newly developed hepatoma cell line

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The absence of a robust cell culture system for hepatitis B virus (HBV) and hepatitis C virus (HCV) infection has limited the analysis of the virus lifecycle and drug development. We have established a hepatoma cell line, HLCZ01, the first cell line, to the authors' knowledge, supporting the entire lifecycle of both HBV and HCV. HBV surface antigen (HBsAg)-positive particles can be observed in the supernatant and the lumen of the endoplasmic reticulum of the cells via electron microscopy. Interestingly, HBV and HCV clinical isolates propagate in HLCZ01 cells. Both viruses replicate in the cells without evidence of overt interference. HBV and HCV entry are blocked by antibodies against HBsAg and human CD81, respectively, and the replication of HBV and HCV is inhibited by antivirals. HLCZ01 cells mount an innate immune response to virus infection. The cell line provides a powerful tool for exploring the mechanisms of virus entry and replication and the interaction between host and virus, facilitating the development of novel antiviral agents and vaccines.

Significance

More than 500 million people worldwide are persistently infected with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) and are at risk of developing chronic liver diseases (1). There is no vaccine against HCV, and many patients who are persistently infected by HBV or HCV do not respond to currently available therapies (2, 3). Improved understanding of the biology and pathogenesis of these infections is required for the development of vaccine and antiviral drugs (4). The inability to grow HBV and HCV efficiently in cell culture has presented a major obstacle to understanding the virus lifecycle and pathogenesis and to developing improved therapeutics.

HBV is a member of the hepadnavirus families, and its genome is a relaxed circular, partially double-stranded DNA molecule. The negative strand has an invariable length of ~3.2 kb, and the positive strand is 50–100% of this length. Several key issues about the biology of HBV remain to be explored, including the identification of the cellular receptors, the role of the X gene, and the mechanisms by which the viral minichromosome is formed. Covalently closed circular DNA (cccDNA) is responsible for the establishment of viral infection and persistence. Understanding the mechanisms underlying cccDNA formation and regulation is critical for understanding the HBV pathogenesis and finding a cure for hepatitis B. HepG2.2.15 cells derived from the hepatoma cell line HepG2 transfected with the full genome of HBV have been used to study HBV replication (5). Primary human hepatocytes (PHH) are susceptible to HBV infection (6, 7), but the use of this model is hampered by the limited availability and unpredictable variability of human liver. Several human hepatoma cell lines support HBV replication after HBV DNA transfection, and overexpression of sodium-taurocholate cotransporting polypeptide (NTCP) in HepG2 and Huh7 cells can render these cells able to support HBV produced in cell culture at low efficiency (5, 8, 9). HepaRG is a hepatoma cell line that is susceptible to HBV infection (10), but the susceptibility of HepaRG cells to HBV is strictly dependent on the differentiation state induced by DMSO, causing variable toxic side effects and irreproducible results for HBV replication levels. Moreover, poor viral replication, low viral yields, the absence of reinfection, and lack of cccDNA amplification in HepaRG cells make the study of HBV lifecycle and pathogenesis difficult (10). For reasons that still are unknown, HBV clinical isolates do not propagate in cell culture.

HCV is a positive-strand RNA virus of the Flaviviridae family. Its genome encodes a large polyprotein that is processed to produce viral structural proteins including core, E1, and E2 proteins as well as nonstructural proteins consisting of p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Genotype 2a, 2b, and 1a isolates were used to develop infectious cell culture systems for HCV study (11–15). However, other HCV genotypes cannot propagate in vitro. The permissive human hepatoma cell HuH7.5 may not mount an innate immune response to HCV infection, so it is difficult to explore the interaction between the virus and host cells. Moreover, clinical isolates of HCV do not propagate in cell culture. Although primary culture systems have been established for HCV (16, 17), these do not seem to extend the range of isolates that can be studied. We now report the establishment of a novel hepatoma cell line, HLCZ01, supporting the entire life cycle of HBV and HCV. Interestingly, the cells can be infected by the sera from patients with different HBV and HCV strains.


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Results

Establishment of a Novel Hepatoma Cell Line, HLCZ01. To understand better the virus lifecycle and the interaction between host and virus, we attempted to replicate HBV and HCV in hepatoma cells, which have histological features characteristic of hepatocellular carcinoma (Fig. 1A). Tumor tissue was harvested and cultured in DMEM/F12 medium, and several clones survived for long-time culture. We One of these clones, HLCZ01 (Fig. 1B), was derived from the grade 2 differentiated hepatocellular carcinoma of an HCV-infected male patient. To obtain hepatoma cell lines, we injected clones into immunodeficient mice and cultured tumor tissues from the mice in DMEM/F12 medium. HLCZ01 cells express liver-specific genes, such as human albumin (ALB), α1-antitrypsin (AAT), hepatocyte nuclear factor 4 (HNF4), cytochrome P450 3A4 (CYP3A4), and microRNA122 (miR-122) (Fig. 1C), and the liver-specific proteins human ALB and AAT (Fig. 1D). The presence of HCV RNA is not detectable by RT-PCR after the establishment of HLCZ01 cells in culture.

Infection of HLCZ01 Cells by HBV. To determine whether the various hepatoma cell lines we established are permissive for HBV infection, we inoculated those cell lines with the filtered supematant of HepG2.2.15 cells producing infectious HBV. Intracellular viral DNA and cccDNA could be detected only in HLCZ01 cells (Fig. 2A and Fig. S1A). When we attempted to infect Huh7 cells under identical conditions, no signal corresponding to viral DNA could be observed (Fig. S1A and B). Similarly, we confirmed the presence of the pregenomic RNA of the virus in HLCZ01 cells inoculated with HBV (Fig. 2B and Fig. S1C), whereas viral RNA could not be detected in Huh7 cells under similar conditions (Fig. S1 C and D). For comparison with other HBV-permissive cells, we inoculated PHH with the supernatant of HepG2.2.15 cells. HBV DNA could be identified in PHH inoculated with the supernatant of HepG2.2.15 cells (Fig. S1F). HBV surface antigen (HBsAg) and HBV core antigen (HBeAg)-positive HLCZ01 cells could be observed clearly (Fig. 2C). Around 3.2% of HLCZ01 cells were infected at 5 d postinfection (dpi), and HBV-infected cells increased up to 30% at 65 dpi (Fig. S2). HBsAg-positive particles were observed in the supernatant and in the lumen of the endoplasmic reticulum of HBV-infected HLCZ01 cells under electron microscopy (Fig. 2D and Fig. S1G).

To test whether HLCZ01 cells support the entire HBV life cycle, we examined the production of viral particles in the supernatant by analyzing both the secretion of HBsAg or HBeAg and the production of virions. HBsAg and HBeAg increased and reached a plateau at 60 dpi (Fig. 2E). HBV DNA could be identified in the supernatant of HBV-infected HLCZ01 cells and reached at plateau at 65 dpi (Fig. 2F and Fig. S1E). To explore whether the virus particles from HBV-infected HLCZ01 cells could be transferred to naive HLCZ01 cells, we harvested the supernatant of HBV-infected HLCZ01 cells and inoculated naive HLCZ01 cells. Viral DNA and cccDNA could be detected in HLCZ01 cells inoculated with the supernatant of HBV-infected HLCZ01 cells (Fig. 2G). The titers of HBV preparations applied to and released from HLCZ01 cells inoculated with the supernatant of HepG2.2.15 cells are shown in Fig. S1H. HBsAg and HBeAg also could be identified in the supernatant of HLCZ01 cells inoculated with the supernatant of HBV-infected HLCZ01...
HBV Clinical Isolates Do Propagate in HLCZ01. To determine whether clinical isolates of HBV can propagate in HLCZ01 cells, we inoculated HLCZ01 cells with the sera from HBV-infected donors. HBV DNA and cccDNA could be detected in HLCZ01 cells (Fig. 3A and B). When we attempted to infect Huh7 cells under same conditions, no signal corresponding to viral DNA could be observed (Fig. S3A). When the infectious HBV sera were heat-inactivated, only HBV DNA and cccDNA at the background level were observed in HLCZ01 cells (Fig. S3C). The levels of HBV DNA and cccDNA in HLCZ01 cells were dose-dependent when the cells were inoculated with a dilution series of sera from hepatitis B patients (Fig. S3D and E). For comparison with other HBV-permissive cells, we inoculated PHH with the sera from HBV-infected donors. HBV DNA and cccDNA could be detected in PHH (Fig. S3D and E). We also ascertained the presence of viral pregenomic RNA within HLCZ01 cells inoculated with the sera from HBV-infected PHH.
donors (Fig. 3C and Fig. S3F). Similar to viral DNA, viral RNA was undetectable in Huh7 cells inoculated with HBV sera (Fig. S3F). Flow cytometry results showed that HBsAg-positive HLCZ01 cells increased from ∼3.5% at 7 dpi to 7% at 10 dpi (Fig. S3G), and when the cells were inoculated with the serum of patient #2465, which had high viral titer, HBsAg-positive HLCZ01 cells increased from 8.7% at 7 dpi to 10.9% at 10 dpi (Fig. S3G). HBsAg could be found in the supernatant of HLCZ01 cells inoculated with the sera from hepatitis B patients (Fig. 3D). Moreover, viral particles in the supernatant of HLCZ01 cells infected by HBV sera could be transferred to naive HLCZ01 cells (Fig. 3E). However, when we attempted to infect Huh7 cells under similar conditions, no signal corresponding to viral DNA could be observed (Fig. S3F). These findings clearly show that HBV clinical isolates can propagate in HLCZ01 cells.

Inhibition of HBV Infection in HLCZ01 Cells by Antiviral Drugs in Vitro and in Vivo. To verify further that infection of HLCZ01 cells by HBV follows the authentic entry pathway, we incubated HBV with various concentrations of the anti-HBsAg antibody before incubation with HLCZ01 cells. Anti-HBsAg antibody blocked viral infection (Fig. 4A). In addition, we examined the inhibition effect of myristoylated peptide representing the N-terminal 48 amino acids of the HBV large-surface protein required for HBV infectivity. As expected, HBV infection could be inhibited in HLCZ01 cells when the peptide was added during the infection process (Figs. 2 and 4B). One recent study showed that NTCP is a putative HBV receptor (9). The expression of NTCP protein in HLCZ01 and PHH is comparable (Fig. S4A). Silencing of NTCP inhibited HBV infection in HLCZ01 cells (Fig. S4B and C). A previous study indicated that IFN-α inhibits HBV transcription and replication in vitro and in vivo (18), so we examined the
ability of IFN-α to inhibit HBV replication in HBV-infected HLCZ01 cells. In our culture system IFN-α inhibits HBV replication by decreasing the transcription of viral pregenomic RNA (Fig. 4C). Lamivudine is widely used for hepatitis B treatment, so we tested the effect of lamivudine on HBV DNA replication in our cultured cells and in HBV-infected mice. Lamivudine markedly decreased the HBV DNA level in our cell culture system in a dose-dependent manner (Figs. 2A, 3A, and 4D). Moreover, lamivudine inhibited HBV infection in mice implanted with HBV-infected HLCZ01 cells (Fig. 4E). All these data indicate that HBV infection of HLCZ01 may be a powerful model studying for HBV and for the development of antivirals and therapeutic vaccines.

Infection of HLCZ01 Cells by HCV. Infection of the host cell by HCV is initiated by the interactions between the viral envelop protein and several previously identified HCV entry receptors, including CD81, scavenger receptor class B type I (SR-BI), claudin-1 (CLDN1), and occludin (OCLN) (19–23). ApoE is critical for HCV assembly (24). The expression of these receptors in HLCZ01 and permissive Huh7.5 cells is comparable (Fig. S5). To understand better the interaction between HCV and host cells, we attempted to replicate HCV in HLCZ01 cells. We inoculated HLCZ01 cells with HCV in cell culture (HCVcc) and found that NS5A-positive HLCZ01 cells could be observed readily (Fig. 5A). Intracellular viral RNA was detectable in the cells at 1 dpi and increased rapidly (Fig. 5B). Viral replication was slightly less efficient in HLCZ01 cells than in Huh7.5 cells (Fig. 5B). Extracellular viral RNA and core protein levels in the supernatant of HLCZ01 and Huh7.5 cells were comparable (Fig. 5C and D). To prove that HCV-infected HLCZ01 cells indeed release infectious viruses, we inoculated naive HLCZ01 cells with the supernatant collected from HCV-infected HLCZ01 cells and detected NS5A-positive signal and viral RNA in the infected naive cells (Fig. 5E and Fig. S6A). Moreover, HCV-infected HLCZ01 and Huh7.5 cells produced comparable infectivity titers (Fig. S6B).

To examine whether clinical isolates of HCV can propagate in HLCZ01 cells, we inoculated HLCZ01 with different genotypes of sera from patients with hepatitis C. HCV RNA and core protein could be observed in the cells (Fig. 5C and Fig. S7A). Recently, the protease NS3 inhibitor telaprevir has entered...
clinical use for treatment of HCV-infected individuals (25). IFN-α or telaprevir decreased viral RNA levels in HLCZ01 cells under identical conditions (Fig. S7B and C). When the infectious HCV sera were inactivated by UV, viral RNA could be detected in HLCZ01 only at the background level (Fig. S7B).

Viral particles in the supernatant of HLCZ01 cells infected by sera from HCV-infected donors could be transferred to naive HLCZ01 cells (Fig. S7D). However, IFN-α or telaprevir inhibited the passage of sera-derived HCV from sera-inoculated HLCZ01 cells to naive HLCZ01 cells (Fig. S7D). The quantification of infectious units in HLCZ01 cells infected with HCV sera is shown in Fig. S7E. Flow cytometry results further demonstrated that HLCZ01 cells...
cells can be infected by sera from hepatitis C patients (Fig. S7F). All these data suggest that HLCZ01 cells indeed can be infected by HVCVc and by the sera from hepatitis C patients.

It has been reported that human CD81 is an essential coreceptor for HCV entry (19). To provide evidence that the uptake of HCV into HLCZ01 cells follows the authentic entry pathway, we performed infection competition assays using mouse monoclonal anti-CD81 antibody or soluble CD81 large extracellular loop (hCD81-LEL). HVCVc infectivity could be blocked with either anti-CD81 antibody or hCD81-LEL (Fig. 5 G and H), indicating that the virus enters via the authentic HCV entry pathway.

The current standard of care for chronic hepatitis C involves IFN-α–based therapy. We found that IFN-α significantly inhibited HCV RNA replication in HLCZ01 cells (Fig. 5f and Fig. S7B), demonstrating that HLCZ01 cells infected by HCV may be useful for testing novel drugs.

To understand better the interaction between HCV and host cells, we infected HLCZ01 cells with HVCVc and tested the innate immune response in the cells. IFN-β and IFN-stimulated genes (ISGs) were induced in viral-infected cells (Fig. 5f and Fig. S8), indicating that HLCZ01 cells mount an innate immune response to HCV infection.

HBV/HCV Coinfection in HLCZ01 Cells. HBV/HCV coinfection is common, with an estimated 7–20 million individuals affected worldwide. Patients with HBV/HCV coinfection have an increased risk for cirrhosis, hepatocellular carcinoma, and death (26). The virological and molecular aspects of HBV/HCV coinfection are poorly understood. The lack of appropriate model systems has made the study of the interactions between HBV and HCV difficult. Our novel cell culture system allows us to investigate the interactions between HBV and HCV. HCV infection did not affect HBV replication in HLCZ01 cells (Fig. 6A and B and Fig. S9A). HCV RNA and core protein in the supernatant of the cells could be verified (Fig. 6C). Interestingly, HBV and HCV infected the same cells (Fig. 6D and E and Fig. S9A), providing new insights into the pathogenesis of HBV/HCV coinfection. The frequency of coinfected cells increased when HLCZ01 cells were infected with HBV and HCV at higher virus titers (Fig. S9A). Moreover, HBV had little effect on the innate host response of HLCZ01 cells to HCV infection (Fig. 6F). HBV infection did not affect HCV replication in HLCZ01 cells (Fig. 6G). HCV infection was confirmed in the cells (Fig. 6H). Finally, simultaneous infection of HLCZ01 cells by HBV and HCV did not affect either HBV (Fig. S9B) or HCV (Fig. S9C) replication. These data indicate that HBV and HCV replicate in the same cells without evidence of direct interference in vitro (27). Superinfection exclusion generally is restricted to homologous viruses (28), whereas nonrelated viruses are able to replicate normally. In agreement with the general concept, our results showed that there is no superinfection exclusion of HBV by HCV or of HCV by HBV.

Discussion

We have established that HLCZ01 is a robust cell culture model of HBV infection by showing the kinetics of several markers of viral infection, including viral DNA replication, the formation and amplification of cccDNA, newly synthesized pregenomic viral RNA, the secretion of HBSAg and HBeAg, and the production and release of infectious viral particles from HBV-infected HLCZ01 cells. In addition, evidence that HBV infection is blocked by specific anti-HBsAg antibody or by pre-S1–blocking peptide strongly indicates that HBV infection of HLCZ01 cells follows the authentic entry pathway and that the process of viral adsorption and entry of HBV can be studied in this system. That the expression of NTCP protein is comparable in HLCZ01, HepG2, and HuH7 cells and in PHH but only HLCZ01 and PHH are susceptible to HBV infection suggests that other HBV receptors exist. Our data show that HBV infection in HLCZ01 cells results in the formation of foci of infected cells and that the percentage of HBV-infected cells increases, indicating that HBV may spread via cell-to-cell transmission and/or by attaching preferentially to the adjacent cells after secretion. Interestingly, HBV clinical isolates can propagate HLCZ01 cells, providing a very useful tool for the analysis of clinical isolates of HBV and for the development of antiviral drugs and vaccines. The HLCZ01 cell line provides a powerful tool for improving our understanding of the HBV life cycles, including the identification of the still unknown receptors and the mechanisms by which cccDNA is formed and amplified.

The HLCZ01 cell line also is susceptible to HCV infection, as shown by the kinetics of intracellular viral RNA replication, the expression of viral protein, and the production and release of infectious virus particles. HVCVc infectivity could be blocked with either anti-CD81 antibody or hCD81-LEL, indicating that virus enters via the authentic HCV entry pathway. The remarkable feature of HLCZ01 cells is their susceptibility to different genotypes of sera from hepatitis C patients, providing a useful tool for the analysis of clinical isolates of HCV and for the development of vaccines.

Our novel culture system allows us to investigate the interactions between HBV and HCV. Interestingly, the two viruses can infect the same cells without evidence for direct interference, providing new insights into the pathogenesis of HBV/HCV coinfection.

In summary, we have established a robust cell culture model of HBV and HCV infection in which infectious HBV and HCV can be produced and passaged to naïve cells. Supporting the entire life cycles of both viruses, the HLCZ01 cell line provides a powerful tool for addressing aspects of the virus life cycles, including the identification of the yet unknown receptors of HBV, virus entry, formation and amplification of cccDNA, virus assembly, the analysis of clinical isolates of HBV and HCV, and HBV/HCV coinfection. It will enable us to explain functionally the role of viral surface proteins in the entry process and consequently will facilitate the development of antiviral drugs interfering with the early steps of viral infection. Evidence that type I IFN and ISGs are induced in HCV-infected HLCZ01 cells and that different genotypes of HBV and HCV clinical isolates can propagate in HLCZ01 cells suggests that this cell culture system may be useful for the analysis of host–virus interactions that should facilitate the discovery of antiviral drugs and vaccines.

Materials and Methods

Isolation and Establishment of a Novel Hepatoma Cell Line. Cells were isolated from the tissue of a liver tumor from a male patient with chronic HCV infection. Experimental procedures were performed in accordance with the provisions of the Ethics Committee of Hunan Provincial Tumor Hospital (Changsha, China). The cells were isolated and cultured as described previously (29). Briefly, immediately after surgical resection, the tumor tissue was stored in PBS, and cells were dissociated within 1 h by two-step perfusions. Visible vessels were perfused first for 15 min with Liver Perfusion Medium (In vitro) to eliminate the blood cells. A second perfusion was performed with collagenase- and dispase-containing Liver Digest Medium (In vitro) until the tissue was digested. Then the liver tissue was cut into small pieces and shaken gently in Hepatocytes Wash Medium (In vitro). The cells and small pieces of liver tissue were cultured with DMEM/F12 medium supplemented with 10% (vol/vol) FBS (In vitro). The cell clones were selected and cultured in a six-well plate until cell growth filled the culture well. To obtain the hepatoma cell line, 5 × 10^6 cells were injected into NODScid immunodeficient mice. Two months later, the tumor tissues were removed from the mice, cut into small pieces, and cultured in DMEM/F12 medium supplemented with 40 ng/mL of dexamethasone (Sigma) and 10 ng/mL of EGF (BD). Several clones were obtained, one of which we designated HLCZ0. All cells were cultured in collagen-coated tissue culture plates. HLCZ0 cells were passaged every 5 d (1/3 dilution) by trypsinization.

Isolation of PHH. All procedures were performed in accordance with the provisions of the Ethical Committee of Hunan Provincial Tumor Hospital. PHH were obtained from healthy peritumoral liver resection specimens from...
non-HBV–, non-HCV–, and non-HIV–infected patients undergoing partial hepatectomy for primary hepatocellular carcinoma or secondary metastatic lesions caused by other cancers. PHH were isolated and cultured as described previously (30).

**Serum Samples.** HBV and HCV sera were collected between 2009 and 2011 in Xiangya Hospital of Central South University, China, with the approval of the Ethics Committee of Xiangya Hospital. Information regarding these donors can be found in Tables S1 and S2.

**HBV Infection of Cell Culture.** The supernatant of HepG2.2.15 cells derived from human hepatoma cell line HepG2 transfected with the full genome of HBV was collected and filtered. HLCZ01 cells were inoculated overnight with the filtered supernatant at a multiplicity of infection (MOI) of 20 genome equivalents (Geq) per cell. The cells were washed three times with PBS, were maintained in DMEM/F12 medium, and were harvested at the indicated times. HLCZ01 cells were inoculated with the different strains of sera from hepatitis B patients diluted at 1:20 and were cultured for the indicated time periods.

**Quantitative Assay for HBV-Specific Proteins.** HBsAg and HBV e antigen (HBeAg) in the supernatant were quantified by a chemiluminescent microparticle immunoassay using the Architect i2000SR Analyzer according to the manufacturer’s instructions. Quantitative HBsAg levels are reported in infectious units (IU) per milliliter, with a reactive range of 0.05–250.00 IU/mL. Samples with HBsAg titers beyond 250 IU/mL were diluted at 1:20 and 1:500 before further testing. In the Architect platform, the HBeAg assay is designed to be semi-quantitative and is reported as sample per cutoff (S/CO). Samples with S/CO values greater than 1.0 were considered to be HBeAg positive.
Isolation and Analysis of Viral DNA. DNA was isolated from the whole-cell lysates and supernatant. Real-time PCR for total HBV DNA and cccDNA was performed as previously described (31). Intracellular hepatic HBV DNA and cccDNA were measured using real-time PCR analysis. The primers used for PCR to detect HBV DNA were 5′-CACCTCTGCATATACTC-3′ (sense) and 5′-GGAAAAAGTCTGAGGACCA-3′ (antisense). The primers used for PCR to detect cccDNA were 5′-GGTCTCTTACATGGCTCGG-3′ (sense) and 5′-GGAAAGTCTGAGGACCA-3′ (antisense). Plasmid-safe ATP-dependent DNase (Epitect) was used to digest the single-strand region of HBV genome, allowing enrichment of cccDNA for subsequent real-time PCR detection.

Reverse Transcription and Analysis of HBV-Specific Transcripts. Total RNA was extracted from the cells using TRizol reagent (Invitrogen) as recommended by the manufacturer. RNA samples were treated with RNase-free DNase (Promega) for 1 h at 37 °C to remove genomic DNA. Quantitative measurement of HBV pregenomic RNA was performed using real-time PCR as described previously (31). The primers for the detection of pregenomic were 5′-CTCAATCTGGGGAAATCTAATG-3′ (sense) and 5′-TGGATGAAAAACCTGACGACCAAT-3′ (antisense).

HCV Infection of HLCZ01 Cells. Huh7.5 cells were kindly provided by Charles Rice (Rockefeller University, New York). pJFH1 was a gift from Takaji Wakita (National Institute of Infectious Diseases, Tokyo) (13). HCVcc was generated as described previously (12). HLCZ01 cells were inoculated overnight with HCVcc at an MOI of 0.1, and the inoculum was removed. The cells were maintained and harvested at the indicated times. HLCZ01 cells were infected with the sera from hepatitis C patients diluted at 1:20 and were maintained and harvested at the indicated times. HLCZ01 cells were inoculated with the sera from hepatitis C patients diluted at 1:20 and were cultured for various time periods.

Detection of HCV RNA and IFN-β by Real-Time PCR. Total cellular RNA was isolated using TRizol. The primers targeting HCV, IFN-γ, IFN-α, G1P3, 5′-8U, and GAPDH have been reported, and real-time PCR was performed as described previously (30). The primers for amplification of ALB, AAT, HNF4, and CYP3A4 are given shown in SI Materials and Methods. The CDNA of mR-122 was synthesized from total RNA using the stem-loop reverse-transcription primer 5′-GTGTCATCAGCTGGTGTCGTCAGCTGTCGGTGGTGGATGCTGTCAGCAGCTGGCACATAACTGAGCCTGG-3′ (sense) and 5′-GCTTCGGCAGCACATATGGAATCTCAATGT-3′ (antisense). The cDNA of miR-122 was synthesized from total RNA using the stem-loop RT primer 5′-GCTTCGGCAGCACATATGGAATCTCAATGT-3′ (antisense). Fold variations were calculated after normalization to U6.

Southern Blot Analysis. HBV DNA was isolated from whole-cell lysates. Cells in a 60-mm dish recovered after trypsinization and one wash were lysed overnight at 37 °C in 1 mL lysis buffer [50 mM Tris HCl (pH 8.0), 10 mM EDTA, 1% SDS, 150 mM NaCl] supplemented with proteinase K (200 μg/mL). The cccDNA was selectively extracted from cells in a 60-mm dish recovered by trypsinization. Cells were lysed for 1 h at 37 °C in 1 mL lysis buffer not supplemented with proteinase K, followed by the addition of 0.25 mL of 2.5 M NaCl and incubation at 4 °C overnight. The lysate then was clarified by centrifugation at 12,000 × g for 30 min at 4 °C. Both samples were then treated with phenol and phenol:chloroform, followed by ethanol precipitation. For cccDNA detection, the prepared DNA sample was treated with plasmid-safe, ATP-dependent DNase (Epitect Technologies) following the manufacturer’s instructions. HBV viral particles in cell supernatants were concentrated by ultracentrifugation at 28,000 rpm in a SW28 rotor (Beckman Coulter) for 16 h at 4 °C. Fifteen milliliters of supernatant per sample were used for the concentration and extraction of HBV viral DNA. Nucleic acids were separated on 1% agarose gel and analyzed by Southern blot procedures with modifications (32). HBV-specific nucleic acids were detected with a digoxigenin (DIG)-labeled probe obtained by random priming (DIG High Prime DNA labeling and detection kit; Roche Diagnostics) on a 32-kb EcoRI fragment containing a complete linear HBV genome from HepG2.2.15 cells, according to the manufacturer’s instructions. Biodyne B Nylon transfer membranes (0.45 μm) were from Pall.

Northern Blot Analysis. Total RNA was isolated by using the TRizol reagent and treated with RNase-free DNase I. Thirty micrograms of total cellular RNA per sample denatured for 5 min at 100 °C was separated on a 1.2% agarose gel and analyzed by Northern blot according to the procedures published previously (33) and using the DIG-labeled HBV probe described above.

Immunofluorescence of Viral Protein and Human Hepatocyte-Specific Markers. Cells were seeded on glass coverslips and fixed with ice-cold acetone for 10 min. Cells were blocked with 1:50 goat serum for 30 min and then incubated for 1 h with mouse monoclonal anti-NS5A (HL1126), a gift from Chen Liu (University of Florida, Gainesville, FL), mouse monoclonal anti-HBsAg (S26) or anti-HBcAg (10E11) antibody (Pierce), mouse monoclonal anti-CDB1 antibody (a8) (Santa Cruz Biotechnology), mouse monoclonal anti–claudin-1 (2H10D10) or anti-occludin (OC-3F10) (Invitrogen) antibody, or rabbit anti-SR-BI antibody (ab137829) (Abcam). Cells were washed three times with PBS and stained with fluorescein-labeled secondary antibodies (Invitrogen) for 45 min. Finally, the coverslips were washed with PBS, and the nuclei were counterstained with DAPI (Vector Laboratories, Inc.). Fluorescent images were obtained with a fluorescent microscope (Olympus). Titration of infectious HCV was reported previously (12).

Statistical Analyses. The data were analyzed using a two-tailed Student t test and are presented as means ± SD.

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