Original Article

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Modeling of the hepatitis B virus life cycle and the efficacy of antivirals in human iPSC-derived hepatic organoids

Tanbin Liu[1,§], Junming Xu[2,§], Xiaoni Chen[3], Jingyi Li[1], Jie Ke[3], Jiasen Xu[1], Hongzhou Lu[1,*], Fenfang Wu[1,3,*]

[1] The Third People's Hospital of Shenzhen and the Second Affiliated Hospital, Southern University of Science and Technology, Shenzhen, Guangdong, China.
[2] Department of Hepatobiliary and Pancreatic Surgery, the Eighth Affiliated Hospital of Sun Yat-sen University, Shenzhen, Guangdong, China.
[3] Central Laboratory, Shenzhen Hospital, Beijing University of Chinese Medicine, Shenzhen, Guangdong, China.

SUMMARY: Hepatitis B virus (HBV) infection remains a major global health burden, affecting approximately 296 million people worldwide, and yet progress in mechanistic studies and development of antivirals has been limited by the lack of physiologically relevant and sustainable *in vitro* models. This study established a human induced pluripotent stem cell (hiPSC)-derived multilineage hepatic organoid system that robustly supports the complete HBV life cycle, including viral entry, replication, covalently closed circular DNA (cccDNA) formation, antigen secretion, and production of infectious progeny virus. These organoids exhibit stable expression of sodium taurocholate cotransporting polypeptide (NTCP), a key receptor for HBV entry, and remain viable long term under infection conditions for at least 20 days, with sustained secretion of HBsAg and HBeAg. Importantly, the model recreates key pathological features of chronic HBV infection, including downregulation of hepatocyte functional genes (*e.g.*, ALB and CYP3A4) and induction of fibrosis-associated markers such as COL1A1, reflecting early extracellular matrix remodeling. Moreover, results indicated the utility of this platform in the evaluation of antivirals. Treatment with tenofovir effectively reduced viral DNA and antigen production without affecting cccDNA levels, whereas bulevirtide resulted in stage-specific inhibition of viral entry, highlighting the model's capacity to resolve mechanism-of-action differences. At the same time, drug-induced hepatotoxicity was assessed within the same system. Collectively, this hiPSC-derived hepatic organoid model provides a scalable and physiologically relevant platform that bridges the gap between conventional cell culture and *in vivo* systems, offering a powerful tool for studying HBV pathogenesis, host–virus interactions, and preclinical antiviral discovery.

Keywords: human iPSC-derived hepatic organoids, hepatitis B virus (HBV), cccDNA persistence, liver fibrosis, antiviral screening

1. Introduction

Hepatitis B virus (HBV) infection is a major pathological factor leading to liver fibrosis, cirrhosis, and hepatocellular carcinoma and it remains a substantial global health burden, with HBV infection affecting approximately 296 million people (*1*). HBV is a hepatotropic DNA virus with a natural infection that is highly restricted to humans and non-human primates, while common rodent animals are non-permissive hosts (*2*). Consequently, the development of pathophysiologically relevant experimental systems for HBV has been particularly challenging. Currently available models for studying HBV-related liver diseases—including various animal models, immortalized cell lines, and primary hepatocytes (*3,4*) — fail to faithfully recreate the process of long-term HBV infection in the liver. These models exhibit notable limitations: they neither support sustained long-term infection nor replicate the human liver microenvironment. Moreover, factors such as the high cost, prolonged modeling time, and ethical concerns associated with animal models significantly hinder progress in HBV research.

Hepatic organoids (HOs) derived from hiPSCs possess a three-dimensional physiological architecture and exhibit the cellular composition and functional characteristics of human liver tissues (*5*). These organoids have been widely used in disease modeling, mechanistic studies, and drug testing (*6,7*). In recent years, significant progress has been made using HOs in research on HBV infection, replication, and tumorigenesis (*8,9*). For

instance, De Crignis *et al*. established an HBV infection model using primary hepatocyte-derived HOs and they evaluated the anti-HBV activity and drug-induced toxicity of two compounds (*10*). Nie *et al*. established an HBV infection model using HOs co-cultured from hiPSC-derived hepatocytes, umbilical vein endothelial cells, and mesenchymal cells, and they demonstrated that the level and duration of HBV replication in these organoids surpassed those in conventional cell culture models (*11*). While these studies represent preliminary explorations of hiPSC-derived HOs to model HBV infection, the existing models still fail to recreate the pathological processes following HBV infection, and particularly the liver fibrosis that develops during long-term HBV infection. Thus, there is a pressing need for a human-relevant HBV organoid model that not only sustains persistent infection but that also displays captures the progressive fibrotic remodeling of the liver.

Building on our previously established *in vitro* organoid platform, we have generated functionally mature hepatobiliary organoids (*12*). Using a directed co-differentiation strategy from hiPSC-derived mesoderm and endoderm, we successfully established multicellular hepato-biliary organoids in which hepatic parenchymal and non-parenchymal lineages coexist (*13,14*). Incorporation of our proprietary MIX cocktail further enabled long-term maintenance and stability of these organoids (*12*). Together, these features recreate the complete HBV life cycle, including viral attachment, entry, uncoating, replication, assembly, and production of infectious virions. Importantly, the non-parenchymal cell compartment within the organoids was configured to permit the emergence of fibrosis-like pathological changes that arise during chronic HBV infection, thereby enabling parallel analysis of viral and fibrogenic responses.

Thus, reported here are multi-lineage hiPSC-derived HOs that recreate persistent HBV infection and concomitant fibrosis *in vitro*. This model was further used to assess the efficacy and hepatotoxicity of antiviral compounds. This approach overcomes the inability of traditional animal models to emulate the human HBV infection microenvironment, thereby providing a potential platform for drug screening and mechanistic studies on hepatitis B fibrosis.

2. Materials and Methods

2.1. Cell lines and culture

Cell culture was performed with the hiPSC line UC (passages 30 to 37), purchased from Beijing Saibei Biotechnology Company Ltd., Beijing, China. Undifferentiated hiPSCs were cultured on qualified Matrigel Basement Membrane Matrix Growth Factor Reduced (Corning, NY, USA)-coated 6-well plates (Thermo Fisher Scientific, MA, USA) and maintained in mTeSR medium (STEMCELL Technologies, BC, Canada) at 37°C with 5% CO_2 and 95% air. The medium was replaced every 24 h.

The HepAD38 cells and HepG-Puro-NTCP cells were maintained on 10 cm dishes with DMEM and 20% fetal bovine serum (Life Technologies, Gaithersburg, USA). All cells were maintained at 37°C in an incubator with 5% CO_2.

2.2. Differentiation and generation of HOs

HOs were generated from hiPSCs following previously described protocols (*12*) in which a small fraction of mesodermal cells is intentionally retained during definitive endoderm specification. Upon subsequent directed differentiation, definitive endoderm cells give rise to hepatic parenchymal cells, while the residual mesodermal cells differentiate into non-parenchymal lineages, including endothelial cells. HOs were used for HBV infection experiments as well as other analyses.

2.3. HBV preparation and infection

HBV stocks were derived from supernatants of HepAD38 cells (*15*), which were stably transfected with a complete HBV genome (genotype D). HiPSCs-HOs and HepG-puro-NTCP cells (*16*) were infected with HBV [100 genome equivalents (GEq)/cell or 3,000 GEq/cell] in the presence of 4% polyethylene glycol 8000 in 24-well plates. After the indicated days post-infection, cultured cells were then harvested. Pre-genomic RNA (pgRNA) was quantified using SYBR Green (Vazyme Bio, Nanjing, China) with specific primers (Thermo Fisher Scientific). The expression of pgRNA was normalized against expression of GAPDH.

2.4. RNA isolation and quantification

RT-qPCR was used to determine pgRNA levels. Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. RNA was reverse-transcribed with Superscript III reverse transcriptase (Thermo Fisher Scientific). Real-time PCR was performed using the CFX96 Touch System (Bio-Rad, CA, USA). The primers used are available upon request.

2.5. DNA isolation and quantification

Infected HOs were collected after infection. Total DNA in the cells was purified using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). The concentration of total DNA was determined using a NanoDrop spectrophotometer (Thermo Fischer Scientific), and the DNA concentration was adjusted to 20 ng/μL for further experiments. Two μL of adjusted DNA sample was used to quantify vDNA with SYBR Green using a standard curve plotted using plasmid pUC-HBV over

a range of 10^7-10^2 copies. To quantify HBV covalently closed circular DNA (cccDNA) copies, adjusted DNA samples of 25 μL [20 ng/μL] of HBV DNA were treated with plasmid-safe DNase. After digestion, two μL of the sample was used to quantify cccDNA copies with SYBR Green using a standard curve plotted using plasmid pUC-HBV over a range of 10^7-10^2 copies. The primers used for HBV DNA and cccDNA quantification are available upon request.

2.6. Detection of HBsAg and HBeAg

A total of 450 μL of the HOs or cell supernatant was collected after infection and used for the detection of HBsAg and HBeAg. The HBsAg and HBeAg ELISA Kit (Wantai Bio, Beijing, China) were used to detect of HBsAg and HBeAg, respectively, from the supernatant of the infected organoids according to the manufacturer's instructions.

2.7. Immunofluorescence analysis

HOs were washed with PBS at the indicated time points post-infection, fixed with 4% paraformaldehyde for 30 min at room temperature, and permeabilized with 0.15% Triton X-100 for 30 min. After blocking with 10% normal donkey serum in PBS for 60 min at RT, the organoids were incubated with rabbit Hepatitis B Virus Core Antigen antibody (1:200) and mouse Hepatitis B Virus Surface Antigen antibody (1:200) (Thermo Fischer Scientific) for 12 h at 4 ℃, and they were then washed with PBS three times. The organoids were then incubated with secondary antibodies - Alexa Fluor™ 488 Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody and Alexa Fluor™ 647 Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody (1:400) - for 1 h at RT. After they were washed three times with PBS, the organoids were examined by using the ZEISS LSM 980 Confocal Laser Scanning Microscope and Airyscan.

2.8. Statistical analysis

Statistical significance was analyzed using the two-tailed unpaired Student's *t*-test and the software GraphPad Prism 8.0 (GraphPad, San Diego, CA, USA). A *p*-value of < 0.05 was considered significant.

3. Results

3.1. Generation of functional HOs from hiPSCs

Building on our previous work on HO models (*12,17*), we differentiated hiPSCs into HOs using the established protocol (Figure 1A). Morphological analysis indicated that, relative to day 0, hepatocytes on day 25 exhibited pronounced aggregation and a more mature hepatic morphology, characterized by polygonal cell shapes and frequent multinucleation (Figure 1B). Before differentiation, flow cytometry indicated that more than 90% of hiPSCs on day 0 co-expressed the pluripotency markers SSEA4 and TRA1-81. By day 4, over 70% of the cells co-expressed the definitive endoderm markers SOX17 and FOXA2, as confirmed by flow cytometry (Figure 1C). Immunofluorescence analysis on day 15 indicated that a substantial proportion of cells expressed SOX9 and HNF4α, a hallmark feature of bipotent hepatoblasts. During liver development, signals from the endothelial lineage not only promote liver bud formation but also guide biliary morphogenesis (*18,19*).

To investigate this in our system, we tracked the mesoderm-derived endothelial lineage and observed a significant upregulation of the mature vascular endothelial marker CD31 by day 15. The cultures were subsequently treated with HBM medium for an additional 10 days to further support maturation. By the end of this stage, mature hepatocyte markers including CYP3A4 and HNF4α were detectable (Figure 1D). Moreover, quantitative polymerase chain reaction (qPCR) analysis of mature HOs indicated robust expression of key liver-specific genes (*20-23*), including *ALB* (albumin), *G6PC* (glucose-6-phosphatase catalytic subunit), and *RBP4* (retinol-binding protein 4), as well as cytochrome P450 enzymes (*CYP3A4, CYP3A7* and *CYP2C9*) (Figure 1E). Collectively, these findings demonstrate that our defined *in vitro* culture system reliably generates well-differentiated, functionally competent HOs with appropriate lineage-specific marker expression from a single hiPSC line.

3.2. Modeling HBV infection in hiPSC HOs

HOs derived from hiPSCs were cultivated in HCM for 25 days before being infected with recombinant HBV produced from HepAD38 cells, a subclone of the HepG2 cell line that stably expresses HBV (Figure 2A). Sodium taurocholate co-transporting polypeptide (NTCP) is a crucial transporter located on the basement membrane of hepatocytes and is vital for HBV infection (*16*). To determine whether HOs can serve as a source of HBV infection, RT-PCR was used to detect NTCP expression in HepG-puro-NTCP, hiPSCs, and HOs. Results indicated that NTCP was highly expressed in HOs as of 20 days after post-differentiation and the level of expression was significantly higher than that in HepG-puro-NTCP cells (Figure 2B). Immunofluorescence was used to detect NTCP, and results indicated that the HOs exhibited high levels of NTCP on days 20 and 25 post-differentiation (Figure 2C). Next, the sensitivity of HBV generated by HepAD38 in HOs was examined in order to model the HBV life cycle in HOs. After the HOs were infected with varying viral doses (ranging from 0 to 3,000 GEq/cell), the alterations in HBV cccDNA, pgRNA, and supernatant vDNA were examined 7 days post-

infection (dpi). As the results indicated, elevated levels of HBV cccDNA, pgRNA, and supernatant vDNA were associated with infection in a dose-dependent manner (Figure 3D-F). HBeAg and HBsAg were also detected and quantified in supernatants of infected organoids as an additional indicator of active HBV replication (Figure 2G-H). The presence of foci of HBV replication in HBV-infected organoids, was confirmed by immunostaining with antibodies that recognize the HBcAg, resulting in distinct nuclear and cytoplasmic staining in infected HOs 10 dpi (Figure 2I). Therefore, differentiated HOs offer a viable *ex vivo* HBV infection platform that enables for the analysis of the roles played by distinct host and virus factors.

3.3. HOs remain viable long term in an infected state and produce infectious HBV

Next, the capacity of differentiated HOs to serve as a model of chronic infection was assessed. Following

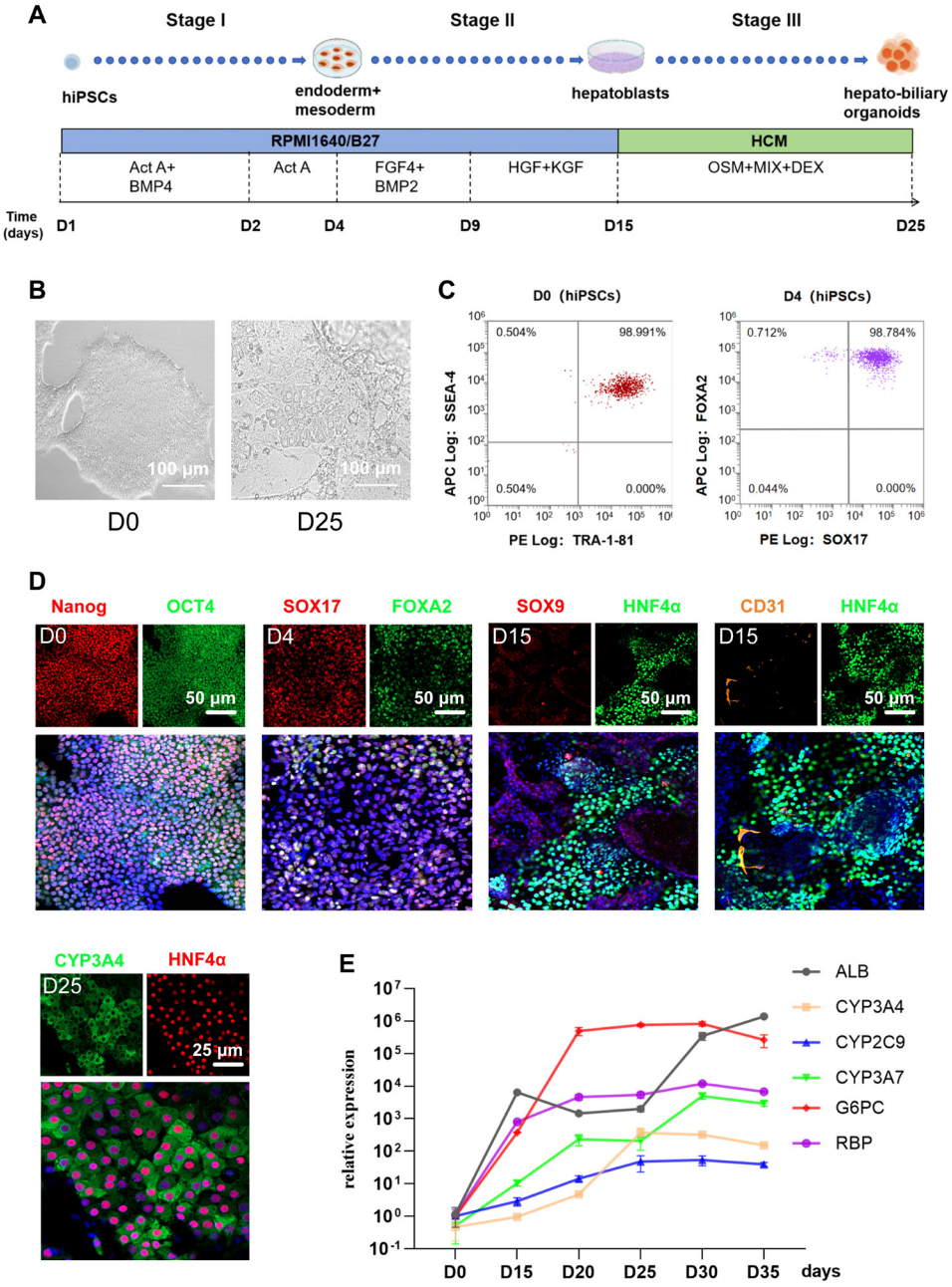

Figure 1. Generation of functional hepatic organoids from hiPSCs. (A) Schematic representation of the differentiation procedure. **(B)** Sequential morphological changes (day 0 and 25) as hiPSC differentiated into hepatic organoids. Scale bars = 100 μm. hiPSCs, human induced pluripotent stem cells. **(C)** Flow cytometry confirmed that most day-0 hiPSCs were positive for SSEA4 and TRA1-81, and most day-4 cells were positive for FOXA2 and SOX17. **(D)** Immunofluorescence analysis of stage-specific markers and differentiation efficiency at the end of each key stage. Scale bars, 50 μm. (E) qPCR analysis of hepatic genes (*ALB, G6PC, RBP4, CYP3A4, CYP3A7* and *CYP2C9*) in day-25 hepatic organoids ($n = 3$).

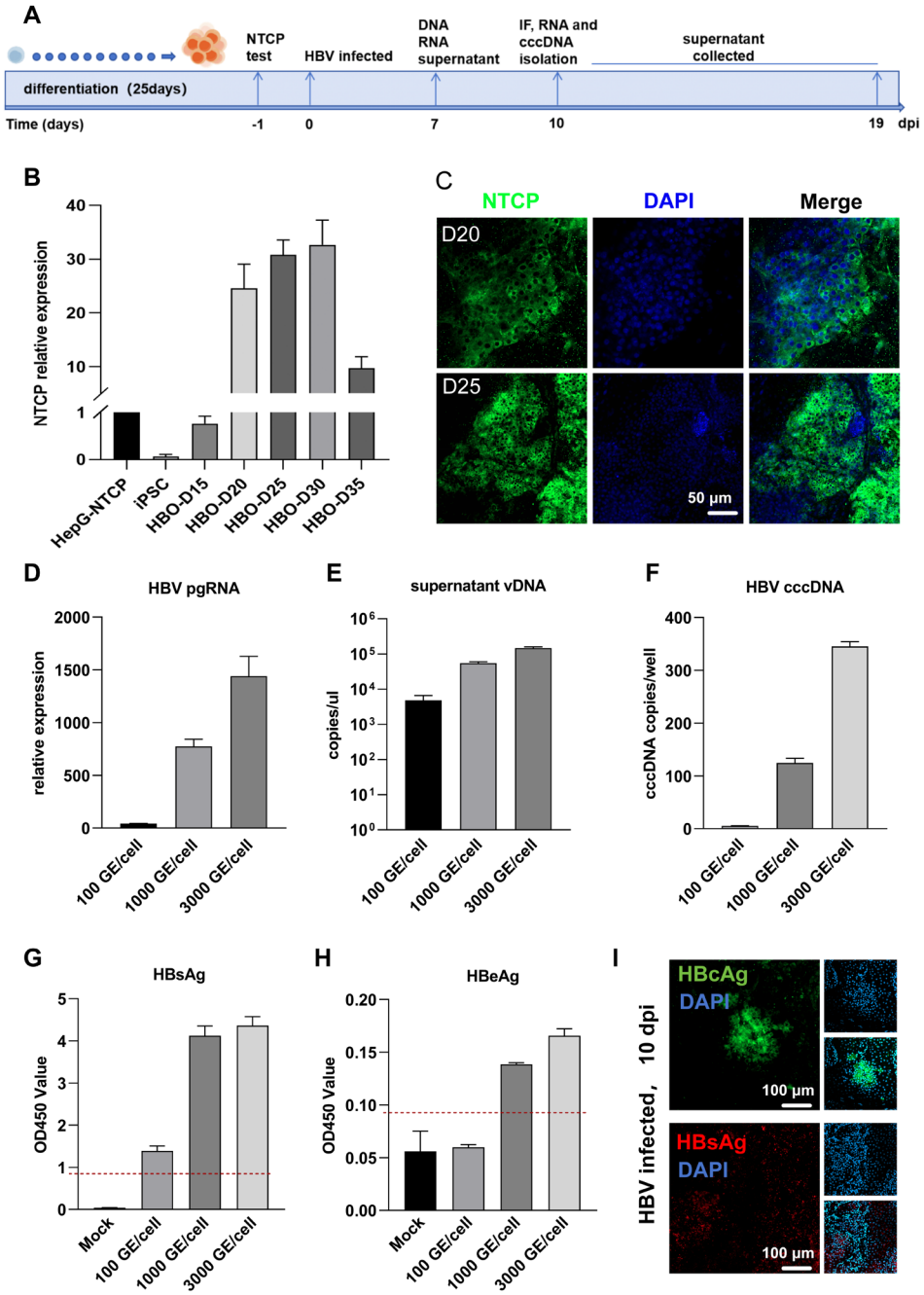

Figure 2. Modeling HBV infection in hiPSC hepatic organoids. (A) Experimental design of infection experiments. Arrows indicate the time points for HBV detection. **(B)** Q-PCR analysis of NTCP expression in HepG-puro-NTCP cells, iPSCs, and differentiated HOs (n = 3). **(C)** Immunofluorescence analysis of NTCP in differentiated HOs. Scale bars = 50 μm. **(D-F)** Q-PCR quantification of HBV pgRNA **(D)**, supernatant vDNA **(E)** and cccDNA **(F)** in HOs (n = 3) infected with a range of doses of 100-3,000 GE/cell 7 days post-infection (dpi). **(G-H)** HBeAg and HBsAg from the supernatant of infected HOs were quantified with an enzyme-linked immunosorbent assay (ELISA). Negative bars correspond to positive and negative controls provided by the kit manufacturer. **(I)** Immunofluorescence analysis of HBcAg and HBsAg in HO sinfected at a dose of 1,000 GEq/cell 10 dpi, Scale bars = 100 μm.

infection of HOs with low doses of HBV, HOs were found to remain viable for at least 20 days (Figure 3A). Dynamic monitoring indicated that HBsAg are constantly secreted in HOs for a minimum of 19 days (Figure 3B). Immunofluorescence also revealed HBcAg and HBsAg 20 dpi (Figure 3C). Chronic HBV infection poses a serious risk to human health because it quickly leads to liver fibrosis, which can then develop into cirrhosis, liver failure, and hepatocellular cancer. The development of HBV-induced liver fibrosis, however, is difficult for current HBV research models to faithfully reproduce. Remarkably, immunofluorescence revealed considerably higher levels of COL1A1 expression 2 weeks after HOs were infected with HBV compared to the control group (Figure 3D-E). HBV-infected HOs develop liver dysfunction during the infection

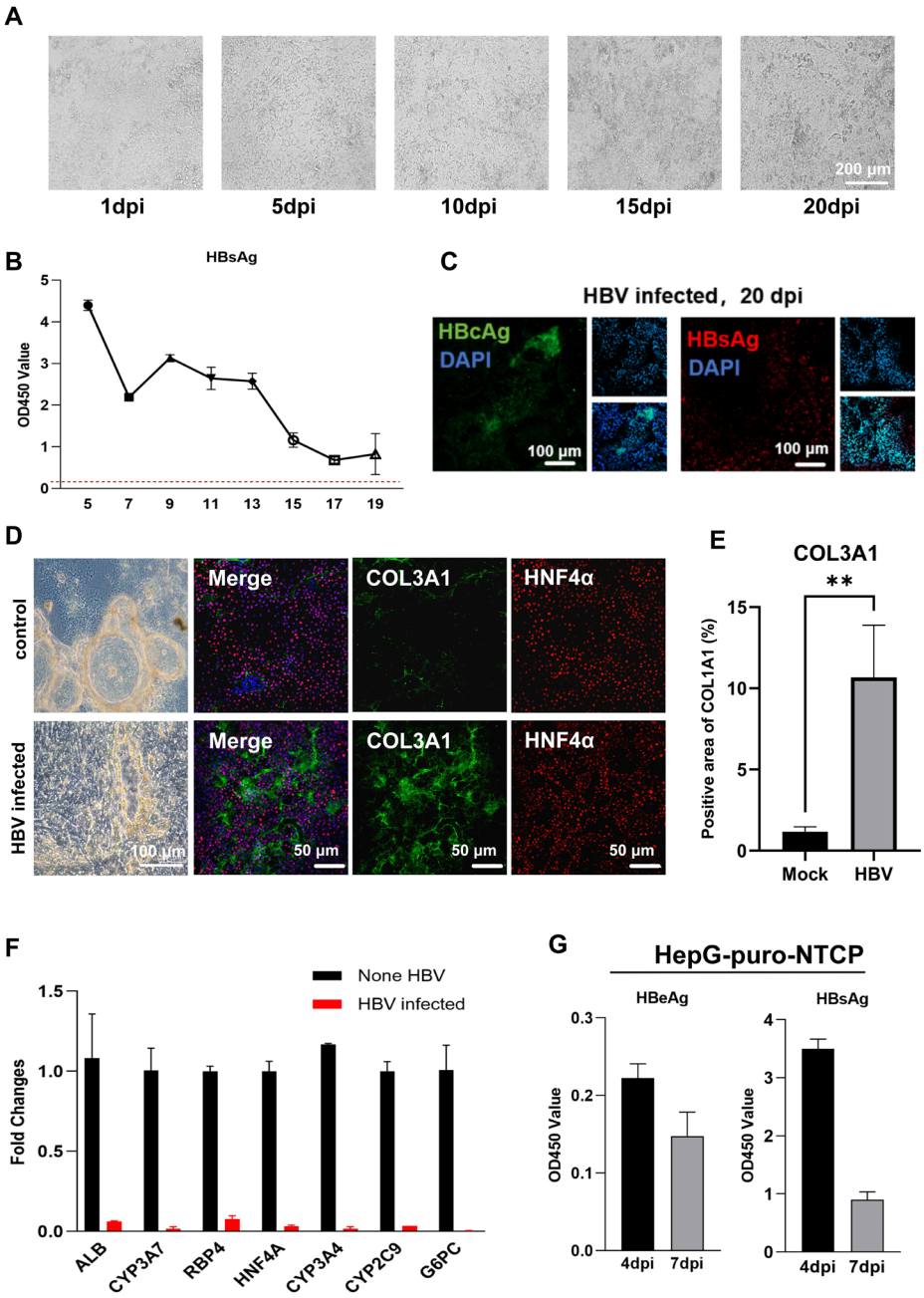

Figure 3. Hepatic organoids remain viable long term in an infected state and produce infectious HBV (A) Representative bright-field images of HOs infected with HBV for 1 to 20 days as indicated. **(B)** HBeAg from the supernatant of infected organoids for 1 to 20 days was quantified with ELISA. **(C)** Immunofluorescence analysis of HBcAg and HBsAg in HOs infected at a dose of 1,000 GEq/cell 20 dpi, Scale bars = 100 μm. **(D-E)** Immunofluorescence analysis of COL3A1 in HOs 20 dpi, Scale bars = 100 μm. **(F)** Q-PCR quantification of the hepatic genes *ALB, G6PC, RBP, CYP3A4, CYP3A7*, and *CYP2C9* in HOs 20 dpi, $n = 3$. **(G)** ELISA analysis of HBeAg and HBsAg in HepG-puro-NTCP cells infected with the supernatant of infected hepatic organoids (10-20 dpi).

process, as evinced by the significantly lower mRNA levels of ALB, CYP3A7, RBP4, HNF4α, CYP3A4, CYP2C9 and G6PC in the infected group compared to the normal group, according to RT-PCR measurement of mRNA levels of substances relevant to liver function (Figure 3F). To further investigate whether infectious progeny viruses could be produced by infected HOs, the supernatant was collected from infected hiPSC-HOs at 20 dpi (20 dpi-Sup, from day 10 to day 20), and its infectivity was examined in HepG-puro-NTCP cells. Notably, the progeny virus displayed infectivity, confirmed by detection of HBeAg and HBsAg in HepG-puro-NTCP cells (Figure 3G). These findings suggest that this organoid system can serve as a model of chronic *ex vivo* infection.

3.4. HBV-infected HOs are a viable platform for evaluation of antivirals

Over the past few years, organoids have been widely used as drug screening instruments and have been crucial to the creation of novel drugs. Next, using the setup shown in Figure 4A, we investigated whether the *ex vivo* infected HO platform would be suitable for anti-HBV drug evaluation to track the antiviral activity and drug-induced toxicity of two distinct medications, tenofovir and bulevirtide. Tenofovir is a nucleoside reverse transcriptase inhibitor that prevents HBV pre-genomic RNA from being reverse-transcribed to DNA (*24*). Bulevirtide, an NTCP inhibitor, can inhibit HBV entry into liver cells and block HBV infection in hepatocytes (*25*). Due to the well-established detrimental effects of tenofovir and bulevirtide on the viability of primary human hepatocytes, we sought to evaluate their drug-induced toxicity on HOs. The decreased cell viability was apparent in the phenotype of the 10 μM of tenofovir-treated and 4 μM of bulevirtide-treated organoids compared to controls according to microscopy (Figure 4B). Treatment with 5 μM of tenofovir after HBV infection of HOs indicated that production of HBeAg (Figure 4C) and HBV viral DNA (Figure 4F) in the culture supernatant was inhibited by tenofovir, whereas, as expected, HBV cccDNA (Figure 4D) and pgRNA (Figure 4E) levels remained the same. After HOs were infected with HBV and treated with bulevirtide either 1 day before infection or 1 day after infection, results indicated that the pre-treatment group significantly suppressed HBeAg, cccDNA, pgRNA, and vDNA, while the post-treatment group did not (Figure 4C-F). Thus the organoid *ex vivo* infection platform not only allows the quantification of the antiviral efficacy of drugs but it also provides information on how they work by enabling the identification of specific stages of the HBV life cycle that are blocked and targeted.

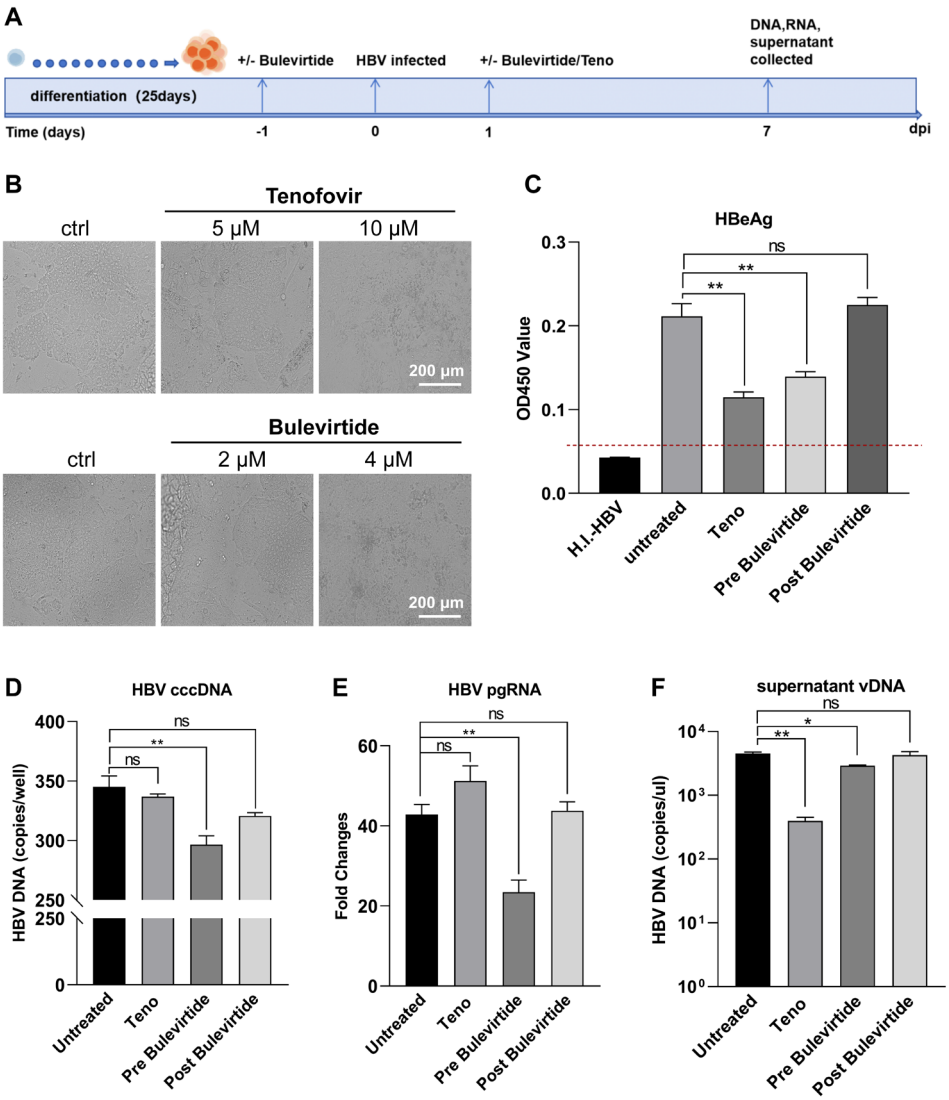

Figure 4. HBV-infected hepatic organoids are a viable platform for evaluation of antivirals. (A) Experimental design of the drug treatment of HBV-infected hepatic organoids followed by assessment of antiviral activity. **(B)** Representative bright-field images of hepatic organoids treated for 4 days with the control or increasing concentrations of the antivirals tenofovir or bulevirtide as indicated. **(C)** ELISA analysis of HBeAg in hepatic organoids (pre- or post-drug treatment) 7 dpi. **(D-F)** Q-PCR quantification of cccDNA **(D)**, HBV pgRNA **(E)**, and supernatant vDNA **(F)** in hepatic organoids ($n = 3$) at 7 dpi with drug treatment. *$p < 0.05$, **$p < 0.01$, ns, not significant.

4. Discussion

This study, differentiated functionally mature HOs from human-derived iPSCs. This single-cell-derived differentiation system successfully generated multilineage HOs that not only exhibited efficient liver differentiation but that also displayed a high level of susceptibility to HBV. The HOs can simulate the complete cycle of HBV infection, remain viable long term under infectious conditions, and secrete infectious viruses. This organoid system also mimics virus-induced liver dysfunction and fibrosis, suggesting that viral infection in HOs may authentically reproduce the *in vivo* virus-host interaction mechanism. Moreover, this organoid system serves as a platform with which to screen the antiviral activity of HBV drugs.

HBV infection affects over 250 million people worldwide (*26*), posing a major health challenge. Conventional cell lines and primary human hepatocyte (PHH) cultures struggle to replicate the liver's complexity, limiting research on host-pathogen interactions (*27,28*). Animal models exhibit a high level of species specificity, limiting their broad applicability (*29*). Hepatocellular carcinoma cell lines have undergone tumorigenic changes, failing to mimic the progression from normal hepatocytes to cancer (*30*). Primary hepatocytes are difficult to obtain and have limited expansion capacity, making them unsuitable for large-scale analysis. Compared to PHHs, HepaRG cells, and iPSC-derived HOs as were previously reported, the current system offers several advantages, including single-cell-derived multilineage differentiation, stable NTCP expression, long-term viral persistence, and the emergence of fibrosis-like features. These characteristics collectively position this model as a complementary and scalable tool for research on HBV pathogenesis and development of antivirals.

Although the current organoid system does not incorporate immune components such as Kupffer cells or lymphocytes, this feature provides a unique advantage for distinguishing hepatocyte-intrinsic responses to HBV infection. The absence of immune-mediated cytotoxicity enables the model to depict early hepatocellular alterations, including metabolic dysfunction and ECM remodeling, which are often masked in immune-competent systems. Future integration of immune cells or microfluidic co-culture platforms may further extend the utility of this model to the study of immune-driven liver injury and chronic inflammation.

Notably, NTCP was found to be expressed at high levels in HOs. Since NTCP is the specific receptor for HBV entry into hepatocytes, only cells or organoids stably expressing high levels of it can facilitate the complete viral infection cycle, making them suitable for disease modeling and antiviral screening. Numerous drugs enter hepatocytes *via* NTCP transport and interact with bile acid metabolism, potentially causing drug-induced liver injury (*31*). Models with sustained, high levels of NTCP expression are crucial to evaluating such chronic or cumulative toxicity. Levels of NTCP expression serve as one of the gold standards for hepatocyte maturation and functionalization (*32*). Its long-term stable expression indicates that organoid models possess stable, primary-like hepatic metabolic functions. This characteristic makes these organoids an ideal platform for studying HBV infection, bile acid metabolism, and the long-term hepatotoxicity of related drugs, providing a powerful tool for liver disease modeling and drug screening.

Chronic hepatitis B virus infection is a major global cause of chronic cirrhosis and hepatocellular carcinoma (HCC). The fibrosis-like phenotype observed in HBV-infected organoids, characterized by COL1A1 upregulation, likely represents an early-stage ECM remodeling response. While this does not fully recreate advanced fibrosis, it provides a valuable window into the initial fibrogenic events triggered by persistent viral infection. Incorporating hepatic stellate cells or modulating TGF-β signaling in future studies may enable reconstruction of a more complex fibrotic architecture.

The evaluation of antivirals as was performed in this study focused on tenofovir and bulevirtide as representative inhibitors targeting distinct stages of the HBV life cycle. This proof-of-concept design demonstrates that the organoid platform is capable of recreating stage-specific antiviral mechanisms. Future expansion to capsid assembly modulators, siRNA-based therapies, and cccDNA-targeting agents will further validate the platform's potential for preclinical drug screening.

In summary, functional HOs have been successfully established. This organoid system can mimic the whole life cycle of HBV and exhibit HBV-induced liver failure and fibrosis. These findings imply that the model serves as a viable and feasible model for study of HBV infection.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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[§]These authors contributed equally to this work.
*****Address correspondence to:*
Fenfang Wu, Biotherapy Clinical Research Center, The Third People's Hospital of Shenzhen and the Second Affiliated Hospital of Southern University of Science and Technology, Shenzhen, Guangdong, China.
E-mail: wufenfang19@126.com

Hongzhou Lu, The National Clinical Research Center for Infectious Diseases, the Third People's Hospital of Shenzhen and the Second Affiliated Hospital of Southern University of Science and Technology, Shenzhen, Guangdong, China.
E-mail: luhongzhou@szsy.sustech.edu.cn

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